Delivery to Intracellular Targets by Nanosized Particles

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Abstract Nanosized drug carrier systems, including liposomes and nanoparticles, have the potential of delivering their contents to the interior of cells. However, engineering of the particle size and surface properties is necessary to achieve targeting to particular cell types. Conventional particles with hydrophobic surfaces are rapidly engulfed by phagocytic cells. Modification of the surface with hydrophilic polymers yields so-called "Stealth" particles which avoid phagocytosis and remain in the circulation longer after intravenous injection. The addition of specific ligands to the surface of these particles can confer more specific targeting to particular cell types. Nanoparticles and liposomes are normally taken up by endocytosis in non phagocytic cells, leading to their delivery to the lysosomal compartment. In order for the cargo to reach other cell compartments, a mechanism of endosomal escape is necessary. Examples are given of drug delivery in two particular applications: delivery to macrophages for immunomodulating and anti-infectious functions, and delivery of antisense oligonucleotides and small interfering RNA to cells.

Keywords Liposome • Nanoparticle • Endocytosis • Macrophage • Nucleic acids

Abbreviations

AS-ODN	antisense oligo deoxynucleotide
CHEMS	cholesteryl hemisuccinate
DC-Chol	3ß-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol
DNA	deoxyribonucleic acid
DOGS	dioctadecylamidoglycylspermine
DOPE	dioleoylphosphatidylethanolamine

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DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	1,2-di-O-octadecenyl-3-trimethylammonium propane
EPR	enhanced permeation and retention
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
HUVEC	human umbilical vein endothelial cells
IgG	immunoglobulin G
LDL	low density lipoprotein
LHRH	luteinizing-hormone-releasing hormone
MDP	muramyl dipeptide
MTP-Chol	muramyl tripeptide cholesterol
MTP-PE	muramyl tripeptide phosphatidylethanolamine
NC	nanocapsules
PACA	poly (alkylcyanoacrylate)
PAMAM	poly (amido amine)
PEG	poly (ethylene glycol)
PEG-PMMA	poly (ethylene glycol) – poly (methyl methacrylate) copolymer
PEI	poly (ethyleneimine)
PIBCA	poly (isobutylcyanoacrylate)
PIHCA	poly (isohexylcyanoacrylate)
PLA	poly (D, L-lactide)
PLGA	poly (lactide –*co*-glycolide)
RGD	Arg-Gly-Asp tripeptide
SAINT-2	N-methyl-4(dioleyl)methylpyridinium chloride
SiRNA	small interfering ribonucleic acid
VEGF-R2	vascular endothelial growth factor receptor 2

1 General Remarks About Drug Carriers

The use of drug carrier systems to improve the efficacy of therapeutic molecules has been attracting attention over the last five decades. Among the different constructions that can be made to modify the distribution of an active molecule, colloidal or nanosized particles present a number of advantages (Barratt 2003). They are large enough to transport a large number of guest molecules but small enough (typically between 20 and 200 nm) to pass through some biological barriers and to be taken up by cells.

The first form of colloidal particle to be considered as a potential drug delivery system was the liposome (Ryman and Tyrell 1980). These particles, which were first proposed as models of cell membranes, consist of one or more phospholipid bilayers surrounding aqueous compartments. Water-soluble drugs can be entrapped in the aqueous phase and lipophilic or amphiphilic ones can be inserted into the lipid bilayers. According to the application, the particle diameter can be adjusted

from a few microns down to about 25 nm by the choice of preparation technique and the phospholipid composition can also be chosen to provide adequate stability (Szoka 1990). Other supramolecular assemblies based on amphiphilic molecules have also be proposed for drug delivery. For example, oil-in- water micelles can be used to solubilize lipophilic drugs for delivery. Of particular interest are micelles formed from amphiphilic copolymers which are very stable to dilution (Kakizawa and Kataoka 2002).

Emulsified systems have either an oil phase dispersed in an aqueous phase (o/w) or an aqueous phase dispersed in an oil phase (w/o) with the aid of surfactants; o/w systems are more suitable for biological applications. Despite the name, the so-called microemulsions that are attracting much attention as delivery systems for water-insoluble drugs have droplet sizes in the sub-micronic range. They are thermodynamically stable and form with a minimum of energy input, because they contain a high proportion of surfactant and often a co-surfactant. On the other hand, nanoemulsions, with a similar droplet size but a different internal structure are only kinetically stabilized and require a large energy input to generate their small droplet size (Heuschkel et al. 2008). Self micro-emulsifying drug delivery systems (SMEDDS) have been developed recently in attempt to improve the oral bioavailability of some water-insoluble drugs. A mixture of drug, oil, surfactant and co-surfactant is administered and the micro-emulsion forms on dilution in the intestinal fluid (Kyatanwar et al. 2010). Apolar lipids that are solid at physiological temperatures can be formed into nanoparticulate systems with the aid of surfactant and an energy input; these are called solid lipid nanoparticles (Wissing et al. 2004).

