REVIEW

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Targeted intracellular delivery of therapeutics: an overview

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During the last decade, intracellular drug delivery has become an emerging area of research in the medical and pharmaceutical field. Many therapeutic agents such as drugs and DNA/oligonucleotides can be delivered not just to the cell but also to a particular compartment of that cell to achieve better activity e.g. proapoptotic drugs to the mitochondria, antibiotics and enzymes to the lysosomes and various anticancer drugs and gene to the nucleus. The lipidic nature of biological membrans is the major obstacle to the intracellular delivery of macromolecular and ionic drugs. Additionally, after endocytosis, the lysosome, the major degradation compartment, needs to be avoided for better activity. To avoid these problems, various carriers have been investigated for efficient intracellular delivery, either by direct entry to cytoplasm or by escaping the endosomal compartment. These include cell penetrating peptides, and carrier systems such as liposomes, cationic lipids and polymers, polymeric nanoparticles, etc. Various properties of these carriers, including size, surface charge, composition and the presence of cell specific ligands, alter their efficacy and specificity towards particular cells. This review summarizes various aspects of targeted intracellular delivery of therapeutics including pathways, mechanisms and approaches. Various carrier constructs having potential for targeted intracellular delivery are also been discussed.

1. Introduction

Recent advances in molecular and cellular biology have led to the development of new classes of therapeutic agents, which are required to be delivered at their cellular/subcellular targets. Most of the newer agents developed have their site of action in the cytosol or cellular organelle of the cells, e.g. glucocorticoids such as dexamethasone; enzymes for the lysosomal compartment and various anticancer proapoptotic drugs to the mitochondria (Torchilin 2006).
While proximity of the bioactive substance to the cell could be achieved by various currently developed transport strategies, the plasma membrane of the cellular target provides a formidable obstacle for large and charged molecules and hence getting a drug across the plasma membrane into the cytosol is considered one of the biggest rate limiting steps, as the majority of cells are not phagocytic and fusion of carriers with target cells is a very rare phenomenon (Simkiss 1998). Cellular targeting of drugs and therapeutic materials to designated cellular locations relies upon their release from the carrier, either at the extracellular or intracellular level, and subsequent passage across the biological membranes (Moghimi and Rajabi-Siahboomi 2000). In general, various levels of cellular drug targeting can be categorized according to their level of specificity (Table 1).

To develop an effective and successful carrier to deliver the therapeutic agent at the cellular level, an understanding of differences in membrane function, properties, and structure among cellular organelles as well as the basic mechanism(s) by which cells internalize extracellular material is essential (Moghimi and Rajabi-Siahboomi 2000). Following endocytosis, the lysosome is also a major obstacle to the delivery of drugs and DNA to the cytosol owing to lysosomal membrane permeabilizing properties and the lysosomal degradation pathway. The delivery of bioactive molecules/macromolecules to the intracellular site can be achieved by various strategies, which are termed 'cytosolic approaches' (Fig. 1). These include both direct entry to the cell cytosol and entry by endosomal escape. Cell-penetrating peptides follow the first pathway (Gupta et al. 2005), and various approaches such as vesicle membrane destabilization or buffering of the compartment have been used to avoid the de-

Table 1: Various levels of specificity of cellular drug targeting

Specificity	Examples
Cell type	Macrophages, hepatocytes
Cellular compartment	Mitochondria, nuclei, lysosomes, cytosol
Cellular component	DNA, cationic proteins
Specific molecule	Single mRNA, reverse transcriptase

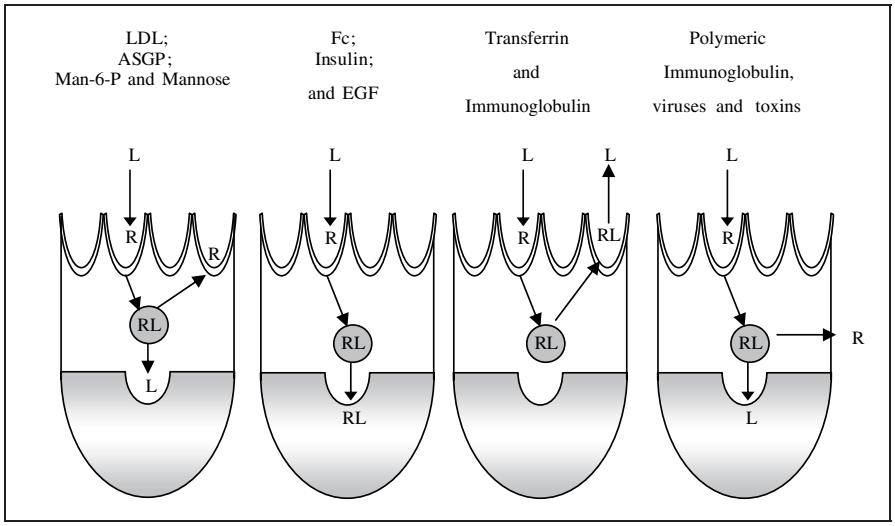


Fig. 1:
Pathways of receptor internalizing and recycling. Subsequent to entry into acidic endosomes, ligand and receptors are sorted and trafficked independently, which may result in degradation, recycling or transcytosis of either molecule. L = Ligand, R = Receptor. (Adapted from Vyas and Khar, 2002)

gradation in the lysosomes. The carriers that have been used successfully are pH-sensitive liposomes (Venugopalan et al. 2002), cationic liposomes (Bailey and Cullis 1997), cationic lipids (Martin et al., 2005) and polymeric nanoparticles (Vasir and Labhateswar 2006) etc. For more efficient and specific delivery, various targeting moieties can be attached to the surface of the delivery system e.g. folate and transferrin for tumor cells, polyschacharides for hepatoma cells, etc. Various endogeneous ligands and their respective receptors have been discussed elsewhere (Vyas and Shihorkar 2000).

2. Rationale for cellular drug delivery

2.1. Organeller disease

Lysosomes, the digestive organelles of animal cells, contain approximately 50 different hydrolytic enzymes. Lysosomes vary insize from large (over 1 μm) to very small vesicles (25–50 nm). An important property of lysosomal enzymes is that they all show their optimum activity at acidic pH' thus they are all acid hydrolases. The pH of these organelles is approximately 4.6. Two major roles of lysosomes include phagocytosis and autophagy. Autophagy means the destruction of the cells own organelles and their replacement. As the lysosomal enzymes can digest every type of biological macromolecules, disturbance of this function can affect human health. There are various genetic disorders, which are caused by the absence of one or more lysosomal enzymes. These are termed lysosomal storage disorders (Neufeld 1991). In these disorders various glycolipids and extracellular components accumulate in lysosomes as large inclusions. I-cell disease is a lysosomal storage disorder in which multiple enzymes are missing from the lysosomes. Enzyme replacement therapy is in prospect for the treatment of lysosomal storage disorders (Karp 1999). Enzymes can be efficiently delivered to the specific site in the cell where the deficiency is manifested because lysosomes are the natural target site of materials in endocytosis. Liposomes have been used as "lysosomotropic carriers" to convey the enzyme for therapeutic supplementation in enzyme deficiency diseases like Gaucher's disease or Pompe's disease. A variety of lysosomal enzymes can be entrapped in liposomes for delivery to patients suffering from lysosomal storage disorders (Belchetz et al. 1977).

There are a number of pathogenic bacteria, which reside in the endosomal, phagosomal or phagolysosomal compartment of phagocytic and non-phagocytic cells, for example, tuberculosis and leprosy bacteria, and other protozoans such as Trypanosoma and Toxoplasma. The need for antibiotics with greater intracellular efficiency has led to the development of endocytosable drug carriers such as liposomes and nanoparticles, which mimic the entry path of bacteria by penetrating into phagosomes or lysosomes. Fattal et al. (1998) tested the effectiveness of ampicillin loaded polyisohexylcyanoacrylate (PIHCA) nanoparticles and found increased localization of the drug in compartments, where there were bacteria. In another study it was found that the dose of nifurtimox required decreased when it was delivered through ethylcyanoacrylate nanoparticles, a lysosomotropic carrier and it also showed high trypanocidal activity on both free trypomastigates and intracellular amastigates of *Trypanosoma cruzi*, an intracellular parasite for Chagas' disease (Sanchez et al. 2002). Liposomes loaded with antibiotics can also used as a suitable lysosomotropic carrier to target facultative intracellular bacteria (*Brucella*, *Listeria*, *Mycobacterium*, *Legienella*, *Salmonella*, *Klebsiella* and *Escherichia* sp.) (Gregoriadis 1978).

