British Journal of Pharmacology (2009), 157, 179–194

0007-1188/09
www.brjpharmacol.org

THEMED SECTION: VECTOR DESIGN AND DRUG DELIVERY

REVIEW

Nanotechnologies and controlled release systems for the delivery of antisense oligonucleotides and small interfering RNA

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Antisense oligonucleotides and small interfering RNA have enormous potential for the treatment of a number of diseases, including cancer. However, several impediments to their widespread use as drugs still have to be overcome: in particular their lack of stability in physiological fluids and their poor penetration into cells. Association with or encapsulation within nano- and microsized drug delivery systems could help to solve these problems. In this review, we describe the progress that has been made using delivery systems composed of natural or synthetic polymers in the form of complexes, nanoparticles or microparticles.
British Journal of Pharmacology (2009) **157**, 179–194; doi:10.1111/j.1476-5381.2009.00148.x; published online 2 April 2009

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Keywords: antisense oligonucleotides; siRNA; pharmacokinetics; cell penetration, nanoparticles; microparticles, controlled release

Abbreviations: AS-ODN, antisense oligonucleotides; CTAB, cetyltrimethylammonium bromide; DC-Chol, 3β [N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol; DOTMA, N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride; EPR, enhanced permeability and retention effect; GFP, green fluorescent protein; MPS, mononuclear phagocyte system; mRNA, messenger RNA; NS, nanospheres; PACA, poly(alkyl-cyanoacrylates); PAMAM, poly(amidoamine); PEG, poly(ethylene glycol); PEI, poly(ethyleneimine); PIBCA, poly(isobutylcyanoacrylate); PLGA, poly(lactide-co-glycolide); RISC, RNA-induced silencing complex; RNase H, ribonuclease H; siRNA, small interfering RNA; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor

Introduction

The aim of this review is to describe advances that have been made in the delivery of antisense oligonucleotides (AS-ODN) and small interfering RNA (siRNA) by the use of natural and synthetic polymers. Over recent decades, much hope, and research effort, has been invested in nucleic acids as therapeutic agents. In particular, AS-ODN and siRNA, which are able to inhibit expression of specific genes by interfering with transcription or, more usually, translation, have enormous potential for the treatment of many diseases, including cancer, in which they can be used to silence oncogene expression. However, several impediments to their widespread use as drugs still have to be overcome. The most serious of these are their lack of stability in physiological fluids and their poor penetration into cells. Association with or encapsulation within nano- and microsized drug delivery systems could help to solve these problems but, as we attempt to describe below, the design of the carrier is important to achieve the correct tissular, cellular and subcellular localization of the AS-ODN or siRNA.

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Received 28 August 2008; revised 30 October 2008; accepted 5 January 2009

Mechanism of action of AS-ODN and siRNA

An understanding of the mechanisms of action of AS-ODN and siRNA is a prerequisite for designing efficient therapeutic systems. Although the activity of both types of molecule is due to their base-pairing capacity with complementary cellular nucleic acids, the method by which they inhibit gene expression is different.

Antisense oligonucleotides are single-stranded DNA sequences of 13–25 bases, which are complementary to