Nanoparticulate systems can also be prepared from macromolecules. Proteins (e.g. albumin), poly (amino acids), polysaccharides (dextran, chitosan) and synthetic polymers have all been used (Vauthier and Bouchemal 2009). Obviously for drug delivery applications, the polymer chosen to prepare nanoparticles should be biodegradable to non toxic products. Therefore, the polyesters poly (D,L-lactide) (PLA) and poly (glycolide-*co*-lactide) (PLGA) are very often chosen as the basis for nanoparticles. A particulate system can be formed from a single highly branched polymer molecule: a dendrimer. Guest molecules can be attached by absorption or covalent linkage. The most usual type of nanoparticle is a matrix of entangled polymer chains. Depending on the affinity of the drug for the polymer, it can be included in the matrix or adsorbed on the surface. These nanoparticles are sometimes referred to nanospheres to distinguish them from another type of organization, the nanocapsule (Couvreur et al. 2002). This is a reservoir form consisting of an oily or aqueous core surrounded by a polymer shell. An appropriate drug molecule can be dissolved in the core liquid.

The major role of a drug carrier is to modify the distribution of the drug, re-routing it away from sites of toxicity and delivering more to the site of action. The carrier can also protect a fragile molecule from degradation in physiological fluids. It follows that the biodistribution of the carrier is primordial in determining its range of application (Gregoriadis and Senior 1982). Simple colloidal particles are "recognized" by the immune system in the same way as other foreign bodies such as bacteria. That is, when they are introduced into the blood stream some components

of the complement system, in particular C3b, and other proteins known as opsonins, adsorb onto the surface of the particles. This makes the particles susceptible to phagocytosis by macrophages, especially those of the liver and spleen (Szebeni 1998). Thus intracellular delivery occurs, but only in a particular cell type. As described below, there are some therapeutic scenarios in which intracellular delivery to macrophages is a useful strategy. However, it is often desirable to deliver drugs to other cell types; for example cancer cells. Opsonization and interaction with complement proteins can be reduced by decorating the surface of the drug delivery system with end-attached hydrophilic polymer chains (Jeon et al. 1991). Poly (ethylene glycol) (PEG) is the most commonly used hydrophilic polymer (Woodle 1998). The resulting liposomes or nanoparticles persist in the circulation after intravenous injection and are often referred to as "Stealth" particles. They are then able to carry drug to other cell types. In particular, they are able to extravasate into solid tumors by the "EPR" effect (Jain 1987) and also penetrate into infected or inflamed tissue (Oyen et al. 1996).

Cell-specific delivery with colloidal drug carriers can be achieved by attaching a ligand to the surface (ideally at the far end of a PEG chain). Antibodies and fragments thereof have often been used for this purpose, although a smaller ligand has some advantages. Small molecules that have been used for targeted drug delivery systems include folic acid and RGD-containing peptides. Carrier systems that bind to cell-surface receptors in this way will be internalized if receptor ligation normally leads to internalization and if the particle size is consistent with the size of the endocytic vesicle formed (in non phagocytic cells generally 150–200 nm). However, uptake by endocytosis or phagocytosis results in the carrier system being sequestered in endosomes or phagosomes, which are then acidified and fuse with lysosomes containing hydrolytic enzymes. Labile, hydrophilic drugs which cannot escape from this compartment may be destroyed without reaching their target. In response to this, pH-sensitive carrier systems which destabilize the endosome membrane and allow the drug to reach the cytoplasm have been developed. These are discussed in the section dealing the intracellular delivery of nucleic acids.

2 Interactions Between Colloidal Drug Carriers and Cells

As explained above, colloidal drug carriers have the potential to deliver their cargo to the interior of cells under certain conditions. Whether intracellular delivery occurs and to which compartment depends on the cell type and the composition of the carrier. The interactions of nano-sized carriers with cells have recently been reviewed by Hillaireau and Couvreur (2009). Whatever the mechanism of uptake, it must be preceded by contact and binding between the particle and the cell surface. Both hydrophobic and electrostatic interactions can occur, and increased binding is seen with charged particles, whether they are negatively or positively charged. As explained in the section above, binding to the cell surface is enhanced by the presence of proteins adsorbed on the cell surface. The presence of PEG or other

hydrophilic polymers at the surface of the particle can reduce protein absorption and in this way hinder binding to the cell surface. This is a useful property when the aim is to reduce clearance by phagocytic cells, but will also reduce binding to target cells. Specific binding to the target can be achieved by the attachment of a ligand to the particle surface. Many different types of molecule have been proposed as targeting ligands for liposomes and nanoparticles. Antibodies are obvious candidates because of their extreme specificity; however saccharides and other small molecules may also be employed. The efficiency of ligand-mediated binding will depend on the orientation of the ligand on the surface. For example, when liposomes are covered with PEG, the ligand should be attached to the distal end of the PEG chain, to allow unhindered interaction with the receptor (Mercadal et al. 1999). It should also be noted that the binding of a ligand to a receptor will not necessarily lead to internalization; this depends on the nature of the receptor and also on the size of the particle carrying the ligand (Allen and Moase 1996).

When the particle is a liposome, there are several theoretical possibilities after binding to the cell surface: internalization of the intact particle by endocytotic processes; fusion of the liposome membrane with the cell membrane, leading to delivery of its contents directly to the cytoplasm; exchange of lipids between the liposome membrane and the plasma membrane. A very early study by Pagano and Huang (1975) using Chinese hamster V79 cells and liposomes prepared from dioleylphosphatidylcholine and cholesterol gave evidence for the last two mechanisms. However, subsequent studies have shown that liposome-cell fusion is quite rare unless specific fusogenic peptides are included (see for example Fattal et al. 1994; Pecheur et al. 1997). Koning et al. (2002) exploited lipid exchange between liposomes immobilized at the surface of colon carcinoma cells by means of a specific monoclonal antibody to deliver a lipophilic prodrug of 5-fluorodeoxyuridine.