Mitochondria, the power house of the cell, is another organelle which also plays a major role in apoptosis. Any defect in these processes may contribute to disease in the cell, e.g. neuromuscular and neurodegenerative disease, aging, and cancer (Weissig et al. 2004). Mitochondria also have 1% of the total cellular DNA and various somatic mutations have been observed in the mitochondrial genome of cancer cells (Modica-Napolitano and Singh 2002). Drugs may be required to be delivered to the mitochondrial site for the induction of apoptosis, specifically in cancer cells, necessitating a mitochondria specific delivery system (Dias and Baily 2005). Various strategies have been used for the delivery of drugs to mitochondria. Advantage can be taken of the large negative inner membrane potential of mitochondria for targeted delivery of therapeutic agents to the mitochondria. Various lipophilic cations, e.g. triphenylphosphonium, dequalinium chloride etc., accumulate inside the mitochondria and this property of lipophilic cations has been used to deliver the antioxidant vitamin E to the mitochondria (Smith et al. 1999). Boddapati et al. (2005) attached lipophilic cations to the liposome surface and found that the modified liposome could be rendered mitochondriotropic.

2.2. Gene delivery

In the cells two types of DNA are found, i.e. mitochondrial DNA (mtDNA) and nuclear DNA. mtDNA is a circu-

lar 16.5 kbp DNA which encodes 13 polypeptides, 22 tRNA and 2 rRNA. All 13 polypeptides are the components of mitochondrial enzymes (Murphy and Smith 2000). So any mutation in the mtDNA may cause disease and mtDNA is highly susceptible to oxidant produced by oxidative phosphorylation. Many mtDNA mutations have been observed that cause neural and muscular dysfunction and spontaneous mutation may also play role in normal aging (Pulkes and Hanna 2001). Various somatic mutations have also been found in a variety of cancers. Thus, gene therapy, by delivering DNA specifically to mitochondria, is a new approach to treat these diseases. Due to the sensitivity of DNA to enzymatic degradation, genetic material cannot be introduced unprotected *in vivo*. To achieve efficient transfection, two types of vectors have been studied. For the most part, viral vectors are more effective than non-viral vectors for achieving high efficiency gene transfer, but they have associated problems that hinder their application in gene therapy, such as immunogenicity, risk of infection, targeting and/or the duration and level of gene expression, limitation of DNA encapsulation, size and difficulty in scaling up of production. Such obstacles have driven the search for the development of non-viral DNA carriers, which obviate the shortcomings associated with viral vector systems (Felgner et al. 1987).

Numerous approaches using non-viral (synthetic) methods of gene transfer have been developed so far. Among them cationic liposomes have shown good potential in cultured cells (Gao and Huang 1995; Zhu et al. 1993). A major problem with gene transfer by cationic liposomes is inefficient delivery to tissues *in vivo*. Other problems with cartionic liposomes are their toxicity to cells and their property of forming aggregates with negatively charged serum proteins (Senior et al. 1991).

3. Pathways for cellular drug delivery and cellular transport

All eukaryotic cells exhibit one or more forms of endocytosis. Endocytosis has been defined as the internalization of plasma membrane with concomitant engulfment of extracellular cargo/fluid. The process can be divided into two types: phagocytosis and pinocytosis.

Phagocytosis involves the internalization of particulate matter such as bacteria, erythrocytes, beads and colloidal particles. It is carried out only by a few specialized cell types called phagocytes, i.e. phagocytic cells of the reticuloendothelial system (RES) including the Kupffer cells of the hepatic sinusoids, tissue fixed macrophages (histocytes) and blood macrophages or monocytes (Mukherjee et al. 1997). Sequential steps for this process are

(i) Recognition: – mediated by the coating of blood components, mainly by opsonin and high density lipoproteins.

(ii) Adhesion: – attachment of the particles to the macrophage cells of the RES.

(iii) Digestion: – in this the particles are transferred to phagosome, phago-lysosome and finally to digestive vacuoles.

On the other hand, pinocytosis or "cell drinking" involves the continuous internalization of small solutes and small droplets of extracellular fluid. It occurs through nearly all nucleated cells (fluid phase pinocytosis).

In adsorptive pinocytosis a solute binds to an external phase of the plasma membrane and is drawn into the cell interior forming a pinosome with a solute concentration higher than that in the ambient liquid.

Adsorptive pinocytosis can be categorized as substrate-specific (receptor-mediated) or non-specific.

In the former, the cell surface recognizes and internalizes a liquid of narrowly defined structural composition; whereas in the latter substrate specificity is much broader. Free drug enters the cell interior via transmembrane diffusive transport or non-specific adsorptive pinocytosis, while cellular uptake of a drug-carrier composite is mostly restricted to receptor mediated endocytosis. When transported by diffusion, the drug will reach the cytoplasm of the cell, whereas all pinocytic processes are lysosomotropic (Molema and Meijer 1994). Cellular uptake of many endogenous and exogenous ligands takes place via receptor mediated endocytosis. Recently Khalil et al. (2006) described various processes of cellular internalization.

Clathrin dependent endocytosis, an energy dependent process, is the best characterized phenomenon of cellular internalization (Takei and Haucke, 2001). Clathrin coated pits have been proposed as molecular 'filters' and are ~100 to 150 nm in size. This process is used for the uptake of specific ligands such as transferrin, low density lipoprotein (LDL) and alpha-2-globulin. Initial uptake of ligands into coated vesicles is followed by fusion with early, tubulo-vesicular endosomes near the plasma membrane (Hansen et al. 1991; Takei and Haucke 2001). The pathway appears to be independent of the cellular cytoskeleton, such as cytochalasin D and colchicines (Watts and Marsh 1992). There are also clathrin-independent mechanisms for endocytosis of which caveolae-mediated endocytosis has been widely investigated. Caveolae are small, coated invaginations of plasma membrane that are rich in cholesterol and glycosphingolipids (Harris et al. 2002). Caveolae are present in many cell types and primarily involved in transcytosis in blood vessel wall endothelial cells (Conner and Schmid 2003). Caveolae are smaller in size (50–80 nm) and differ in receptor disposition from clathrin-coated vesicles in that they do not separate from the plasma membrane while unloading their cargo (Panyam and Labhasetwar 2004). This pathway is advantageous for drug delivery because it avoids acidic compartments (endosomal/lysosomal pathways). However, some of these uncoated vesicles deliver their contents to endosomes and lysosomes (Hansen et al. 1993). The process has been termed *potocytosis*, being exemplified by folate, which is the best characterized cargo molecule undergoing potocytosis (Rijnboutt et al. 1996). Macropinocytosis is another type of actin-dependent endocytotic pathway in which irregular sized and shaped vesicles are formed (Panyam and Labhasetwar 2004). The macropinocytosis process is apparent, however, it can be stimulated by growth factors such as epithelial growth factor or other signals. Macropinosomes formed after stimulation have no coat and do not concentrate receptors and are generally 0.5–2.5 μm in diameter but can sometimes could be as large as 5 μm (Khalil et al. 2006). This is a non-selective phenomenon for the uptake of large volumes of fluid (Conner and Schmid 2003). Recently macropinocytosis has been demonstrated as an entry route for gene and drug delivery and it has been reported that TAT peptide uptake occurs by macropinocytosis (Nakase et al. 2004; Wadia et al. 2004; Kaplan et al. 2005). In macropinocytosis, the vesicles (macropinosomes) do not fuse with endosomes or lysosomes despite fusing with each other; however, sometimes macromolecules internalized in macropinosomes have been found to be delivered to lysosomes (Racoosin and Swanson 1992; Racoosin and Swanson 1993).