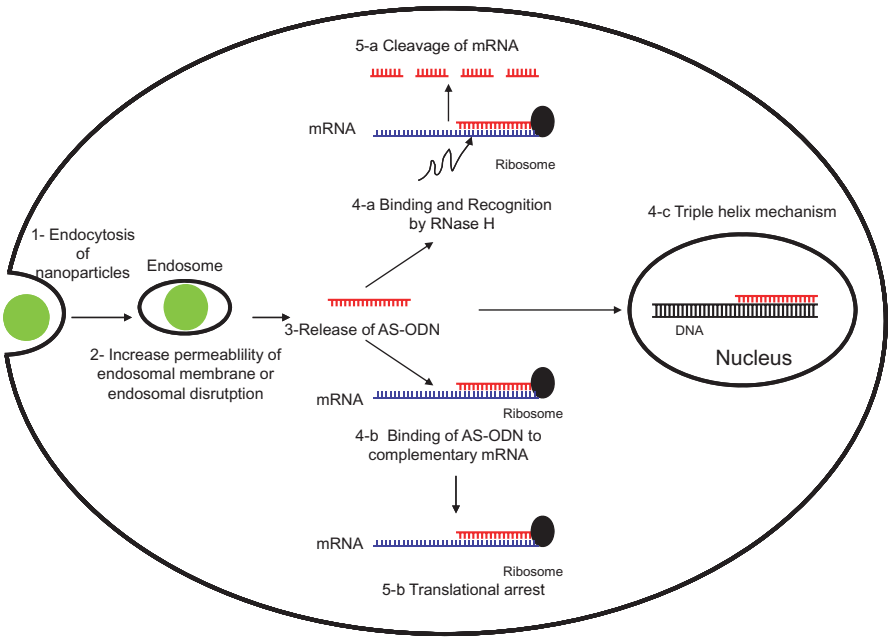

Figure 1 Intracellular penetration of nanoparticulate systems containing antisense oligonucleotides and their subsequent mechanisms of action after release in the cytoplasm. AS-ODN, antisense oligonucleotides; mRNA, messenger RNA; RNase H, ribonuclease H.

specific messenger RNA molecules (mRNA). Their binding to this mRNA by Watson-Crick base-pairing stops translation and thereby reduces synthesis of the encoded protein (Loke *et al.*, 1989). The first explanation of this was a steric blockage of the process of translation, in which the presence of the bound AS-ODN prevents the binding of translation factors and modulators and impedes the movement of the ribosome along the mRNA strand (Baker *et al.*, 1997; Dias *et al.*, 1999) (Figure 1). A second mechanism that comes into play is the action of ribonuclease H (RNase H). This enzyme specifically cleaves the RNA strand of RNA-DNA duplexes (Figure 1). This releases the AS-ODN, which can then bind to a new mRNA strand; this mechanism is therefore catalytic (Walder and Walder, 1988). However, chemically modified AS-ODN may not be recognized by the enzyme (see below).

The mechanisms described above involve mRNA and occur in the cytoplasm. However, AS-ODN can also exert activity in the nucleus, where they are able to form triple helixes with DNA, inserting into the major groove of the double helix. This occurs preferentially when a sequence composed entirely of purines is base-paired with one composed of pyrimidines (Moser and Dervan, 1987). The formation of a triple helix leads to cleavage of the DNA and will therefore inhibit gene expression at the transcription stage.

Small interfering RNAs differ from AS-ODN in being made up of ribonucleotides rather than deoxyribonucleotides and in being double-stranded rather than single-stranded. These double-stranded RNAs of 21–23 base pairs bind to a multi-protein complex in the cytoplasm, known as RISC for RNA-induced silencing complex (Figure 2). During assembly of RISC, one of the two siRNA strands, referred to as the passenger strand, is released, whereas the other strand (guide strand) binds to the argonaute protein, Ago2 (Meister *et al.*, 2004). This strand then guides RISC to its complementary target RNA, which is finally cleaved by the RNase activity located in the Ago2 protein, triggering its destruction (Song *et al.*, 2004). The mechanism is therefore an antisense, catalytic one. The RISC contains elements that search for homology, which means that siRNA is more efficient than AS-ODN and a smaller number of molecules need to be delivered to the cell (Corey, 2007). The site of action of siRNA is always the cytoplasm. As they are double-stranded, they have better *in vivo* stability than AS-ODN and therefore give more prolonged gene-silencing activity; however, they share with AS-ODN the drawback of poor penetration into cells, being negatively charged hydrophilic molecules (Table 1).

Similar to siRNA, miRNA, which stands for microRNA (Lee *et al.*, 1993) constitutes a class of evolutionarily conserved, endogenously expressed, non-coding RNAs that are 19–24 nucleotides long and which bind with only a partial complementarity to the 3′ untranslated region of mRNA and negatively regulate target mRNAs (Ambros, 2004; Bartel, 2004). All four mammalian argonaute proteins, Ago1 to Ago4, seem to participate in miRNA-mediated repression (Pillai *et al.*, 2004). Because of partial complementarity, in most cases cleavage of mRNA does not occur, as noted for siRNA. miRNAs are therefore important post-transcriptional regulators of gene inhibition and appears to play an important role in a wide range of diseases, including cancer. Indeed a number of miRNAs have been identified to function as oncogenes. These so-called 'oncomirs' are usually found to be aberrantly expressed in various malignancies of different cellular origin (Wiemer, 2007). The use of strategies such as AS-ODN as inhibitors of miRNA has been reviewed recently (Esau and Monia, 2007).