In the case of nanoparticles, fusion with the cell membrane is not possible. Transfer of lipophilic cargo to the cell can occur after binding to the cell surface, as shown by Mosqueira et al. (2001) with a fluorescent marker within the oily phase of non PEGylated nanocapsules. However, most interactions take place by endocytotic mechanisms, including phagocytosis, clathrin- or caveolin-mediated endocytosis and macropinocytosis.

Phagocytosis (“cell-eating”) is restricted to specialized cells of the immune system: monocytes and neutrophils in the blood and macrophages and dendritic cells in the tissues. The physiological role of phagocytosis is to clear foreign bodies and to present antigens from them to other actors in the immune response. The size of particles that can be engulfed by macrophages reaches several microns; senescent red blood cells, for example. Binding to the phagocyte surface is greatly increased by opsonization with plasma proteins, among which immunoglobulins, fibronectin and complement component C3b play important roles. The phagocyte membrane carries receptors for these proteins, as well as receptors for saccharides (mannose/fucose and galactose), apolipoproteins and the less specific scavenger receptors. Pseudopodia grow out around the particle and a “zip fastener” effect of binding to a number of receptors on the same particle encloses it within a vacuole known as a phagosome. This vacuole is subsequently acidified and fuses with lysosomes

(containing a panoply of hydrolytic enzymes), while receptors are recycled to the cell surface. It follows that a drug carrier that is internalized in this way, and its contents, will come into contact with an environment in which it can be degraded. This is an important feature for polymeric delivery systems because it allows drug release and prevents the accumulation of polymer within the cells but can inactivate or sequester the active agent.

Factors which influence the uptake of drug carriers by macrophages include size, surface charge, surface coating and the presence of specific ligands. *In-vitro* studies have often observed more rapid uptake of larger particles, although this may be a consequence of more rapid sedimentation of larger objects in unstirred culture medium (Barratt et al. 1986; Tabata and Ikada 1988). However, more rapid clearance of larger particles is also observed in vivo (Senior and Gregoriadis 1982) and complement consumption also increases with particle size (Vonarbourg et al. 2006), suggesting that the surface of larger particles is more susceptible to opsonization than that of particles with a smaller radius of curvature. Nanoparticles and liposomes with positively or negatively charged surfaces are taken up more rapidly than neutral ones (Tabata and Ikada 1988; Heath et al. 1985) and those with hydrophobic surfaces are phagocytosed more readily than those with a hydrophilic surface. In particular, the presence of end-on hydrophilic chains such as PEG (Woodle 1998; Gref et al. 2000) or dextran (Jaulin et al. 2000) on the surface reduces uptake by phagocytes. Analysis of the kinetics of binding and internalization for various particle types suggests that the rate-limiting step is binding to the particle surface and that once they are bound PEG-covered particles are internalized at the same rate as non PEGylated ones (Mosqueira et al. 2001; Martina et al. 2007).

Some specific ligands can increase particle uptake by macrophages and this phenomenon can be exploited to deliver biologically active material to these cells. Liposomes containing phosphatidylserine are preferentially taken up by macrophages (Schroit and Fidler 1982) by means of a receptor whose primary purpose is to clear senescent erythrocytes and fragments of cells after apoptosis (Fadok et al. 2000). Another receptor which can be utilized to promote capture of drug carriers by macrophages is the mannose/fucose receptor, which allows these cells to capture and destroy a number of microorganisms. One example is the delivery of an immunomodulator in mannose-grafted liposomes, in order to stimulate the antitumoral properties of macrophages (Barratt et al. 1987). More recently, this strategy has been applied to the delivery of Amphotericin B (Vyas et al. 2000; Nahar et al. 2010) and another fungally derived antibiotic (Mitra et al. 2005) to macrophages for treatment of leishmaniasis. Another targeting ligand which has been used in a similar application is the tetrapeptide tuftsin (Thr-Lys-Pro-Arg, Agrawal et al. 2002). This peptide has the advantage of being both a targeting element and a macrophage activator. The anti-leishmanial activity of the drug is thus reinforced by macrophage-mediated effects.

Most other cell types are capable of internalizing carrier systems by endocytic mechanisms. The best documented pathway is that of clathrin-dependent endocytosis, involving so-called “coated pits”. These are invaginations of the plasma membrane enriched in the protein clathrin, to which the intracellular portion of

some membrane-bound receptors can attach. Binding of ligand to these receptors triggers assembly of further clathrin molecules to form a vesicle around the particle which is pinched off to become completely internalized. The clathrin coat is lost and the vacuole becomes an early endosome. As in phagocytosis, acidification, receptor recycling and fusion with endosomes occur. Another vesicular endocytotic pathway has been described more recently: the caveolae pathway. Caveolae are flask-shaped invaginations in the membrane coated with the protein caveolin. The membrane composition is different from the bulk composition, rich in cholesterol and resembling that of lipid rafts; some receptors are particularly associated with these areas, particularly those which have a GPI anchor (Anderson 1998). The vesicles formed after pinching off of the caveolae by the protein dynamin are not acidified and do not fuse with lysosomes. Finally, macropinocytosis involves the actin-driven formation of membrane ruffles which collapse and fuse with the plasma membrane to enclose a vesicle, internalizing a droplet of the extracellular medium without any specific receptor. Unlike clathrin- and caveolin-coated vesicles which are about 200 nm in diameter, vesicles formed by macropinocytosis can be as large as 5 μm (Swanson and Watts 1995), and thus presents a mechanism of uptake for larger-sized particles.