Following endocytosis, macromolecules are initially entrapped in endosomes, an acidic compartment (pH 5.5–6.5). Subsequently to this, entrapped molecules are trans-

Table 2: Various ligands and their respective receptors on various cell types

Ligand	Receptor	Cell type	References
Folate	Folate receptor	Epithelial carcinomas	Lee and Low, 1994; Gabizon et al., 1999; Ni et al., 2002; Zhang et al., 2004; Seow et al., 2007
Insulin	Insulin receptor	Brain	Gupta et al., 2003
Transferrin	Transferrin receptor	Brain	Vinogradov et al., 2004; Mishra et al., 2006
		Tumor cells	Sarti et al., 1996; Maruyama, et al., 2004; Yamada et al., 2005
RGD	Integrins	Vascular endothelial smooth muscles of tumor	Dubey et al., 2004; Schiffelers et al., 2004; Mitra et al., 2005; Suk, et al., 2006;
Galactose	Macrophage galactose lectin	Macrophages	Ishida et al., 2004
Mannose	Macrophage galactose lectin	Macrophages	Yeeprae et al., 2006
Anti-cell surface receptor antibody (or fragment thereof)	Cell surface receptor (transferrin)	Brain	Gosk et al., 2004
		T-cell Leukemia	Hege et al., 1989
VEGF	VEGF receptor	Endothelial cells	Brown et al., 1993
O-Palmitoylated mannan	Macrophage galactose lectin	Macrophages	Vyas et al., 2000
Pullulan	Macrophage galactose lectin	Macrophages	Xi et al., 1996
Endoglin (CD105)	Transforming growth factor-β (TGFβ)	Tumor neovasculature	Burrows et al., 1995
NGR	Aminopeptidase N	Vascular endothelial smooth muscles of tumor	Pasqualini et al., 2000; Curnis et al., 2000; Pastorino et al., 2003; Matteo et al., 2006
Sulfatides	ECM glycoprotein Tenascin-N(TN-C)	Tumor cells	Shao et al., 2006

ferred to lysosomes. This process does not involve passage through the membrane surrounding the vesicles but rather occurs by content mixing between the late endosome and lysosome as a result of 'kiss-and-run' events and/or direct fusion between two organelles. Lysosomes, similar to the endosome, are like the cells 'stomach'; they contain a greater number of hydrolytic enzymes and are acidic in nature. These are membrane bound vesicles and constitute a barrier to the transport of macromolecules and delivery system (Bulmus 2005).

4. Receptors and ligands as delivery portals and modules

4.1. Receptor as delivery portal

Cell surface receptors are complex trans-membrane proteins, which mediate highly specific interactions between cells and their extra-cellular milieu. Receptors however, are cellular markers and play an integral role in the regulation of cellular functions, including growth differentiation, metabolism, secretion, contraction and migration (Molema and Meijer 1994). The two most common functions of receptors are to mediate the trafficking of their specific ligands and to transduce and regulate transmembrane signaling. Since receptors are differentially expressed in various cell types and tissues, they provide a basis for targeted drug delivery (King and Feener 1998). Cell surface receptors have been proven to be excellent ports, which, may be effectively used in selective targeting of drugs, oligonucleotides or even genes by making use of their specific affinity ligands.

4.2. Ligands as targeting tools

Ligands are surface appended group(s), which can selectively direct the carrier to the pre-specified site(s) housing the appropriate receptor units, serving as a 'homing device' to the carrier/drug. The carrier systems interaction serves to assist presentation of ligands to their respective receptors localized on the cellular surface. The various ligands exploited for selective drug targeting include antibodies, polypeptides, oligosaccharides, viral proteins, endogenous hormones, fusogenic residues, etc. The ligands confer recognition and specificity upon the carrier/vector and endow them with the ability to approach the respective target selectively and deliver the drug. Carrier target recognition is a prerequisite for ligand mediated targeting and provides a basis for using cell specific (receptor specific) ligands, attached to the carrier surface as a means of promoting recognition and conferring specificity (Vyas et al. 2001).

Ligands are often covalently anchored or non-covalently associated with the surface of the carrier in such a way that the carrier tends to approach the accessible cells, those expressing surface receptor, with a ligand specific affinity. Various ligands have been reported so far in the pursuit of providing optimal targeting. Endogenous or exogenous ligands can be conjugated with either the drug or drug bearing delivery systems using various non-covalent and covalent techniques. Various ligands and their respective receptors on a variety of cells are summarized in Table 2.

4.3. Intracellular processing of receptor-ligand complex

Intracellular transport and processing after receptor mediated endocytosis and transcytosis vary markedly between different receptor-ligand systems and different cell types, and decide the fate of the drug-carrier composites for specific intracellular destinations. Subsequent to ligand-receptor dissociation, recycling of receptor to the plasma membrane and transportation of ligand to the lysosomal compartment is the most widely executed pathway. In this case, the ligands dissociate from their receptors in the acidic environment of the endosome and eventually end up in the lysosome, while the receptors are recycled via transport vesicles back to the cell surface for reuse (Brown et al. 1983).

Other receptor-ligand complexes follow pathways other than the endosomal compartment. Receptors can follow one of at least four pathways from the endosomal com-

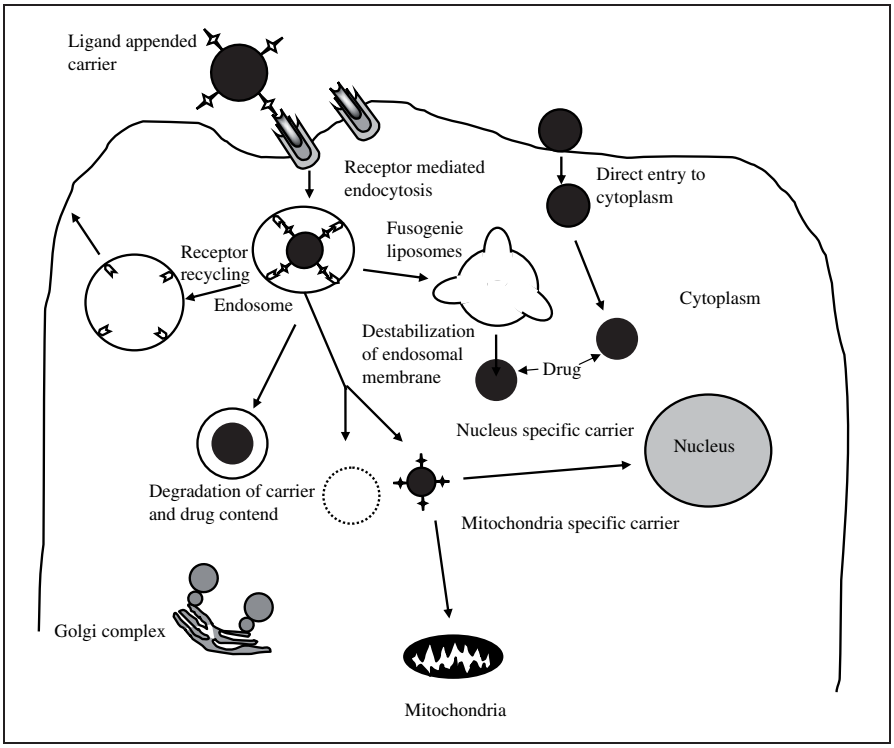


Fig. 2:
Schematic diagram showing various approaches to intracellular delivery

partment (Yamashiro and Maxfield 1984; Wileman et al. 1985) (Fig. 2).

1. Receptors can return to the same plasma membrane domain leaving the ligand to be transported for lysosomal degradation.
2. Receptors can travel to lysosomes and, with ligand bound to them; share the fate of the ligand (lysosomal disposition).
3. Receptors can be recycled along with the ligand back to the site from where the receptor originated.
4. Receptors can return to a different domain of the plasma membrane (transcytosis).

5. The biological membrane: a potential barrier to targeted drugs

When a carrier system reaches the vicinity of the target cells, it needs to penetrate into the cytosol for intracellular targeting. Plasma membrane is the major obstacle for large and charged molecules so it needs to be overcome. Biological membrane is also responsible for the compartmentalization of cellular organelles and acts as a natural barrier for most molecules (Langer 2000; Bulmus 2005). Therefore, the transportation through this is a fundamental requirement for drug therapy and delivery. There are various factors which are responsible for determining the rate of diffusion through membrane the most important being the size and hydrophobicity of the molecules (Belting et al. 2005).

1. Small, nonpolar molecules, such as CO_2, O_2, NO_2 and benzene diffuse rapidly because these dissolve in the lipid bilayer.
2. Low molecular weight, noncharged polar molecules such as H_2O and ethanol, diffuse slowly across the lipid bilayer.
3. Membrane permeability coefficient decreases dramatically with size, e.g. glucose has a membrane permeability coefficient less than one millionth that of H_2O whereas its size is only 10 times that of H_2O.
4. The entry of ions from the lipid phase of the bilayers is prevented by the charge and hydration, and small ions such as Na^+ or K^+ have only one billionth of the capacity of H_2O to cross the lipid bilayer.