Barriers to the use of AS-ODN and siRNA in clinical practice

Despite the obvious promise shown by nucleic acids as therapeutic agents, a number of problems need to be solved before

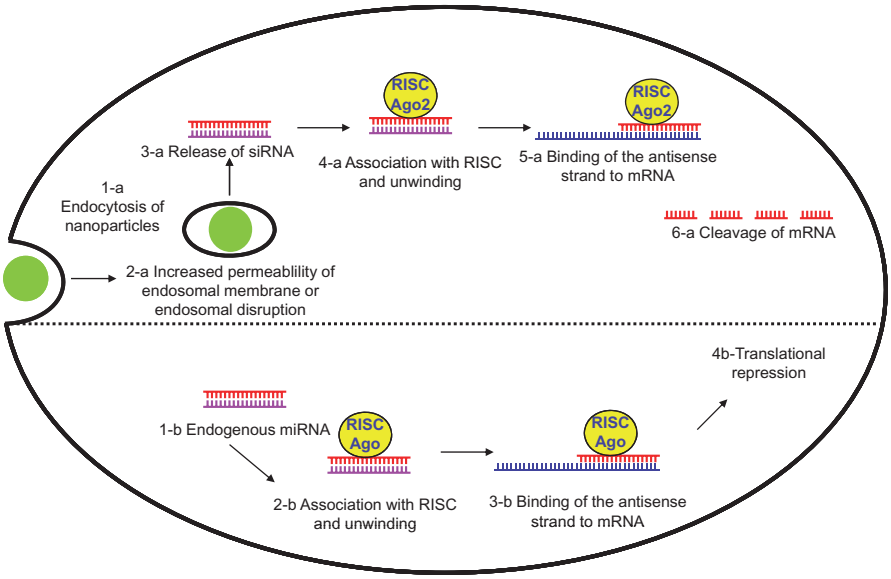

Figure 2 Intracellular penetration of nanoparticulate systems containing siRNA and their subsequent mechanism of action after release in the cytoplasm. The mechanism of action of endogenous miRNA is presented here for comparison purposes. miRNA, microRNA; mRNA, messenger RNA; RISC, RNA-induced silencing complex; siRNA, small interfering RNA.

Table 1 Main differences between unmodified AS-ODN and siRNA

ODN	*siRNA*
Single-stranded	Double-stranded
Short length	Short length
Negatively charged	Negatively charged
Hydrophilic	Hydrophilic
Poorly stable	Stable
Low intracellular penetration	Low intracellular penetration
Target is cytoplasm or nucleus	Target is cytoplasm
Cleavage or blockage of mRNA	Cleavage of mRNA
Short-term activity	Long-term activity

AS-ODN, antisense oligonucleotides; mRNA, messenger RNA; siRNA, small interfering RNA.

their use can become routine. These are essentially their instability in biological media and their poor penetration into cells. The phosphodiester backbone of natural ODN is readily hydrolysed by nucleases present in serum. Thus, when pharmacokinetic studies were first undertaken with an AS-ODN (a 25-mer) by Agrawal *et al.* (1995), the circulating half-life in the monkey was found to be only 5 min. It was shown by PAGE analysis of blood samples that no intact AS-ODN could be detected after 15 min. A similarly short half-life was observed by Sands *et al.* (1994) after administration of a 20-mer to rats. AS-ODN that is not degraded in the plasma is distributed into peripheral tissues, principally the liver (Agrawal *et al.*, 1995).

On a cellular level, AS-ODN, which are large negatively charged hydrophilic molecules, cannot penetrate the plasma membrane of cells by simple diffusion. However, their mechanism of action requires that they reach the cytoplasm. The ways in which AS-ODN are internalized by cells have not been completely elucidated. Given their physicochemical nature, the available pathways would be receptor-mediated uptake and endocytosis. This is consistent with the observations that uptake depends on temperature (Yakubov *et al.*, 1989) and the AS-ODN concentration (Vlassov *et al.*, 1994). There is some evidence that uptake by a membrane-bound receptor occurs at relatively low ODN concentrations (Yakubov *et al.*, 1989). Whether uptake is by a specific receptor or by absorptive or fluid-phase pinocytosis, the outcome for the ODN will be the same; it will be delivered to a lysosome where low pH and the presence of hydrolytic enzymes will combine to degrade it. Lysosomal escape is therefore an important factor for therapeutic effect.