The endocytic pathway taken by drug delivery systems is usually determined by the use of specific inhibitors, such as cytochalasin B for clathrin-mediated endocytosis, filipin for caveolae-mediated endocytosis and amiloride for macropinocytosis. Cholesterol depletion is also used to detect caveolae-mediated processes. Thus, Rejman et al. (2004) were able to reveal the influence of size on the mechanism of uptake of fluorescent polystyrene nanoparticles by non phagocytic B16 cells. While particles smaller than 200 nm were internalized by clathrin-coated pits, larger particles from 200 to 500 nm in diameter were preferentially taken up by caveolae.

A variant of the endocytic pathway is transcytosis. In this pathway, vesicles formed by endocytosis do not fuse with lysosomes but cross the cell, fuse with the plasma membrane in another region of the cell and release their contents into the extracellular medium. In particular, receptor-mediated transcytosis is a mechanism of carrying macromolecules across endothelial cells. For example, transcytosis of insulin, IgG, LDL and iron bound to the transport protein transferrin cross the endothelial cells of the blood-brain barrier by transcytosis. This pathway can be exploited for drug delivery to the brain. The group of Pardridge has reported results using monoclonal antibodies targeting either the transferrin receptor or the insulin receptor conjugated to long-circulating liposomes (Pardridge 2010a). Jallouli et al. (2007) observed that neutral or cationic polysaccharide nanoparticles of 60 nm in diameter without any surface modification underwent transcytosis by the caveolae pathway across a model of the blood-brain barrier. PLGA nanoparticles coated with transferrin followed the same route in this model (Chang et al. 2009). On the other hand, PEGylated poly (alkylcyanoacrylate)-based nanoparticles were taken up by rat brain endothelial cells by a clathrin-dependent pathway (Kim et al. 2007). This uptake was found to be via LDL receptors, since Apolipoprotein E is adsorbed onto PEGylated nanoparticles. These results illustrate the complexity of uptake mechanisms for drug delivery systems.

In the second part of this chapter, two aspects of intracellular drug delivery with colloidal drug carriers will be discussed in more detail: intracellular delivery to macrophages and intracellular delivery of nucleic acids.

3 Intracellular Delivery to Macrophages

The accumulation of colloidal drug carriers within phagocytic cells can be exploited in some drug delivery applications. For example, muramyldipeptide and analogues which stimulate the antimicrobial and antitumoral activity of macrophages can be delivered more efficiently as carrier-associated molecules. Muramyldipeptide (MDP) is a low-molecular-weight, soluble, synthetic compound derived from the structure of peptidoglycan from mycobacteria. Such compounds would be generated within macrophages after the ingestion of bacteria; therefore they act on intracellular receptors but, because of their hydrophilicity, they penetrate poorly into the cells and are eliminated rapidly after i.v. administration. Therefore, muramyl peptides have been associated with both liposomes and nanocapsules. The first studies using using soluble MDP within liposomes showed activity against pulmonary (Fidler et al. 1981) and liver (Daemen et al. 1990) metastases in mice. However, the low molecular weight and water-solubility means that this compound was poorly encapsulated and leaked easily from liposomes. In response to this, lipophilic derivatives such as muramyltripeptide-cholesterol (MTP-Chol; Barratt et al. 1989) and muramyltripeptide-phosphatidylethanolamine (MTP-PE; Asano and Kleinerman 1993) were developed. These systems promoted increased intracellular penetration of muramyl peptides into macrophages in vitro. *In-vitro* studies of nanocapsules loaded with MTP-Chol indicated that nanocapsules were taken up by phagocytosis and that a soluble derivative was released in the lysosomes (Seyler et al. 1999; Mehri et al. 1996). Thereafter, a number of effector mechanisms are induced in the macrophages, such as the production of nitric oxide (Morin et al. 1994), cytokines and arachadonic acid derivatives (Seyler et al. 1997).

Nanocapsules were also active against hepatic metastases in mice; however, the treatment was only curative when the tumour burden was low (Barratt et al. 1994). Similar observations were made with liposomes containing MTP-PE (Asano and Kleinerman 1993). Nevertheless, these liposomes have been proposed for the treatment of osteosarcoma (Mori et al. 2008).

Similar liposomes were also able to activate macrophages to control bacterial infections, for example, *Klebsiella pneumoniae* (Melissen et al. 1994). As well as activating the non specific defence mechanisms of macrophages, muramyl peptides can act as adjuvants facilitate the development of a specific immune response to an antigen and in this respect as well liposomal encapsulation increases their efficiency (Turanek et al. 2006). The potential of liposomes as immunological adjuvants (reviewed by Kersten and Crommelin 2003) was recognized as early as 1974. Both liposomes and nanoparticles can be used to deliver immunological adjuvants or antigens or combinations of the two to antigen-presenting cells

(macrophages and dendritic cells) (Peek et al. 2008). This approach can be applied to both protein antigens (van Broekhoven et al. 2004) and DNA vaccines (Greenland and Letvin 2007).