Thus, the biological membrane prevents the entry of hydrophilic molecules. Eukaryotic cells use a number of different endocytic mechanisms to transport macromolecules and carriers over the plasma membrane barrier as described earlier, whereas small apolar molecules are transported through passive diffusion. Like the plasma membrane, the lysosomal membrane is also a natural barrier to macromolecular ligands and/or ligand appended carrier composites and only low molecular weight products released as a consequence of lysosomal degradation are transported to the cytoplasm. As plasma membrane uses endocytosis for transportation of molecules, the lysosomal membrane uses substrate-specific porters for the efflux of end-products of lysosomal metabolism (Lloyd 2000).

6. Design of a Cellular Targeted Drug Carrier

A cellular targeted drug carrier should deliver the drug not just to the target cells but also to the particular cellular compartments or microenvironments where it is most required. Degradation of ligand coupled drug-carrier complexes in lysosomes along with the encapsulant, as well as the inability of the encapsulant to cross the lysosomal lipidic membrane, which constitute a 'lysosomotropic' approach to drug targeting, are the major barriers to achieving effective cellular delivery. These barriers must be overcome before the targeted delivery of cellular biomolecular therapeutics to specific cells and tissues becomes a widespread clinical reality. An ideal drug carrier for cellular targeting should: (a) bypass the anatomical barriers (b) be recognized only by the target cells (c) release the drug at or inside the target cells and not elsewhere (Fig. 3).

Targeting to the appropriate cells is not enough for therapy involving drugs acting intracellularly. The initial extracellular recognition step is usually followed by internalization

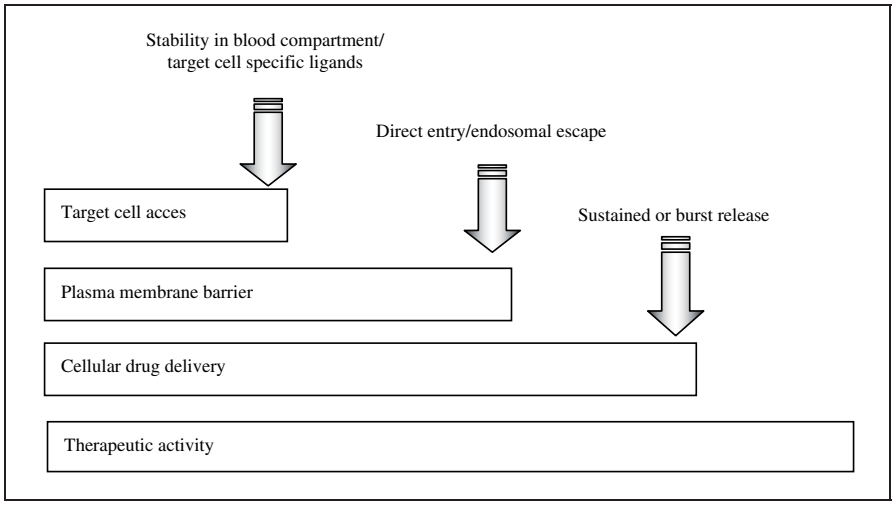


Fig. 3:
Biological barriers that must be crossed for a cellular targeted system to be therapeutically useful

via receptor-mediated endocytosis. The efficacy of several important proteins and DNA therapeutics is subsequently limited by nonproductive intracellular trafficking. For example, the release of immunotoxins and gene therapeutics from endosomes can represent the dose-limiting step in getting the drug to the site of action.

The current challenge is to manipulate or circumvent the dominant non-productive trafficking pathways, such as routing of drug(s), proteins and DNA to the lysosomes where they are degraded. The formulation attributes for the successful design of cellular targeted carrier constructs are as follows:

(a) Cell specific specialized ligand/recognition moieties.
(b) Cellular compartment specific module/strategy to achieve further specificity e.g. cytosol, cell organelles.
(c) Should not cause toxic or immune reactions, and
(d) The drug-carrier complex should be capable of being manufactured under sterile and apyrogenic conditions (Fig. 3).

There are various strategies to deliver the drug through carrier complexes directly to cytoplasm scavenging the late endosomal and lysosomal compartments. These strategies can be subdivided into two broad classes: (a) Direct entry to cytosol and (b) Entry by escaping the endosomal compartment.

6.1. Direct entry to cytosol

Various macromolecular drugs such as proteins and peptides, DNA and a number of drugs which are unstable at endosomal/lysosomal pH, are required to bypass the endocytic pathway of internalization for efficient activity in the cytosol or other organelles (Gupta et al. 2005). The lipidic nature of biological membranes (plasma membranes) restricts the entry of such drugs to the cell (Varga et al. 2000).

A novel approach for transferring molecules directly to the cytosol bypassing the endocytic pathway makes use of various peptides called cell penetrating peptides (CPPs) (Deshayes et al. 2005). The most actively studied peptides are derived from HIV-TAT (Green and Lowenstein 1988; Frankel and Pabo 1988), HSV-VP22 (Elliott and O'Hare 1997) and antennapedia (Antp) (Joliot et al. 1991). These peptides are structurally similar in that they all contain a short sequence of less than 20 amino acids with a positively charged arginine and lysine residues. This sequence is called the "protein transduction domain" (PTD) and is significant for contact with the cell membrane (Panyam and Labhasetwar 2004; Torchilin 2006).

The mechanism of internalization of PTD is not well understood, but it excludes the classical endocytic pathway and occurs efficiently at both 37 °C and 4 °C (Vives et al. 1997; Pooga et al. 1998). Wadia et al. (2004) showed Tat-mediated intracellular delivery of protein and nanoparticles via energy-dependent macropinocytosis followed by escape from the endosome into the cytosol. This has been used for the delivery of various large and small molecule drugs (Rinne et al. 2007). The use of that CPPs for the efficient intracellular delivery of various carrier systems like nanoparticles (Lewin et al. 2000; Liu et al. 2001; Dodd et al. 2001), liposomes (Torchilin et al. 2001; Levchenko et al. 2003) and Quantum Dots (Xue et al. 2007) has also been studied. Recently it has been reported that plain and PEGylated liposomes of 200 nm size could be delivered to various cells by attaching multiple TAT molecules to the surface of the liposomes (TATp-liposome) via a p-nitrophenylcarbonyl PEG-phosphatidylethanolamine spacer group (Torchilin et al. 2001; Levchenko et al. 2003) and they have been found to be intact in the cytosol for 1 h after translocation (Torchilin et al. 2003).

However, there are some limitations to using PTDs as carriers since they require crosslinking. Some of them, such as those derived from HIV-1 TAT proteins requires denaturation of the protein before delivery to increase the accessibility of the PTD domains. Recently a short synthetic amphipathic carrier Pep-1 was developed which delivers protein and peptide intracellularly without the need for crosslinking or denaturation (Panyam and Labhasetwar, 2004). The Pep-1 peptide carrier has been shown to be extremely efficient in the targeting of proteins into cells independently of endocytosis (Rozenzhak et al. 2005).

6.2. Entry by escaping endosomal compartment

6.2.1. Fusogenic peptides or proteins

Following uptake of a bioactive loaded carrier system by receptor-mediated endocytosis, it reaches the endosome. The next step, of releasing the bioactive substance from the endosome before degradation, is the rate limiting step. When a virus reaches the endosome by cell adsorption it releases its genome to the cytosol by activation of capsid protein at the acidic pH of the endosome. This active protein fuses with the endosomal membrane resulting in membrane rupture or pore formation. A number of viral

fusion peptides have been identified, e.g. C503-I609/ ADM-1 proteins of *C. elegans* (Podbilewicz 1996), G1-G29/HA-2 influenza virus (Lear and Degrado 1987), D149-D166/E2 glycoprotein of rubella virus (Blobel et al. 1992), M1-Q16/Sprotein of Hepatitis B virus (Rodriguez-Crespo et al. 1995), G524-E540/ Ebola virus (Ruiz-Arguello et al. 1998), INF7/influenza virus (Funtoff et al. 2004).

Commonly, the active fusion peptides of viral proteins are located at the N-terminus of the protein and contain alternating clusters of hydrophilic and hydrophobic residues, which form an α-helical structure. The majority of fusogenic peptides described show pH-dependent fusogenic and endosomolytic activity and are believed to mimic virus like entry into the cell. These peptides are random coil at pH 7.0 but undergo a conformational change into an amphipathic α-helix at pH 5.0. This conformational change induces the fusion and lysis of endosomal membrane (Lear and Degrado 1987; Plank et al. 1994, 1998).