Small interfering RNAs face similar pharmacokinetic and cell penetration limitations. As a result of their small size, they are rapidly eliminated by the kidneys and show circulating half-lives of the order of seconds to minutes (Soutschek *et al.*, 2004). They are also susceptible to degradation by nucleases in the plasma. A study of the biodistribution of radiolabelled siRNA indicated accumulation mainly in the liver and spleen and to a lesser extent in the heart, spleen and lung, in a similar pattern to AS-ODN (Braasch *et al.*, 2004). Within the tissues, they do not cross cell membranes readily because of their negative charge, hydrophilicity and molecular size. Although uptake into cells of invertebrates such as *Caenorhabditis elegans* has been observed, siRNAs are not taken up by most mammalian cells in a way that preserves their activity (Aagaard and Rossi, 2007) and therefore an effective delivery system is a prerequisite for effective gene silencing (Dykxhoorn and Lieberman, 2006).

Beside the barriers related to stability and bioavailability, several classes of off-target effects have been reported for AS-ODNs and siRNA, some of which are related to specific sequence motifs. Unmethylated CpG motifs have been shown to stimulate the immune system (Krieg *et al.*, 1995). In fact, the mechanism occurs through binding to the Toll-like receptor (TLR) 9 in the endosomal compartment and further activation of this receptor (Bauer *et al.*, 2001; Ishii *et al.*, 2002; Rutz *et al.*, 2004). Although CpG motifs have

beneficial effects in stimulating host defence, it can also induce inflammation and may promote tissue injury during infection or local administration (Schwartz *et al.*, 1999). However, these effects generally appear at higher doses of nucleic acids than those at which therapeutic effects are seen. At present, CpG oligonucleotides are considered as effective and well-tolerated vaccine adjuvants. Nevertheless, the effects of CpG on the immunity are strictly sequence-dependent (Krieg, 2006) and are not enhanced by polymeric or lipid non-viral carriers (Bonnet *et al.*, 2008). Other off-target effects can result from guanine quartet sequences, which form planar structures that stack together to make a four-stranded helical structure have been shown to bind many cellular proteins, resulting in non-antisense effects (Dapic *et al.*, 2002).

Two, not mutually exclusive, approaches can be adopted to improve the therapeutic potential of AS-ODN and siRNA. One is a chemical approach that consists in synthesizing nucleic acids with variations in the natural structure, which give them better resistance in physiological media while conserving the specificity of binding to the target. The second is a pharmaceutical technology approach that aims to associate the nucleic acid with appropriate delivery systems, which can protect it from degradation and modify its tissue and cellular distribution to reach the target. This review will concentrate on the second approach; however, a brief overview of modified nucleic acid structures will first be given, as these non-natural ODN and siRNA also benefit from a targeting strategy and the most efficient systems are most likely to be those that use a combination of the two approaches.

Modification of AS-ODN and siRNA structure

Modification of phosphate groups

The first type of modification to be attempted was replacement of the non-bridging oxygen of the phosphodiester backbone by a sulphur atom, resulting in phosphorothioate AS-ODN (Figure 3). These are more resistant to degradation by nucleases while retaining susceptibility to RNase H. This results in a prolongation of their circulating half-life to 30–60 min (Agrawal *et al.*, 1991; Cossum *et al.*, 1993; Zhang *et al.*, 1995; Geary *et al.*, 2001), although some variations between species were seen, with the clearance in mice being twice as rapid as that in monkeys in one study (Yu *et al.*, 2001). They are characterized by high kidney uptake (Lendvai *et al.*, 2005). Accumulation of phosphorothioate AS-ODN within cells is observed 24 h after administration, but the mechanism of uptake has not yet been elucidated (Butler *et al.*, 1997; Butler *et al.*, 2000). The efficacy of the modified AS-ODN, compared with the native structure, is still a matter for debate. For siRNA, two studies found that the silencing efficiency was not diminished (Braasch *et al.*, 2003; Harborth *et al.*, 2003), whereas Chiu and Rana (2003) clearly observed reduced activity.

The non-bridging oxygen in AS-ODN can also be replaced by a methyl group (yielding a methylphosphonate) or a boron atom (resulting in a boranophosphonate) (Figure 3). These structures are more hydrophobic than the natural molecule and show reduced activation of RNase H (Agrawal, 1999). Boranophosphonate derivatives of siRNA have also been prepared. These are much more resistant to nucleases than phosphodiester or phosphorothioate siRNA and consequently