A number of microorganisms – bacteria, viruses and parasites – are able to live within macrophages. Colloidal drug carriers loaded with antibiotic drugs can be used to reach these infections (Pinto-Alphandary et al. 2000). Poly (isohexylcyanoacrylate) nanospheres loaded with Ampicillin allowed a large increase in efficacy compared with free antibiotic in mice infected with *Salmonella typhimurium* and *Listeria monocytogenes* (Fattal et al. 1989; Youssef et al. 1988). Studies using electron microscopy and confocal fluorescence microscopy with labeled *S. typhimurium* and nanospheres revealed the carrier system and the bacteria in the same intracellular compartment (Pinto-Alphandary et al. 1994).

A fluoroquinolone antibiotic, ciprofloxacin, was encapsulated within poly (isobutylcyanoacrylate) (PIBCA) and PIHCA nanospheres in an attempt to kill both dividing and non-dividing bacteria; however, the formulation was not effective against persistent Salmonella (Page-Clisson et al. 1998). More recently, the same antibiotic was encapsulated in PLGA nanospheres (Jeong et al. 2008). PLGA nanospheres are also able to deliver gentamicin to the liver and spleen of *Brucella melitensis*-infected mice (Lecaroz et al. 2007). Nanospheres prepared from poly (D,L-lactide) containing the antiparasitic drug primaquine were also efficient at delivering this drug to the liver (Rodrigues et al. 1994). Co-localization of nanospheres and *Leishmania donovani* parasites in Kupffer cells was observed.

Another infectious disease in which the organism responsible is to be found in macrophages is visceral leishmaniasis. One of the effective drugs against this disease is Amphotericin B, a polyene antibiotic. This amphiphilic molecule has been associated with several lipid-based drug delivery systems (Barratt and Bretagne 2007). Although the main advantage brought by nanoencapsulation is the reduction of Amphotericin B's dose-limiting toxicity, the use of colloidal carriers also means that the drug can reach the same intracellular compartment as the parasite.

4 Intracellular Delivery of Nucleic Acids

A second application in which colloidal drug carriers can provide intracellular delivery is that of the administration of nucleic acids. Progress in molecular biology has led to the availability of therapeutic genes and shorter nucleic acid sequences, in particular anti-sense oligonucleotides (AS-ODN) and small interfering RNA (siRNA). However, these large, negatively charged molecules cannot penetrate cell membranes and are also susceptible to degradation by nucleases, particularly the single-stranded AS-ODN. The stability problem for AS-ODN can be overcome by chemical modification, yielding structures such as phosphorothionate, methylphosphonate and boranophosphonate analogues that are resistant to enzymes but retain the capacity to bind to messenger RNA. However, the barrier of intracellular penetration remains. Furthermore, passage of the cell plasma membrane is not the last

barrier to nucleic acid delivery. Therapeutic genes and oligonucleotides which form triple helices with DNA must be transported into the nucleus, while AS-ODN and siRNA act in the cytoplasm at the level of protein synthesis on the ribosomes. However, if the formulation containing the nucleic acid is internalized by phagocytosis or endocytosis, it will be sequestered in a membrane-bound vacuole, which is subsequently acidified and fused with a lysosome containing acid hydrolases. Therefore, some mechanism must be included to allow the nucleic acid to escape from the endosome before it is degraded.

Viral vectors have been developed for gene therapy, but have serious drawbacks in terms of toxicity, immunogenicity and the size of the plasmid which can be inserted. A number of other strategies have been adopted: physical methods such as the "gene gun" and electroporation, complexes with cationic polymers such as chitosan, poly (lysine) and poly (ethyleneimine) (PEI) and complexes with cationic lipids, known as lipoplexes. This section will concentrate on recent developments in intracellular delivery systems for AS-ODN and siRNA.

4.1 Lipid-Based Systems

The concept of "lipofection" was advanced by Felgner et al. (1987). Small liposomes, formed from dioleoylphosphatidylethanolamine (DOPE) and a cationic lipid, DOTMA, were mixed with DNA and the resulting "lipoplexes" were able to introduce reporter genes into various cell lines. The cationic lipid is able to condense the linear DNA into a complex, but the original lipid vesicle morphology is not conserved. DOTMA is available commercially as Lipofectamine®. Many other cationic lipids have been developed, including DOTAP, DOGS, DC-cholesterol and SAINT-2. These lipids are usually mixed with a "helper" lipid, such as DOPE or cholesterol which improves their stability and may aid cellular penetration. However, these complexes show low transfection efficiency compared with viral systems and in particular do not perform well in vivo. The net positive charge of the complexes is probably responsible for their high toxicity and also promotes the adsorption of plasma protein which leads to their rapid elimination. As a result, attempts have been made to modify the surface of these complexes. The surface charge can be modified by the addition of anionic lipids (Lee and Huang 1996) or by the inclusion of PEGylated lipids (Fenske et al. 2001).