Various hemagglutinin (HA)-derived peptide and synthetic analogs have been studied for their pH-dependent membrane disruptive property and the transfection efficiency has been found to increase in vitro (van Rossenberg et al. 2002). Synthetic membrane-active peptides offer an opportunity to increase the intracellular delivery of drugs. Thus, peptides can be synthesized which may specifically destabilize endosomal membrane specifically without altering cell membrane integrity, which may cause cytotoxicity. The most commonly used synthetic peptides are INF (Wagner et al. 1992), GALA (Parente et al. 1990), JTS1 (Gottschalk et al. 1996), H_5WYG (Midoux et al. 1998), and Melittin (Benachir and Lafleur 1995) Table 3.

Recently multifunctional peptides have also been synthesized, such as KALA (Wyman et al. 1997) and ppTG20 (Rittner et al. 2002), which bind DNA and also possess endosomolytic activity. In these peptides positively charged lysine or arginine stretch bind DNA and amphipathic membrane-destabilizing domain derived from fusogenic peptide GALA and JTS-1 shows endosomolytic activity (Kichler et al. 2003).

Previous studies signify that by coupling a homing device to a carrier a higher cellular uptake of carrier loaded drug can be achieved. The same strategy was applied by van Rossenberg et al. (2002), by attaching a homing device $K(GalNAc)_2$, for the asialoglycoprotein receptor (ASGPr) on parenchymal liver cells, to fusogenic peptide INF7. Results of the studies showed that the glycoconjugated peptide, INF7-$K(GalNAc)_2$ possesses high lytic activity in cholesterol poor liposome in vitro and accumulates in the liver after in vivo administration, thus indicating that these targeted peptides might be suitable for targeted delivery of drug to the parenchymal cells of the liver (van Rossenberg et al. 2002).

An extension of this approach has been used to form virosomes in which the viral envelope glycoprotein, hemagglutinin was embedded in a phospholipid cholesterol bilayer (Bron et al. 1993, 1994). Sendai virus may be directly fused with preformed liposomes to yield virosomes with improved *in vitro* intracellular delivery. Sendai virus F protein is known to effect intracellular delivery through two independent mechanisms: (i) Galactosylated F protein is a ligand for the cell surface asialoglycoprotein receptor, and (ii) F protein also behaves as a membrane fusogen. The fusogenic activity of F protein containing liposomes was found to be abolished by brief heat treatment without affecting the galactose mediated endocytic pathway (Bagai and Sarkar 1993).

6.2.2. Polymeric delivery system

6.2.2.1. Cationic polymers

Recently, another method for intracellular delivery of DNA based on cationic polymer has been studied. Polymers, bearing groups that are protonated at physiological pH, have been used as gene carriers, forming complexes with DNA called polyplexes by the cationic charge of the polymer and the negative charge of DNA. These complexes protect DNA from enzymatic degradation. Due to the cationic charge of the polyplex it adheres to the cell membrane and is subsequently taken up by the cells via endocytosis (Wiethoff and Middaugh 2003).

Commonly used cationic polymers include poly-L-lysine (PLL), polyethyleneimine (PEI), chitosan, poly (2-dimethylaminoethyl)-methoxy (pDMAEMA), polybrene, tetraminofullerene, cationic polysaccharides and cationic dendrimers (Azzam et al. 2004). Poly-L-lysine (Merdan et al. 2002) and chitosan (Koping-Hoggard et al. 2001, Singla and Chawla 2001) are biodegradable polymers that have been used as a DNA delivery system. However, these have lower transfection efficiency than PEIs due to a lack of rapid release of the complex from the endosome (Patil et al. 2005). When this complex reaches the endosome, degradation of the polyplex takes place and no functional activity is observed. This is the reason for the low transfection of the polyplexes. Such types of polymers require the co-delivery of some endosomolytic agent like fugogenic peptide (Plank et al. 1994), inactivated adenovirus (Wagner et al. 1992) or lysosomolytic drugs such as chloroquine (Mislick et al. 1995). The endosomolytics promote the release of drug or DNA to the cytosol.

In contrast PEI, a branched cationic polymer, has efficient gene transfer capability. This high efficiency is due to the buffering effect or "proton sponge effect" of the polymer by the presence of amino groups in the molecules (Patil et al. 2005). When PEI-based polyplexes reach the endosome, protonation of amine groups occurs in the acidic environment of the vesicles. By protonation, polymer swellings takes place, and at the same time the endosome also swells by osmotic imbalance. The combined swelling ruptures the endosomal membrane and releases the content into the cytosol (Cho et al. 2003). This proton sponge hy-

Table 3: List of fusogenic peptides and their sequences

Peptide	Amino acid sequence	References
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	Parente et al., 1990
INF	GLFEAIAGFIENGWEGMIDGGGC	Wagner et al., 1992; Plank et al., 1994
JST1	GLFEALLELLESLWLLEA	Gottschalk et al., 1996
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	Wyman et al., 1997
Model amphipathic peptides (MAP)	KLALKLALKALKAALKLA	Oehlke et al., 1998
Hel-peptide	KLLKLLLKLWLKLLKLLL	Niidome et al., 1997

pothesis is valid not only for PEI but also for other polymers containing amine groups with pKa at or below physiological pH, i.e. pDMAEMA (Van de Wetering et al. 1999), polyamidoamine (PAMAM) dendrimers (Haensler and Szoka 1993), histidylated polylysine (Midoux et al. 1999) and lipopolyamine (Ahmed et al. 2005).
Ideally these vehicles should be targeted specifically to a cell however, these complexes attach to the cell surface and are internalized by non-specific endocytosis or by destabilizing the plasma membrane. For this reason various cell selective polyplexes have been studied for internalization by receptor-mediated endocytosis. Using this technique, PLL-DNA complexes have been studied with a variety of receptors including asialoorosomucoid (Wu and Wu 1988), transferrin (Zenke et al. 1990) and folate (Mislick et al. 1995). The same techniques have also been applied to other cationic polymers such as PEI (Zanta et al. 1997; Guo and Lee 1999), pDMAEMA (van Steenis et al. 2003) and dendrimers (Choi et al. 2005; Majoros et al. 2006). Dendrimers, a new class of polymer, of well-defined structure with a low polydispersity index and with terminal amine groups, have also been studied for the delivery of drugs and genes to the cellular environment (Haensler and Szoka 1993; Delong et al. 1997). PAMAM dendrimers are the most widely studied dendrimer. Quintana et al. (2002) have designed folate receptor targeted PAMAM dendrimers for the delivery of imaging agents, drugs and genes to tumor cells. The results indicate that a better effect might be obtained by cell specific delivery systems. A further increased effect may be obtained by linking fusion peptides to the polymer backbone, which allows lysosomal escape to the carrier (Wagner et al. 1992).