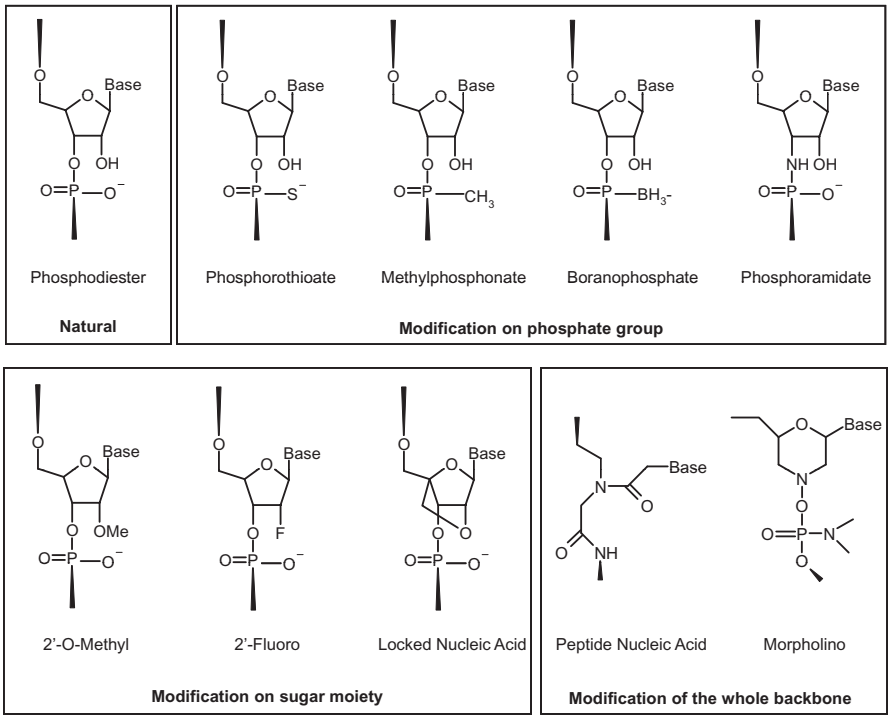

Figure 3 Structure of the main chemically modified antisense oligonucleotides and small interfering RNA.

show higher efficacy (Hall *et al.*, 2004; Hall *et al.*, 2006). Nevertheless, the features of the phosphorothioate backbone modification can also be associated with non-antisense effects through binding of cellular proteins (Levin, 1999).

A more profound modification of the backbone is to substitute every 3′ oxygen by a 3′ amino group, leading to the synthesis of phosphoramidates (Figure 3). These are very stable, nuclease-resistant molecules, which form stable triplexes with double-stranded DNA under near physiological conditions (Gryaznov *et al.*, 1995; Gryaznov *et al.*, 1996).

Modification of the sugar moiety

Attempts have been made to improve the stability of AS-ODN and siRNAs by making substitutions at the 2′ position of the sugar. O-methyl, O-methyoxy-ethyl and fluoro substitutions have been made (Figure 3). These modifications increase the nuclease resistance and the stability of the complexes formed with native RNA. However, the modifications also have consequences for activity, as the substitutions prevent RNase H cleavage of the target RNA in the antisense strategy. Therefore, it can be assumed that the antisense effects observed with these analogues (Kurreck, 2003; Urban and Noe, 2003) are due to steric hindrance of transcription. Increased potency has been claimed for sugar-substituted siRNA (Allerson *et al.*, 2005); however, in another study O-methoxy derivatives were found to have the same activity as the unmodified sequence (Kraynack and Baker, 2006). According to Layzer *et al.* (2004), fluoro-substituted siRNA did not show increased *in vivo* activity, despite better stability in plasma.

The ribose moiety can also be used to produce locked nucleic acid, in which a covalent bridge is formed between the 2′ oxygen and the 4′ carbon of the ribose, fixing it in the 3′-endo configuration (Figure 3). These constructs, which are also referred to as inaccessible RNA, are extremely stable in biological medium and are able to activate RNase H and to form tight hybrids with complementary RNA and DNA (Wahlestedt *et al.*, 2000; Vester and Wengel, 2004). Antisense activity has been obtained in experimental murine tumours (Jepsen *et al.*, 2004), but these molecules have also been observed to produce severe hepatotoxicity (Swayze *et al.*, 2007). Locked nucleic acid also undergo intracellular processing characteristic of siRNA, and therefore represent a useful advance in this technology (Braasch *et al.*, 2003; Elmen *et al.*, 2005).

