

Strategies for Intracellular Drug Delivery

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Introduction

There are two aspects to effective drug design, firstly to develop drugs that induce the desired response against the therapeutic target, and secondly, to ensure that the drugs reach their therapeutic target *in vivo*. While drug delivery is often perceived to be the poor relation in drug research and development, effective drug delivery is vital. No matter how potent the drug *in vitro*, if it cannot reach its site of action *in vivo*, it is rendered useless. Effective drug delivery also has important implications from a business viewpoint. More effective intracellular delivery can provide drug companies with a means of expanding market share or of revitalising therapeutics with previously unrealised potential because of poor pharmacokinetic profiles.

Efficient intracellular delivery can also avoid non-specific effects and reduce toxicity, allow a reduction in dosage levels and minimise wastage (by reducing the quantity of drug excreted or highly metabolised by the liver) and lead to better patient compliance.

Many different technologies have been devised to deliver drugs intracellularly. This article reviews some of the novel technologies that have been developed, which are aimed to overcome the highly selective barrier of the cell membrane. In particular, "last mile" technologies that attempt to deliver drugs to the cytoplasm or the nucleus are discussed.

Cellular Internalisation Mechanisms

Although it is possible for some hydrophobic molecules to pass directly through the cell membrane by a process of simple diffusion, most water-soluble substrates need a means of facilitating their passage into the cell. Facilitated diffusion occurs when membrane proteins form channels, allowing substances to diffuse across the cell membrane. These channels often only open under specific conditions, such as in the presence of chemical messengers (ligand-gated channels). Another class, voltage-gated channels, only open if there is a specific concentration of ions inside and outside the cell.

Although simple and facilitated diffusion allow the transport of molecules from an area of high concentration to one of lower concentration, active transport is required to pump substances against a concentration gradient. This

process uses ATP-derived energy to concentrate substances on one side of the cell membrane.

One such energy dependent mechanism is endocytosis, which allows cellular entry without passage through the cell membrane. Endocytosis results in the formation of an intracellular vesicle through the invagination of the plasma membrane and membrane fusion. There are a number of different endocytosis pathways that differ in their mechanisms of internalisation.

In receptor mediated endocytosis, receptors on the plasma membrane of the target tissue will specifically bind to ligands on the outside of the cell. This is exemplified by the transferrin receptor that internalises iron bound transferrin. Typically, endocytosis is associated with the internalisation of plasma membrane receptors via clathrin-coated pits. The formation of these pits involves the sequential binding of the adapter complex AP-2 to the membrane followed by the assembly of hexameric clathrin subunits. Recent work has suggested that several alternative adapter complexes exist, which work either with, or independently to, AP-2 (e.g. Disabled-2, Dab2 (Mishra *et al.*, 2002)).

Endocytosis can also occur through the caveolar endocytosis pathway, which is involved in signal transduction and intracellular transportation of lipid raft associated molecules (Razami and Lisanti, 2001). Caveolae are found in many cell types including fibroblasts, adipocytes, endothelial and epithelial cells, together with both smooth and striated muscle cells. The pathway is therefore utilised by a wide range of cell types thus making it a useful pathway for internalisation of therapeutic molecules. Like the clathrin dependent pathway, the caveolar dependent endocytosis pathway is energy dependent.

A further mechanism of endocytosis is pinocytosis in which the plasma membrane forms an invagination and the substance found within it is brought into the cell. In general, the internalised molecule is dissolved in water and thus pinocytosis is also referred to as 'cellular drinking'. In contrast, during phagocytosis, the cell firstly sends out membrane projections, or pseudopodia. Once the pseudopodia make contact with the particle, a receptor/ligand interaction occurs between the phagocytic cell surface and the particle to be ingested. The pseudopodia

then surround the particle and when the plasma membranes of the projections meet, membrane fusion occurs, forming an intracellular vesicle.

Physical or Mechanical Methods of Drug Delivery

Drug delivery methods that rely on brute force to traverse cell membranes have been investigated. Particle bombardment, for example, accelerates DNA-coated microparticles (formed from metals such as tungsten or gold) to high velocity in order to penetrate cell membranes (Yang *et al.*, 1990). As it is difficult to control the DNA entry pathway, this method is largely restricted to adherent cell cultures and has yet to be widely employed systemically. In DNA vaccination, however, where localised expression of the delivered DNA is sufficient to achieve an immune response (Qiu *et al.*, 1996), bombardment is more widely used.

Microinjection involves transferring a dissolved substance into a cell using the microscopic tip of a glass capillary. This technique may be perceived as being more accurate than the 'shotgun' approach of particle bombardment, but requires considerable technical skill and can only be performed one cell at a time. Although relatively efficient, the method is slow and laborious and is neither appropriate for use with large numbers of cells nor practical for *in vivo* transfer.