Li and Szoka (2007) have reviewed the development of lipid-based colloidal particles with a diameter of less than 100 nm, which would be better adapted to *in-vivo* nucleic acid delivery. In particular, they describe a detergent dialysis method which allows the different components to be associated in a controlled fashion. They also present a model for the interaction of these lipid articles with cells. It was originally assumed that cationic lipoplexes were able to fuse directly with the plasma membrane, which has a negative charge and deliver their cargo directly to the cytoplasm. However, it is now accepted that the cationic complexes are taken up by endocytosis after electrostatic interaction with the plasma membrane. Within the endosome, endogenous lipids in the endosomal membrane are

transferred into the particle to form ion pairs with the cationic lipid. This destabilizes the endosomal membrane and the nucleic acid is able to escape into the cytoplasm (Xu and Szoka, 1996).

Another, similar, strategy for cytoplasmic delivery is pH-sensitive liposomes (reviewed by Fattal et al. 2004). In this case the nucleic acid is encapsulated in neutral or anionic liposomes including lipids in their composition which undergo a change in organization at the pH of the endosomes, destabilize the endosomal membrane and release the nucleic acid into the cytoplasm. DOPE is one of the lipids used in this context, because it adopts a hexagonal phase at low pH. Oleic acid or cholesteryl hemisuccinate (CHEMS) are often added to the formulation. Some results have been obtained in vitro showing that these liposomes increase nucleic acid delivery to cells; for example, the replication of Friend virus in NIH 3 T3 cells was inhibited by an AS-ODN in pH-sensitive cells (Ropert et al. 1996). Interestingly, this work suggested that virally infected cells preferentially take up particulate carrier systems by reflex endocytosis during virus budding. However, observations in vivo with pH-sensitive liposomes have shown limited efficacy, because interactions with plasma proteins reduce the pH-sensitivity (De Oliveira et al. 2000). Recently, more sophisticated systems have been developed. Thus, Mudhakir et al. (2008) coupled an arginine-rich peptide onto the surface of lipid systems, to direct them to the macropinocytosis uptake pathway. When siRNA was incorporated into this type of particle, which also contained a pH-sensitive lipid combination DOPE/CHEMS, gene expression was effectively inhibited. Another pH-sensitive system, developed by the laboratory of Robert Langer, used a coating of PEG-polycation copolymer on pH-sensitive liposomes encapsulating siRNA (Auguste et al. 2008). At the pH of the endosomes, the protective polymer is desorbed and the nucleic acid released. Efficient knockdown of GFP in transfected HeLa cells and GAPDH in HUVEC cells by these particles has been demonstrated. This is interesting in the light of results reported by Remaut et al., (2007) showing that PEGylation of liposomes fails to protect the nucleic acid cargo from degragation in the lysosomes. On the other hand, an assembly of cationic lipid and AS-ODN conjugated to PEG was able to promote rapid delivery to the nucleus in KB cells (Jeong et al. 2006). Experimental results from Pakunlu et al. (2006) showed that PEGylated liposomes containing both AS-ODN to drug resistance genes and doxorubicin could be taken up by cancer cells and in this way the efficacy of the cytotoxic drug was enhanced.

A alternative strategy for intracellular delivery is the use of so-called “fusogenic” liposomes in which a viral peptide included in the formulation allows cytoplasmic delivery. An example is the promotion of cytoplasmic delivery of DNA oligonucleotides with a system using inactivated Sendai virus (Kunisawa et al. 2005).

The intracellular delivery of antisense oligonucleotides has been reported in a large number of publications. Delivery has been evidenced by detection of fluorescent oligonucleotides within the cells and by an antisense effect on the target gene. Thus, Ruozi et al. (2005) showed the role of DOTAP in liposomes for the intracellular delivery of AS-ODN to COS 1 and HaCaT cells.

In the same way, uptake of siRNA loaded lipid complexes has been demonstrated in many different cells types. For example, Santel et al. (2006) showed

uptake of lipid complexes containing siRNA against CD31 in mouse vascular endothelial cells and also observed inhibition of tumor growth by an antiangiogenic effect. Lavigne and Thierry (2007) were able to quantify the delivery of siRNA directed against cyclin D1 associated with a lipoplex to specific cell compartments in MCF-7 cells. Yadava et al. (2007) compared the performance of lipoplexes with poly(ethyleneimine) (PEI, see below) and saw similar cellular uptake of a model siRNA from the two systems, but a better silencing activity from the lipoplexes, which they attributed to a more rapid dissociation of the lipid complexes.

Recent developments include the use of specific targeting ligands to promote uptake of the nucleic acid complexes by specific cells types. Cardoso et al. (2007) used lipoplexes targeted by transferrin to deliver siRNA silencing marker genes to glioma, hepatocarcinoma and HT-22 cells. Transferrin was also used as a ligand to prepare "Trojan horse" liposomes to carry nucleic acids and other therapeutic agents across the blood-brain barrier, taking advantage of the presence of transferrin receptors promoting transcytosis across endothelial cells (Pardridge 2010a). The insulin receptor has been used for the same purpose (Pardridge 2010b). The folate receptor, overexpressed on many tumor cells, is another target which has been exploited for intracellular nucleic acid delivery (Yu et al. 2009). For example, Bcl2 downregulation in KB cells was shown to be greatly enhanced by the presence of folate acid on the surface of cationic liposomes (Chiu et al. 2006). The ligand is attached to the extremity of PEG chains themselves coupled to phosphatidylethanolamine in the liposome membrane. Delivery of anti HER-2 AS-ODN to head and neck cancer cells was also improved by folate-liposomes and increased the sensitivity of the cells to conventional chemotherapy (Rait et al. 2003).