6.2.2.2. pH sensitive polymers

pH-Sensitive polymers have also been investigated for enhancement of intracellular delivery of therapeutics (Lim et al. 2002; Murthy et al. 2003a, 2003b; Griffiths et al. 2004; Kim et al. 2004,). They can mimic the pH-responsive behavior of fusogenic peptides for endosomal membrane destabilization activity. Zareie et al. (2000) studied the pH-responsive conformational changes of copolymers of acrylic and methacrylic acid with various monomers and used atomic force microscopy (AFM) to investigate the pH- and temperature dependent conformational changes of a copolymer of acrylic acid (AA) and N-isopropyl acrylamide (NIPAAm), and found that decreasing pH from 7.4 to 4.5 at 37 °C brings about a transition from extended chain to globular form. The pH-responsive behavior of a copolymer of methacrylic and with *N*-isopropyl acrylamide and octadecyl acrylate was also studied, and triggered release of fluorescent dye (Meyer et al. 1998; Zignani et al. 2000) and doxorubicin (Leroux et al. 2001) from large unilamellar vesicles composed of egg yolk phosphatidylcholine (EPC) and cholesterol was observed at a pH range between 4.9 and 5.5. pH-dependent conformational change of the polymer might account for such triggered release behaviour. Poly(ethylacrylic acid) (PEAA) also undergoes pH-dependent conformational changes on acidification of an aqueous polymer solution (Eum et al. 1989) and more profound membrane-destabilization activity could be achieved by replacing the ethyl side chain with propyl or butyl groups. Murthy et al. (1999) studied the destabilizing activity of these polymers using red blood cells (RBCs) as model cellular membranes and found that PEAA disrupts RBCs at pH 5.5 and that its hemolytic activity increases as pH decreases from 6.5 to 5.0. On the other hand, disruption of membrane by poly(propylacrylic acid) (PPAA) was 15 times higher than that of PEAA at pH 6.1 but neither showed hemolytic activity at pH 7.4. To assess the potential of PPAA as an intracellular drug delivery vehicle, in vitro experiments were performed in which PPAA was conjugated with a protein (streptavidin). The results showed that this linkage did not affect the pH-dependent behavior of the polymer (Lackey et al. 1999). Moreover, it was also found that the intracellular localization of lysosome tracker dye and PPAA-loaded formulations was not the same, indicating that the PPAA-loaded formulation is not handled through a lysosomal pathway (Kiang et al. 2004).
In a further developments, pyridyl disulfide acrylate (PDSA), a glutathione reactive component, was incorporated in methacrylic acid (MAA) and butyl acrylate (BA) copolymers and it was found that this polymer enhanced the cytoplasmic delivery of fluorescent-labelled oligonucleotides (Bulmus et al. 2003).
Recently, Murthy and associates synthesized a new class of polymer, which they have called encrypted polymer (Murthy et al. 2003a, 2003b). These polymers are terpolymers consisting of a membrane disrupting backbone grafted with hydrophilic PEG through acid degradable linkers (acetal linkages). These polymers are stable at pH 7.4 but hydrolyze at endosomal acidic pH and activate the membrane disruptive backbone (Bulmus 2005). A cell-targeting moiety can also be attached to the encrypted polymer to target a specific cell and direct receptor-mediated endocytosis. It has been found that encrypted polymer can be used for intracellular delivery of oligonucleotide to macrophage cells. In this study lactose was used to target macrophages and rhodamine-labeled oligonucleotide or PEG-FITC was used as a model drug (Murthy et al. 2003a). The encrypted polymeric carriers significantly enhance the delivery of oligonucleotides and peptides to the cytoplasm of cultured macrophages, demonstrating the potential of this approach for delivery of biotherapeutics and vaccines.

6.2.2.3. Polymeric nanoparticles

Polymeric nanoparticles are among the potential carrier systems used for intracellular delivery of cytotoxic agents, proteins, peptides and oligonucleotides (Gutowska et al. 1992; Couvreur and Puisieux 1993; Kim et al., 1994; Kreuter 2001; Park et al. 2006). Drugs interact with the polymeric nanoparticles in one of three ways: First, DNA is complexed with polymer (polyplexes). Second, drugs or DNA are encapsulated in the polymeric matrix. Third, DNA is complexed to the surface of polymeric nanoparticles (Vasir and Labhasetwar 2006) Table 4.
The ideal characteristics of polymeric nanoparticles that are required to improve encapsulation for efficient intracellular delivery of DNA are as follows (Kaul and Amiji, 2002, Vasir and Labhasetwar 2006):

1. Biocompatibility and biodegradability of the polymer
2. Protection of encapsulated drugs from degradation after *in vivo* administration
3. Size permitting access to cell.
4. Ability to target specific cells and avoid uptake by MPS after systemic administration. For this, cell specific ligands and PEG can be attached to the surface of the carrier.
5. Carrier must deliver the drugs or gene in an active form in the cytosol.

Cellular uptake of the polymeric nanoparticles takes place by fluid phase pinocytosis, adsorptive endocytosis and re-

Table 4: Polymeric nanoparticles used in intracellular delivery

Nanoparticles (types)	Polymer used	References
DNA condensed with polymer	Polyethylenimine	Boussif et al., 1995; Kichler et al., 2001; Sweeney et al., 2003
	Poly-L-lysine	Degols et al., 1989; Maruyama et al., 1997
	Cationic dendrimers	Haensler and Szoka, 1993; Delong, et al., 1997
	Chitosan	Koping-Hoggard et al., 2001; Singla and Chawla, 2001
Drugs or DNA encapsulated in the polymeric matrix	Polylactic acid	Emile, et al., 1996
	Poly(lactide-co-glycolide)	Hirouse et al., 2000; Cohen et al., 2000; Panyam and Labhasetwar, 2003; Panyam et al., 2003; Csaba et al., 2005
	Gelatin	Truong et al., 1998; Kaul and Amiji, 2002
DNA complexed with surface of the nanoparticles	Polyalkylcyanoacrylate	Bertling et al., 1991
	Polybutylcyanoacrylate	Chavany et al., 1992; Nakada, 1996; Weyermann, et al., 2004
	Polyhexylcyanoacrylate	Zobel et al., 1997
	Polyisohexylcyanoacrylate	Chawany et al., 1992; Chavany et al., 1994; Fattal et al., 1998

ceptor mediated endocytosis. Uptake depends on the physicochemical properties of the particles i.e. surface charge, size and presence of a specific ligand for the cell surface receptor (Maruyama et al. 1997). Positively charged nanoparticles escape rapidly from the endosome as compared with their neutral or negatively charged counterparts (Blau et al. 2000).

Panyam et al. (2002) reported that poly(lactide-co-glycolide) (PLGA) nanoparticles escape from the endo-lysosome compartment in less than 10 min. and this is attributed to surface charge reversal of the nanoparticles (from anionic to cationic) in the acidic environment of endosomes. As discussed earlier, polymeric nanoparticles should have a long circulation time to avoid MPS. Kaul and Amiji (2002) developed long circulation life gelatin nanoparticles in which gelatin was first PEGylated, and then nanoparticles were formed, and it was inferred that the presence of the PEG chain decreases the release of TMR-dextran (a model hydrophilic macromolecular drug) in the presence of proteolytic enzymes.

7. Liposome based delivery systems for cellular drug delivery

Liposomes are currently the most extensively studied versatile carrier for the intracellular delivery of drugs, antigens and DNA (Felnerova et al. 2004). Liposomes are vesicular systems consisting of a hydrophilic core surrounded by a lipid bilayer. Both types of drug (hydrophilic and hydrophobic) can be entrapped in this system (Gregoriadis 1978). Liposomes have enormous advantages over a viral delivery system for intracellular delivery of DNA (Patil et al. 2005). For example, (i) they are non-immunogenic due to lack of proteinaceous components (ii) liposomes can be tailored to yield the desired size, surface charge, composition and morphology (iii) they protect DNA from nucleases and improve their biological stability. To improve the intracellular delivery of drugs and DNA, various types of liposomes have been developed by varying the lipidic composition, e.g. cationic, anionic, pH-sensitive, fusogenic and combinations of these (cationic-fusogenic, pH-sensitive fusogenic).

7.1. pH Sensitive liposomes

In order to overcome endosomal and lysosomal membrane barriers, pH-sensitive liposomes have been developed that are stable at physiological pH but are destabilized on acidification following cellular internalization, thereby promoting the release of their contents into the cytosol (Chu et al. 1990; Torchilin et al. 1993). Several lipidic variations of pH sensitive liposomes have been shown to improve the cytoplasmic delivery of membrane-impermeable therapeutic agents. The most commonly used lipid in pH-sensitive liposomes is dioleoylphosphatidylethanolamine (DOPE) which determines the membrane stability at neutral pH. At acidic pH, protonation of the amine group induces a lamellar to hexagonal phase transition (non-lamellar structure), and this may constitute a key element in triggering endosomal destabilization (Litzinger and Huang 1992; Venugopalan et al. 2002). It is reported that most commonly used PE based pH-sensitive liposomes are stabilized by the addition of lipid constituents or co-surfactant containing a carboxylic acid group. Various co-surfactants used as stabilizes are palmitoyl homocysteine, fatty acids, N-succinyl PE and cholesteryl hemisuccinate (CHEMS) (Venugopalan et al. 2002). CHEMS is the most commonly used stabilizer, becoming protonated at acidic pH and losing its negative charge and therefore its stabilizing activity, which results in the destabilization and/or fusion of the liposomes (Cullis and De Kruijff 1979).