Oligonucleotide transfection has also been achieved using pressure-mediated techniques that utilise controlled, nondistending pressure (Mann *et al.*, 1999). This method achieved more than 50% successful delivery in cardiovascular tissues.

Another mechanical method, electroporation, uses electric fields to induce temporary permeability of membranes. First used to deliver DNA to mammalian cells in 1982 (Neumann *et al.*, 1982; Wong and Neumann, 1982), the process has since been used to deliver DNA and other molecules to a wide variety of cell types *in vitro*, including yeast and bacteria. Although it is one of the most efficient methods of gene transfer, the technique has been limited due to the high mortality rates of cells after high-voltage exposure and the difficulties involved in optimisation. Despite this, several groups have persevered with the technique, and it has been shown to increase the efficacy of the anticancer drug bleomycin in animal tumour models (Okino and Mohri, 1987).

Drug Smuggling

Research into the cell's own transport mechanisms has enabled a different class of drug delivery methods, which exploit these routes to "smuggle" disguised therapeutics into the cell.

Perhaps the best-known method in this category is that of viral vectors. These are currently by far the most efficient means of delivering DNA, usually achieving success rates of higher than 90% for both delivery and expression. This is reflected by the fact that approximately 72% of clinical

protocols for gene therapy in the past year have used virus-based vectors. To date, however, evidence for the clinical effectiveness of gene therapy has been limited (Hu *et al.*, 2004). This may be attributed to limitations associated with viral vectors including immunogenicity, toxicity, restricted targeting of specific cell types, costs and problems with production and packaging. Hence, increasing research is being carried out into alternative methods of therapeutic delivery.

Liposomal complexes were one of the first chemical delivery systems to be used in animals, with Felgner *et al.* developing the cationic lipid Lipofectin in 1987 (Felgner *et al.*, 1987). Such systems have proved a popular means of delivering oligonucleotides as packaging of the DNA in liposomes confers increased nucleases resistance thus enhancing circulation half-life (Dass, 2002). Continual refinement and experimentation has successfully complexed DNA with cationic, anionic and neutral liposomes, or mixtures of these, and has improved transfection efficiency. Despite this, lipid-based systems do have their disadvantages. Liposomes of conventional formulations are rapidly removed from blood circulation by the reticuloendothelial system, thus preventing them from reaching their target sites, although second generation liposomal complexes have overcome some of these issues. They target cells poorly and variations may arise during their construction, affecting their efficacy. Cationic liposomes may also exhibit cytotoxicity upon systemic administration (Filion and Philips, 1998). In addition, for the most part, they have proved to be inefficient at delivering proteins, transducing less than 5% of cell populations. Although the method displays no significant toxicity under optimal conditions, it does not work in the presence of serum. Additionally, highly positively charged molecules with few, or no, inaccessible hydrophobic domains will not be transported across plasma membranes.

Other hydrophilic molecules have been explored as a means of delivering drugs, such as the colloidal carriers that are comprised of the polysaccharide chitosan. Both DNA-chitosan hybrid nanospheres and ionically crosslinked chitosan nanoparticles have been tested, with the latter found to be efficient vehicles for the transport of peptides across nasal mucosa (Janes *et al.*, 2001).

Cell Penetrating Peptides

Peptides have also been used to facilitate intracellular uptake of molecules. This methodology has been developed following the observation that certain proteins entered cells when added to the surrounding media. From these proteins, short basic peptide sequences (protein transduction domains, PTDs) were identified that could cross the plasma membrane and in so doing take the rest of the protein with them (Schwarze and Dowdy, 2000). The most widely studied PTDs originate from the *Drosophila* transcription factor antennapedia (Lindgren *et al.*, 2000), the HIV-1 transcriptional activator Tat (Cao *et al.*, 2002), and the herpes simplex virus (HSV) protein VP22 (Elliott and O'Hare, 2000). All these peptides show little sequence

homology but are highly cationic and arginine-rich.

The mechanism by which these peptides are internalised has been controversial as initial experiments suggested that internalisation occurred through an energy independent mechanism. More recent data demonstrated that these results are artifactual and result from the fixation methods used that cause redistribution of these highly charged peptides. PTD-mediated intracellular transport occurs by endocytosis and more specifically through caveolar specific endocytosis, which is inhibited at 4°C (Vives *et al.*, 2003). The PTDs interact with glycoaminoglycans (GAGs) that are attached to cell surface heparan sulfate proteoglycans (HSPGs). HSPGs are important cellular modulators involved in cell signalling through a wide range of growth factors, morphogens and chemokins. They also act in combination with membrane integrins to control cell adhesion and migration in the extracellular matrix (Iozzo, 2001). Turnover of HSPGs requires their cellular internalisation by endocytosis resulting in the internalisation of HSPGs without membrane transversion. This process allows intracellular delivery of molecules that form ionic interactions with the HSPGs. Peptide delivery of therapeutics appears to be independent of protein size. PTDs can internalise covalently attached proteins of greater than 700 kDa and Tat has been used to deliver liposomes of over 200 nm in diameter (Torchilin *et al.*, 2001).