4.2 Polymer-Based Systems

As far as polymer-based systems are concerned, three strategies have been adopted for the delivery of nucleic acids: complexation with cationic molecules, adsorption onto preformed nanoparticles and encapsulation within nanoparticles.

The most commonly used macromolecule for complexation of nucleic acids is PEI, which provides a high density of positive charge. Complexes can be formed easily by mixing the polymer and the nucleic acid; the resulting assemblies retain a net positive charge, which leads to electrostatic interactions with the cell membrane and uptake by endocytosis. Within the acidic endosome, the amino groups on the polymer are protonated, leading to osmotic swelling and rupture of the endosome. This so-called proton sponge effect ensures the release of the nucleic acid into the cytoplasm. However, a simple PEI/nucleic acid formulation presents drawbacks in terms of toxicity and an unfavorable biodistribution. PEI, particularly branched or high-molecular weight chains is toxic because of its ability to bind to cell membranes and cause necrotic cell death (Roques et al. 2007). The net positive charge of the complexes also provokes rapid adsorption of plasma proteins after intravenous administration, leading to particle aggregation. These aggregates are

physically trapped in small capillaries, particularly in the lung, or taken up by phagocytosis by macrophages (Chemin et al. 1998). Despite these drawbacks, some success has been obtained in cell culture, including in non dividing cells (Boussif et al. 1995; Dheur et al. 1999; Gomes dos Santos et al. 2006). Some positive results have also been obtained in vivo: Grzelinski et al. (2006) were able to suppress the expression of the growth factor pleiotrophin and Urban-Klein et al. (2005) achieved an anti-tumor effect with a PEI complex of siRNA targeting the HER-2 receptor.

The biocompatibility of PEI-based formulations can be improved by coating them with PEG (Mao et al. 2006) or by glycosylation of the surface (Leclercq et al. 2000). Specific targeting ligands have also been attached. One such ligand is the RGD (Arg-Gly-Asp) peptide, which was used by Schiffelers et al., (2004) to block vascular endothelial growth factor receptor-2 (VEGF-R2) expression in vascular endothelial cells and thereby prevent tumor angiogenesis.

Dendrimers composed of poly (amidoamine) (PAMAM) carry a high density of positive charge and can therefore be used to bind nucleic acids on their surface (Zhou et al. 2006; Shen et al. 2007). Helin et al. (1999) studied the intracellular distribution of a fluorescent ODN adsorbed onto dendrimers and found that this depended on the stage of the cell cycle. Fluorescence was found in the nuclei during G2/M phase while in other phases it seemed to be concentrated in acidic vesicles. Inhibition of target gene expression was observed with these systems. Other examples of inhibition of gene expression by AS-ODN attached to dendrimers are given by Bielinska et al., (1996), Yoo et al. (1999) and Li and Morcos (2008). The subject has been reviewed recently by Raviña et al. (2010). Specific targeting of dendrimers carrying AS-ODN against the Epidermal Growth Factor receptor to glioma cells was achieved by coupling folic acid to their surface (Kang et al. 2010).

SiRNA has also been immobilized on dendrimers (Kang et al. 2005; Zhou et al. 2006). These systems have been rendered more biocompatible by acetylation (Waite et al. 2009; Patil et al. 2008) and they have also been targeted: with the Tat peptide (Kang et al. 2005) and with the peptide hormone LHRH (Minko et al. 2010). Other, more water-soluble, polymers have been used to prepare dendrimers for nucleic acid delivery. Among these are carbosilane (Gras et al. 2009) and poly (L-lysine) (Eom et al. 2007).

Nucleic acids can also be complexed with the cationic arginine-rich polypeptide protamine. Junghans et al. (2001) observed uptake of protamine-AS-ODN particles by endocytosis and delivery to the cytoplasm in Vero cells. More stable particles were prepared using both protamine and human serum albumin (Weyermann et al. 2005). These showed both uptake and an anti-sense effect in mouse fibroblasts. Attempts were made to target these particles to the blood-brain barrier, by coating them with apolipoprotein A-1 to promote transcytosis (Kratzer et al. 2007). More sophisticated delivery systems can be obtained by mixing protamine, lipids and nucleic acids. Thus, Li et al. (2008) were able to deliver siRNA to the cytoplasm of NCI-H460 tumor cells, after modifying the surface with PEG and anisamide to target a tumor receptor. Similar particles have been used to downregulate Bcl-2 expression in cells with AS-ODN, using transferrin as a targeting ligand (Yang et al. 2009).

A positively charged polysaccharide, chitosan, has also been used to complex nucleic acids. Gene silencing in H1299 human lung carcinoma cells has been achieved (Liu et al. 2007). Cyclodextrins, molecular cages of glucose units, are not positively charged but can be coupled to a cationic polymer to form effective systems for complexing nucleic acids. When modified with PEG and a targeting moiety (again transferrin) these particles show gene knock-down in vitro and inhibition of the EWS-Fli-1 gene in Ewing's sarcoma in vivo (Bartlett and Davis 2007; Hu-Lieskovan et al. 2005).