Modification of the whole backbone

A further style of nucleic acid modification is that in which the entire backbone structure is changed. For example, the phosphodiester backbone can be completely replaced by a peptide one consisting of (2-aminoethyl)-glycine residues. These are known as peptide nucleic acids (Hanvey *et al.*, 1992). As AS-ODN, they have the advantages of strong hybridization, low toxicity and good biological stability with respect to nucleases, but also the disadvantages of low solubility in water, poor intracellular penetration and a lack of ability to activate RNase H. Another completely modified structure is the morpholino AS-ODN, in which the ribose is replaced by a morpholino moiety and phosphoroamidate intersubunit linkages are used as a substitute for phosphodiester bonds. They have low toxicity and show affinity for their targets similar to that of unmodified AS-ODN (Summerton, 1999; Summerton and Weller, 1997).

Drug delivery systems for AS-ODN and siRNA

Despite the advances provided by the modification of nucleic acid structure, these molecules require effective delivery systems in order to become useful therapeutic agents. Different formulation strategies have been developed to overcome the problems mentioned above: the susceptibility of these molecules to degradation by extracellular and intracellular nucleases and their inability to cross the cell plasma membrane by diffusion. Drug delivery systems are therefore designed to protect the nucleic acid and to allow it to enter the cell and reach its site of action: the cytoplasm. This review deals only with systems that are based on synthetic or natural polymers. Lipid-based systems have also made an enormous contribution to the field and are covered by other articles in this issue, as well as a recent review by Li and Szoka (2007).

The different polymer-based strategies that have been adopted are complexation with cationic molecules, adsorption onto preformed nanoparticles and encapsulation within nanoparticles (Figure 4). In formulating such systems, it is important to consider their biocompatibility, as most of the polymers are exogenous to the organism. In particular, interactions with plasma proteins at the particle surface may lead to rapid uptake by the mononuclear phagocyte system (MPS). For example, complement activation may be a problem (Plank *et al.*, 1996). Cationic particles are particularly susceptible to forming aggregates after binding of negatively charged plasma proteins, which are then either entrapped in the pulmonary capillary bed or taken up by the MPS (Opanasopit *et al.*, 2002). The use of poly(ethylene glycol) (PEG) to provide a hydrophilic shield over the surface of the particle is the most usual means of minimizing these interactions with plasma proteins, as will be seen in the specific examples below. These particles known as stealth® or long circulating particles have the ability to escape the opsonization process that leads to MPS uptake. As a result, they will be able to circulate in the vascular compartment for a long time without being cleared (Owens and Peppas, 2006). Their main advantage is their ability to pass through leaky endothelium if their size allows (typically 200 nm or less). This phenomenon is called 'EPR' (enhanced permeability and retention effect). It is due to defective capillary endothelium and poor lymphatic drainage in solid tumours (Hashizume *et al.*, 2000). Small particles are able to leave the circulation through the spaces between endothelial cells in the tumour vasculature and reach the interstitial fluid. To reach the tumour cells, they have to cross the tight network constituted by the extracellular matrix composed of a variety of polysaccharides and proteins overlying the surface of the cells that produce them (Li and Szoka, 2007). Some systems incorporate a specific ligand recognizing the target cell. Because the EPR effect is limited to tumour targeting only AS-ODNs used for cancer treatment can take advantage of this specific tissue distribution.