Kinetic study of pH-sensitive liposomes has shown that liposomes release their contents into the cytoplasm within 5 to 15 min, which indicates that cytoplasmic delivery occurs from early and late endosomes (Collins et al. 1989, 1992). The molecular mechanisms by which liposomes release their contents to the cytoplasm by escaping cytoplasmic and endosomal membrane remains to be clarified. In general, three hypothetical mechanisms have been proposed:

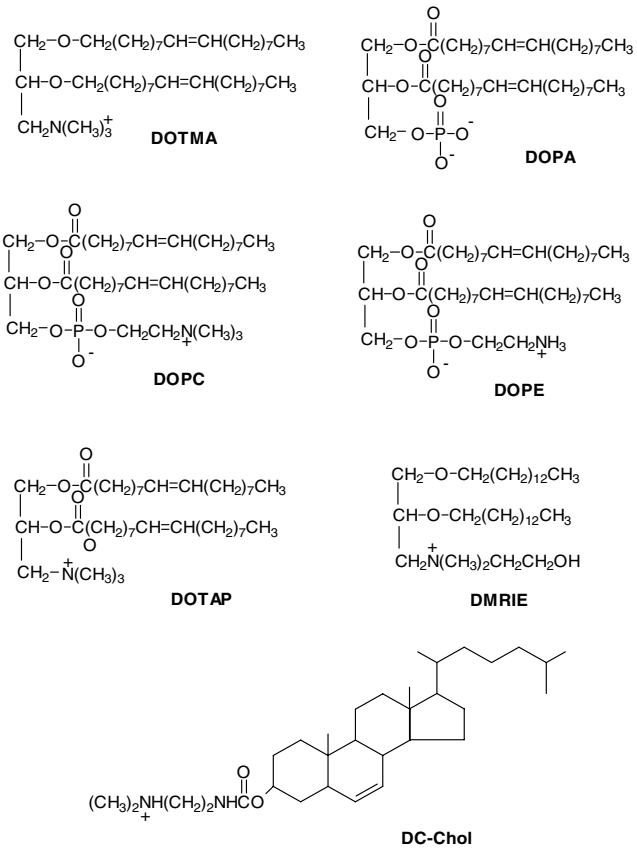
(a) Destabilization of pH-sensitive liposomes, which triggers the destabilization of the endosomal membrane resulting in pore formation, which leads to delivery of their content to the cytoplasm
(b) On liposome destabilization, the encapsulated molecules diffuse through the endosomal membrane to the cytoplasm; and
(c) Fusion between the liposome and the endosomal membranes, leading to delivery of the contents to the cytoplasm.

Of these, (a) and (c) are the most plausible because under certain conditions the fusogenic property of PE is associated with its tendency to form an inverted hexagonal phase (Simoes et al. 2004).

To improve the therapeutic efficacy of a drug it should be targeted to the specific tissue and cell. For this different ligands have been attached to the surface of pH-sensitive liposomes. These include monoclonal antibodies against the H-2Kk receptor (expressed in several types of tumor cells) (Wang and Huang 1987), E-selectin (on activated vascular endothelial cells) (Spragg et al. 1997), CD-19 (on B-lymphoma cells) (Ishida et al. 1997), CD3 (on T-leukemia cells) (Turner et al. 2002), and BCG antigen (Mizoue

et al. 2002). Folate coupled pH-sensitive liposomes have been used to deliver neoplastic drugs (Shi et al. 2002; Sudimack et al. 2002) and plasmid DNA (Reddy and Low 2000). For delivery of neoplastic drugs folic acid has been attached to the distal end of PEG, and for nucleic acid delivery, plasmid DNA has been precondensed with a cationic polymer then complexed with pH-sensitive liposomes (Shi et al. 2002). Fonseca et al. (2005) developed novel human T-leukaemia cells targeted pH-sensitive liposomes by coupling transferrin to the distal end of PEG-phospholipid incorporated liposomes and found that transferrin coupled liposome associated more extensively than non-targeted liposome to human T-leukaemia cells *in vitro*.

Although the development of pH-sensitive liposomes has frequently involved the incorporation of DOPE in the liposome formulation, other alternative strategies to generate liposomes that are fusogenic under acidic conditions have also been explored. Shi et al. (2002) formulated liposomes composed of EPC, DDAB, CHEMS and Tween-80 and found that these liposomes entrap calcein at pH 7.4 but undergo destabilization at acidic pH. They also found that these liposomes showed improved retention of pH-sensitivity in the presence of serum compared with pH-sensitive liposomes incorporating DOPE. Sudimack et al. (2002) also developed pH-sensitive liposomes composed of PC, CHEMS, olelyl alcohol (OAlc) and Tween-80 and evaluated them for cytoplasmic delivery of cytosine-beta-D-arabinofuranoside (araC) in KB human oral cancer cells (folate receptor overexpressing cells). Results revealed that the folate derivatized OAlc-based pH-sensitive liposome showed 17-times greater cytotoxicity than that of folate coupled non-pH-sensitive liposomes. These results clearly indicate the potential of such liposomes for the intracellular delivery of therapeutics.



Commonly used cationic and neutral lipids

7.2. Cationic liposomes

Cationic liposomes, the most promising carrier system for gene therapy, have been used for the delivery of various plasmids, oligonucleotides, DNA and RNA to a variety of cells (Nakanishi and Noguchi 2001; Reddy et al. 2002; Kamiya et al. 2002; Chiu et al. 2006). Cationic liposomes were first reported by Felgner et al. in 1987 for the efficient transfection of eukaryotic cells. These liposomes were composed of cationic lipid [*N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*,-trimethylammoniumchloride, DOTMA] and zwitterionic lipid (DOPE) in 1 : 1 ratio. Cationic lipids in commonly used cationic liposomes are DOTAP, DOTMA, DC-CHOL, DDAB etc., and structures of some of these are shown. Commonly used zwitterionic lipids are DOPE and cholesterol. DOPE shows fusion activity with endosomal/lysosomal membranes and help in the endosomal escape of the liposomal contents. It also reduces the cytotoxicity of cationic lipids

Table 5: Combinations of lipids used in cationic liposomes for intracellular delivery

Combination of lipids	References
SA/cholesterol/PC or DOPC or DOTMA/Cholesterol/DOPC	Senior et al., 1999
DOTMA/DOPE	Felgner et al., 1987
DOTAP/DOPE	Gustafsson et al., 1995; Zelphati and Szoka, 1996; Xu and Szoka, 1996
DODAB/DOPE	Jaaskelainen et al., 1994; Gustafsson et al., 1995
DOGS/DOPE	Behr et al., 1989; Zelphati and Szoka, 1996; Xu and Szoka, 1996
DC-CHOL/DOPE	Gao and Huang, 1995; Wrobel and Collins, 1995; Nakanishi and Noguchi, 2001; Hu et al., 2004
LPLL/DOPE	Zhou and Huang, 1994
DMTAP/DOPE	Zelphati and Szoka, 1996; Xu and Szoka, 1996
CTAC/DOPE	Gustafsson et al., 1995
DC-6-14/Cholesterol/DOPE	Almofti et al., 2003
CTAB/DMPE	Chakarborthy et al., 1999
CDAN/DOPE	Spagnou et al., 2004
DC-CHOL/Egg/PEG-DSPE	Chiu et al., 2006

Abbreviations: DOTMA = {N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammoniumchloride; DOTAP = 1,2-dioleoyl-3-trimethylammoniumpropane; CTAC = cetyltrimethylammoniumchloride; DODAB = dioctadecyldimethylammoniumbromide; DDAB = dimethyldioctadecylammonium bromide; DC-6-14 = o,o′-ditetradecanoyl-N-(α-trimethy ammonium acetyl) diethanolamine chloride; CDAN = N′-cholesteryloxycarbonyl-3,7-diazononane-1,9-diamine; DMRIE = 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide; DOGS = dioctadecylamidoglycylspermine; DMTAP = 1,2-dimyristoyl-3-trimethylammonium-propane; CTAB = cetyltrimethylammoniumbromide; DOPC = dioleoylphasphatidylcholine; LPLL = lipopoly(L-lysine); SA = stearylamine