Micellar systems have also been used to deliver nucleic acids to intracellular compartments. Examples are PEG-poly (aspartic acid) copolymers (Kakizawa et al. 2004) which can exert a "proton sponge" effect in endosomes, lactosylated PEG which can form complexes with poly (lysine) and AS-ODNs and allow targeting to galactose receptors on liver cells (Oishi et al. 2005; Oishi et al. 2007) and PEG-PMMA block copolymers (Kakizawa et al. 2006) which can be used to produce hybrid organic-inorganic nanoparticles.

The adsorption of nucleic acids onto preformed nanoparticles requires a positively charged surface. The first systems described used cationic surfactant on the surface of poly (alkylcyanoacrylates) (PACA) (Fattal et al. 1998) or poly (lactide-co-glycolide) (Singh et al. 2003; Oster et al. 2005). AS-ODN adsorbed onto PACA nanoparticles are protected from nucleases (Lambert et al. 1998) and can be delivered intracellularly (Chavany et al. 1994). These formulations were found to be able to inhibit the proliferation of mutated Ha-ras-transformed cells proliferation and reduce tumorigenicity in nude mice (Schwab et al. 1994). More recently, cationic nanoparticles made up of a biodegradable core of poly (isobutylcyanoacrylate) surrounded by a shell of chitosan were prepared by de Martimprey et al. (2008). A siRNA directed against the Ret/PTC1 junction oncogene was adsorbed onto these particles and was able to silence the gene in papillary thyroid carcinoma cells and exert an anti-tumor effect in mice.

PLGA-based nanoparticles have also been modified to provide a cationic surface. Notably, Nafee et al. (2007) coated them with chitosan and were able increase intracellular uptake of AS-ODN into A549 lung cells. Cationic nanoparticles have also been prepared by co-polymerization of methylmethacrylate and aminoalkylmethacrylate monomers (Zobel et al. 2000). These nanoparticles can also promote intracellular accumulation of AS-ODN (Tondelli et al. 1998; Zobel et al. 2000).

Despite the fact that adsorption onto the nanoparticle surface has produced some results with the intracellular delivery of nucleic acids, better protection from nucleases would be obtained if the nucleic acids were incorporated within the interior of the particles. However, the hydrophilic character of the nucleic acids is not compatible with the hydrophobic polymers; Thus, Lambert et al. (2000) developed a method of producing nanocapsules (NC) with an aqueous core and a PIBCA shell which could encapsulate hydrophilic molecules such as nucleic acids. These nanocapsules were able to protect AS-ODN from nuclease degradation and showed evidence of intracellular uptake by their ability to transfect cells, including vascular smooth muscle cells, which are notoriously difficult to transfect (Toub et al. 2005). The same system was also able to deliver siRNA to cells (Toub et al. 2006). Fluorescent labeling showed a punctate pattern of siRNA within the cells, suggesting

that it was delivered to endosomes. In vivo, these NC containing AS-ODN targeted to the EWS-Fli-1 spliced gene showed a true antisense effect against Ewing's sarcoma in mice (Maksimenko et al. 2003).

Nanoparticles encapsulating nucleic acids have also been prepared from the well known PLGA polymer. Thus, the uptake of AS-ODN directed against VEGF mRNA by ARPE-19 human retinal pigment epithelial cells was increased 4-fold by encapsulation and protein expression was thereby inhibited (Aukunuru et al. 2003). More recently, intracellular delivery of siRNA was achieved by PLGA nanoparticles, silencing the GFP gene in 293 T cells (Yuan et al. 2006).

Coating of PLGA nanoparticles with the positively charged polysaccharide chitosan promoted their uptake by A549 lung cancer cells (Beisner et al. 2010). In this way, 2′-O-methyl RNA was able to inhibit telomerase activity in a sequence-specific way in these cells. Chitosan alone has also been used to form particles for siRNA delivery, by ionic gelation with tripolyphosphate (Katas and Alpar 2006). These particles were efficient at silencing the marker gene luciferase expression in CHO K1 and HEK 293 cells and showed better activity than simple chitosan-RNA complexes. Later, the same group combined PLGA and PEI in nanoparticles. These were able to adsorb siRNA and allow a good gene silencing effect in CHO K1 cells, showing better activity and lower toxicity than PEI alone (Katas et al. 2008).

Finally, methacrylic polymers, such as those in the Eudragit series, are attractive as nucleic acid delivery systems because of their positive charge. Yessine et al. (2006) showed that complexes between these polymers and AS-ODN could deliver the nucleic acid to the cytoplasm, probably because of an endosome-perturbing effect. Recently, nanoparticles for gene delivery have been formulated from Eudragit polymers and shown to be able to transfect some tumor cells lines (Gargouri et al. 2009).

5 Conclusion

Particulate nanocarriers have shown their potential for intracellular delivery of biologically interesting substances in numerous studies over the past decades. However, a number of unresolved issues remain, such as the choice of truly specific ligands for *in-vivo* applications and the formulation of systems which allow the cargo to escape from the lysosomes while remaining non toxic and stable in biological fluids. Therefore, this is likely to remain an active research field in the years to come.

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