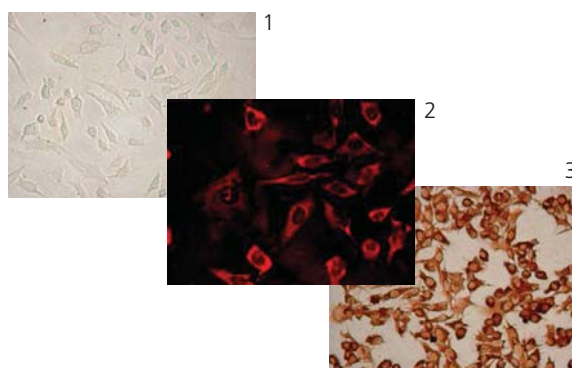
Proteins coupled to Tat have been delivered into cells and tissues both *in vitro* and *in vivo*. The specificity of the process is ensured by the fact that only proteins covalently attached to the PTD can access cells, which may prove important for *in vivo* applications. Tat has also been shown to be capable of delivering functional protein (β -galactosidase) into a live animal model, using intraperitoneal injection (Schwarze *et al.*, 1999).

Structural analysis of PTDs has enabled synthetic versions to be designed and their efficiency improved. A Tat derivative, for example, has been developed that can deliver small molecules *in vivo* and is 33 times more effective than the native Tat peptide itself (Ho *et al.*, 2001).

Peptides from the HSV transcription factor VP22 have also been successfully used to deliver therapeutic molecules to cells, including oligonucleotides, peptides and whole proteins. As VP22 fusion proteins localise to the nucleus, nuclear export signals are added to those fusions that require delivery to the cytoplasm.

One major problem with the above PTDs is that they are derived from non-human (viral or *Drosophila*) proteins and therefore risk provoking an unwanted immune response when used for drug delivery in humans. Indeed, modified Tat proteins have been used to evoke an immune response in patients with HIV (Ramakrishna *et al.*, 2004).

Diatos bypassed this problem by using peptides derived from human anti-DNA antibodies and human heparin binding proteins (Avreameas *et al.*, 1998, 1999; Ternynck *et al.*, 1998). Diatos Peptide Vectors (DPVs, VectoCell © technology) are synthetically made and consist of 15 to 20 amino acids. They contain positively charged, lysine/arginine rich sequences, a feature common to the majority



Internalisation of anti-peroxidase (PO) IgG using DPV technology: (1) Anti-PO-IgG alone does not internalise. Conjugation with DPV allows intracellular delivery of the IgG, which remains active. The antibody is detected intracellularly (2) using an anti-mouse TRITC-conjugated antibody. Activity of the internalised anti-PO IgG is shown (3) by interaction of peroxidase with the antibody.

of PTDs, and bind to cell surface glycosaminoglycans. DPVs have been conjugated to a number of different cargos, including small molecules, proteins, antibodies, RNA and DNA. These conjugates have been shown, both *in vitro* and *in vivo*, to drive intracellular delivery of the molecules and induce a therapeutic response. Once inside the cell, DPVs enable the accumulation of therapeutic molecules in the cytoplasm or nucleus, depending on the molecule and specific DPV used.

Clathrin Specific Endocytosis

Receptor-mediated endocytosis can also be exploited for drug delivery, using the transferrin receptor. A major pathway for cellular iron uptake is through internalisation of the complex of iron-bound transferrin and the transferrin receptor. Transferrin has been widely applied as a targeting ligand in the active targeting of anticancer agents, proteins, and genes to malignant cells that over-express the transferrin receptor. Transferrin specific intracellular delivery is achieved by conjugation of transferrin with drugs, proteins, hybrid systems with macromolecules and as part of liposomal-coated systems. Conjugates of anticancer drugs with transferrin can significantly improve the drug's selectivity and toxicity and overcome drug resistance.

DNA can also be coupled to transferrin via a polycation such as polylysine or via cationic liposomes in order to target proliferating cells. However, transfection efficiency needs are currently insufficient to rival viral vectors for DNA delivery (Li *et al.*, 2002).

The Future

Drug delivery can make or break a promising therapeutic. The techniques described above are not an exhaustive list, but rather represent just a few of the most promising methods in a burgeoning field. One important aspect of these technologies resides in their enabling delivery of large cargo, such as antibodies, to cells opening the possibility of developing biotherapeutics with a catalytic activity, rather than simply an inhibitory action, like the more traditional approach relying on small compounds. Understanding the mechanisms underlying the different types of internalisation and defining tissue targeting is important for the developing of the technology as a clinical approach. Drug delivery based on protein transduction technologies may provide new answers for better targeted, more efficient therapies.

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