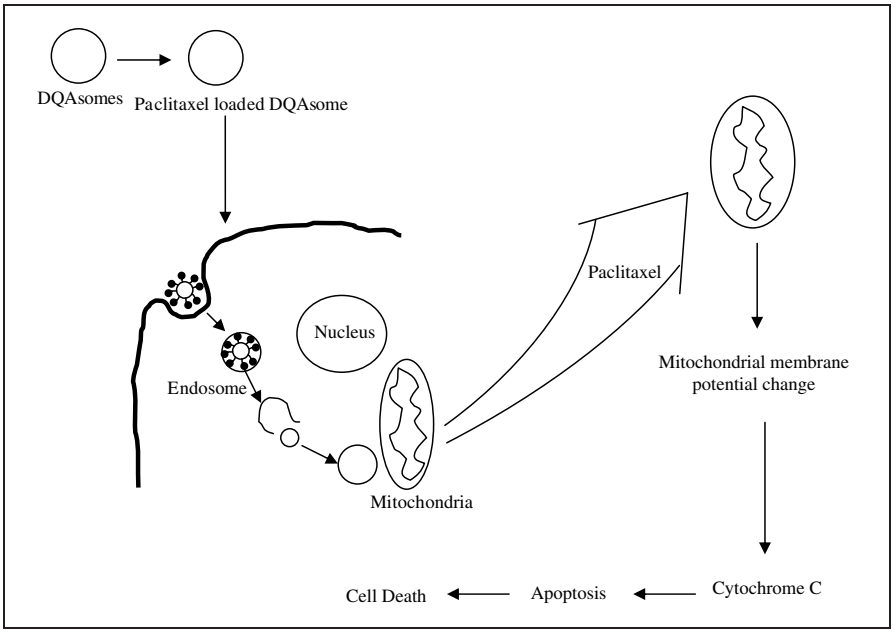


Fig. 4:
Schematic diagram of the hypothetical mechanism of action of paclitaxel loaded DQasomes

(Gustafsson et al. 1995; Lechardeur et al. 2005). Various combinations of lipids that have been studied by researchers are listed in Table 5.

Cationic lipids impart a positive charge to the liposomes that helps in the complexation and condensation of DNA and also in cell interaction due to the negative charge of the cell surface (Ahmed et al., 2005). Previously there were three models for the interaction of a cationic liposome with the cell and the release of DNA and oligonucleotide into the cytosol (Wrobel and Collins, 1995), (i) liposome-cell fusion within or destabilization of the endosome (ii) direct fusion with plasma membrane (iii) transfer of the lipid-DNA complex across the cellular membrane into the cytosol. Later Xu and Szoka, (1996) gave a hypothetical model to explain the mechanism of release of cationic lipid/DNA complexes from endosomes. In this model, the cationic lipid/DNA complex after internalization first destabilizes the endosome membrane. After destabilization, the negatively charged lipids in the cytosolic phase move to the endosomal phase via a flip-flop mechanism. The anionic lipids then diffuse via lateral diffusion to form neutral ion pairs with cationic lipids. As a result, DNA, which was bound to the cationic lipids electrostatically, is displaced and released into the cytosol. Later Zelphati and Szoka (1996) proposed the same flip-flop model for the release of oligonucleotide from cationic liposomes.

It is desirable that DNA carrier systems deliver genes to the cell specifically. This necessitates tagging the surface of the cationic liposome with a targeting moiety. Zhou and Huang (1994) synthesized a cationic lipid lipopolylysine (LPLL), which contains multiple primary amino groups for the convenient conjugation of targeting ligands. They observed 3-fold higher transfection than with lipofectin in mouse L929 cells. They also showed that DNA-liposome complexes are taken up by endocytosis; however, cytoplasmic delivery of DNA involves an endocytic fusion phenomenon. Ahmed et al. (2005) synthesized a new lipopolyamine, N^4,N^9-dioleolyl spermine, and found that this condensed calf thymus DNA and circular plasmid DNA efficiently, and transfects skin cells and cancer cell lines at a low charge ratio (+/−) of 2.5. Since cationic liposome/DNA or ONDs complexes are thought to enter cells primarily via endocytosis, it has been hypothesized that the use of peptides that can destabilize endosomes or facilitate the fusion of the liposome/DNA or ODNs complexes with the endosomal membrane would enhance gene delivery (Kamata et al. 1994; Hu et al. 2004).

Various ligand appended cationic liposomes have been formed and studied for their cell selective property and it was found that they show high transfection efficiency compared to their plain counterparts (Reddy et al. 2002). Recently Chiu et al. (2006) evaluated the transfection efficiency of folate targeted cationic liposome composed of DC-Chol, egg PC and folate-PEG-DSPE using G3139, a phosphorothioate antisense ODN against human bcl2 mRNA, and found that the folate-conjugated liposome showed promising transfection activity in KB cells (folate receptor overexpressing cells) that was up to 6-fold more efficient than that of the non-targeted liposome.

Cationic liposomes have good transfection efficiency; however, they show cytotoxicity *in vitro* and *in vivo*. Lappalainen et al. (1994) studied the cytotoxicity of a cationic liposome composed of DDAD-DOPE and the commercially available transfection reagent DOTAP and found that both are cytotoxic to CaSki cells, a human cervical cancer cell line. In another study it was found that toxicity was reduced by replacing DOPE with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). After intravenous injection cationic liposomes may accumulate in the phagocytic cells of RES avoiding delivery of their contents to all targets in vivo, but local delivery to the skin, mucosa, lungs, tumors etc. can be envisaged as possible targets (Lappalainen et al. 1997).

7.3. Liposome and liposome like vesicular systems for mitochondrial targeting

Mitochondrial research is nowadays the most exciting area of research in the field of biomedicine (Weissig et al. 2004, 2006; Paliwal et al. 2007). Studies have shown that mitochondria play a major role in apoptosis and it has been found that various apoptosis inducers act by interacting with the mitochondrial permeability transition pore complex on the mitochondrial membrane (Li et al. 2002; Dias and Bailly 2005). To induce apoptosis

in the cancer cell, drugs should be targeted to the mitochondrial membrane. This demands systems which have an affinity for mitochondria. In 1998, Weissig and associates reported for the first time liposome like vesicular systems, DQAsomes, that have endosomolytic properties and can release plasmid DNA upon contact with the outer mitochondrial membrane in vitro (Weissig et al. 2001; D'Souza et al. 2003). DQAsome is a vesicular system made by sonicating the dispersion of dequalinium chloride, a dicationic amphiphilic compound, in the aqueous phase (Weissig et al. 1998). It is reported that cancer cells possesses an elevated mitochondrial and a higher plasma membrane potential compared to normal cells, and also DQAsomes may be suitable carriers for double targeting, i.e. both on the cellular level (cancer cell vs. normal cells) and on the subcellular level (mitochondria vs. rest of the cells). Cheng et al. (2005) studied on DQAsome encapsulation of paclitaxel, a drug which induces apoptosis by direct action on mitochondrial membrane, and found that paclitaxel-loaded DQAsomes inhibited the growth of human colon cancer in nude mice by 50% over controls. The hypothetical mechanism of action of paclitaxel loaded DQAsomes is summarized in Fig. 4. When DQAsomes reach the endosome by the process of endocytosis they disrupt the endosomal membrane and are attracted towards the mitochondrial membrane, and their membrane destabilizes on contact with the mitochondrial membrane releasing paclitaxel. The released paclitaxel acts the mitochondrial membrane resulting in the release of cytochrome C which induces apoptosis and ultimately cell death. Later the same group developed another system for targeting mitochondria (mitochondriotropic liposomes). This system is formed by attaching triphenylphosphonium ion to the surface of liposomes, because triphenylphosphonium ion shows specificity toward mitochondria (Boddapati et al. 2005).

8. Conclusion

There are a number of therapeutic agents, which have their specific site of action in the cell and are required to be delivered to specific target sites within the cell. Apart from the discovery of new therapeutic agents, the discovery of new targets and the pathophysiology of various diseases require the delivery of these agents to particular compartments in the cell. Cellular organelles play a major role in the pathogenesis of various diseases so, by understanding their role in the diseases therapeutic agents can be targeted to the particular organelles for better activity. New therapeutic agents have also been exploited for their specific activity when they reach a particular compartment or organelle in the cell. The cellular environment plays a major role in the design of a carrier system that can deliver its contents intracellularly e.g. the pH of the endosome and negative inner membrane potential of mitochondria. Various versions of liposomes and other novel carriers have also been exploited for intracellular delivery provided that they can deliver the contents to a specific organ within the body. This can be achieved by better understanding of the pathophysiological role of a particular organelle/compartment in the disease and knowledge of various drug delivery approaches. Drug delivery to a particular organelle/compartment of a specific organ at therapeutic concentration will open new therapeutic strategies for the treatment of various diseases like cancer, Alzheimer's disease etc.

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