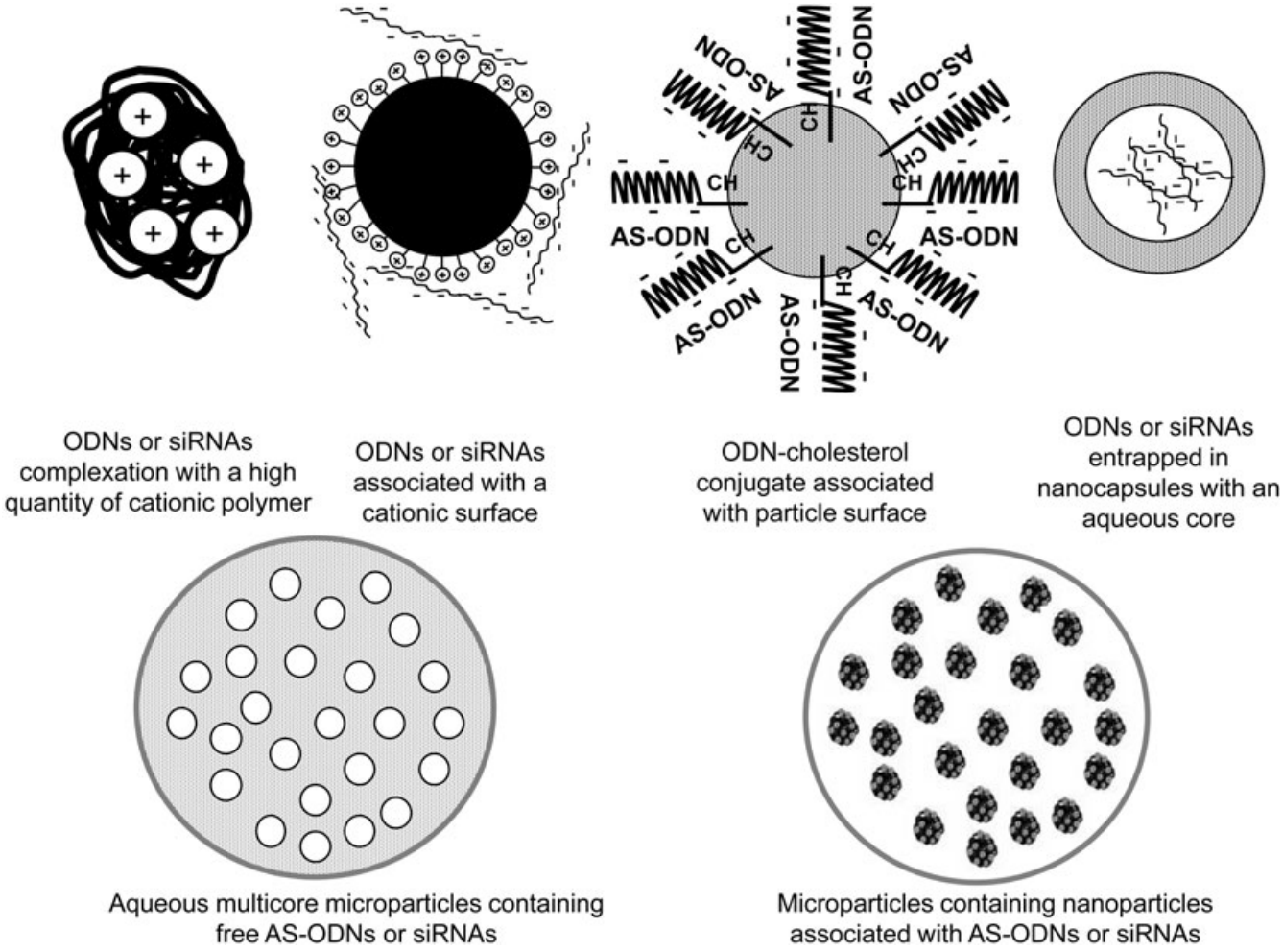

Figure 4 Different types of nano- and microtechnologies for the delivery of antisense oligonucleotides and siRNA. AS-ODN, antisense oligonucleotides; siRNA, small interfering RNA.

Nanoparticles obtained by complexation with cationic molecules

Nucleic acids have a high density of negative charge at physiological pH, which means that they can be readily associated with positively charged polymers forming nanoparticles (Figure 4). One such polymer that has been extensively used is poly(ethyleneimine) (PEI). This synthetic polymer is available with a range of molecular weights and in linear and branched forms. The complexes formed between nucleic acids, and this polymer have a net positive charge, which allows interaction with negatively charged cell membranes, followed by internalization by endocytosis. Delivery of the nucleic acid to the cytoplasm occurs by the 'proton sponge effect', in which the protonation of the numerous amino groups in the polymer causes osmotic rupturing of the endosome and release of the nucleic acid (Boussif *et al.*, 1996; Akinc *et al.*, 2005). The ability of nucleic acid to escape from the endosome is an important factor in its efficacy, because it would otherwise be destroyed. However, PEI poses some problems of toxicity (Roques *et al.*, 2007), and biodistribution *in vivo* is restricted to organs of the MPS (Chemin *et al.*, 1998). As explained above, the rapid adsorption of plasma proteins onto the positively charged polymers can lead to aggregation. Toxicity can be reduced by using lower molecular weight PEI, and by modifying the surface with PEG or by glycosylation. Specific targeting groups such as the Arg–Gly–Asp peptide can also be coupled to PEI. PEI has been shown to deliver AS-ODN and siRNA efficiently to cells in culture including non-dividing cells that are notoriously difficult to transfect (Boussif *et al.*, 1995; Dheur *et al.*, 1999; Gomes dos Santos *et al.*, 2006b). As far as the siRNA strategy is concerned, a very narrow window of conditions and PEI structure (25 kDa branched) are required for successful delivery (Grayson *et al.*, 2006). It has been shown by atomic force microscopy that PEI forms complexes that condense and completely cover chemically unmodified siRNA (Grzelinski *et al.*, 2006). After complexation, siRNA are efficiently protected against nucleolytic degradation both *in vitro* in the presence of RNase and *in vivo* in the presence of serum nucleases (Urban-Klein *et al.*, 2005).

Tumour cells internalized these PEI/siRNA complexes rapidly in cell culture and, as full biological activity of the siRNA molecules was preserved, it was assumed that they can be released intact from the complexes. One practical application that has been investigated *in vitro* and *in vivo* is the knock-down of the growth factor, pleiotrophin (Grzelinski *et al.*, 2006). Anti-tumour effects have also been observed *in vivo* with siRNA directed against the HER-2 receptor com-

plexed with PEI (Urban-Klein *et al.*, 2005). Ligands can be added to the particles to improve targeting. For example the Arg–Gly–Asp peptide ligand was attached at the distal end of the PEG, which was itself attached to PEI (Schiffelers *et al.*, 2004). The resulting nanoparticles had affinity for tumour neovasculature expressing integrins and were able to deliver siRNA inhibiting vascular endothelial growth factor (VEGF) receptor-2 expression and thereby block tumour angiogenesis.

However, the addition of PEG has consequences for the stability of siRNA/PEI complexes, as shown by Mao *et al.* (2006). A high degree of PEG modification increased the size of the complexes to 300–400 nm, and condensation of siRNA only occurred at high polymer to nucleotide ratios. However, PEGylation increased the stability of the resulting complexes against displacement of the nucleic acid by heparin and degradation by nucleases. Furthermore, polyplexes with PEG showed a better gene-silencing effect than those without PEG in an experimental systems involving NIH/3T3 cells transfected with β-galactosidase, with an effect of the PEG chain length. According to the authors, one hypothesis to explain this observation might be that the looser complexes formed in the presence of PEG facilitated the escape of the siRNA into the cytoplasm (Mao *et al.*, 2006).

Dendrimers (highly branched synthetic polymers) consisting of poly(amidoamine) (PAMAM) also have a high density of positive charge on their surface and are able to condense nucleic acids, thus protecting them from nuclease degradation. Although they have often been used for DNA, their use for AS-ODN and siRNA is less well documented. The size-to-charge ratio and the dendrimer generation have been found to be an important factor in the preparation of stable, homogeneous nanoparticles (Zhou *et al.*, 2006; Shen *et al.*, 2007). With short RNA sequences, only higher generation dendrimers provide sufficient electrostatic interactions to form stable particles, whereas with longer RNA, dendrimers from generation 1 to 7 readily form nanoparticles. Dendrimers have been used to deliver AS-ODN to clones of D5 mouse melanoma and Rat2 embryonal fibroblasts expressing luciferase cDNA (Bielinska *et al.*, 1996). Gene expression was inhibited in a specific and dose-dependent fashion. Similar results were obtained by Yoo *et al.* (1999). According to Helin *et al.* (1999) the intracellular distribution of a fluorescein isothiocyanate-labelled ODN delivered by PAMAM was dependent on the phase of the cell cycle, with a nuclear localization predominantly in the G2/M phase.

Dendrimer technology can also be applied to siRNA. Nanosized particles are produced, which protect the siRNA from nuclease activity and allow sustained release, leading to efficient gene silencing in A549Luc cells stably expressing the GL3 luciferase gene (Zhou *et al.*, 2006). The dendrimer was effective when loaded with the specific nucleotide sequence, whereas free siRNA and the G7-dendrimer carrying a non-specific siRNA had no effect on gene expression. Kang *et al.* (2005) have attached the Tat peptide to the surface of PAMAM dendrimers, in an attempt to improve cell penetration, producing particles carrying both AS-ODN and siRNA. However, the Tat peptide did not improve the delivery of the nucleic acid to NIH 3T3 MDR cells. Although MDR1 gene expression was partially inhibited by the antisense complex and weakly inhibited by the siRNA complex when both were tested at non-toxic levels of dendrimer, this effect was not increased by conjugation with the cell-penetrating peptide (Kang *et al.*, 2005). More recently, Pan *et al.* (2007) have grown PAMAM dendrimers on the surface of magnetic iron oxide nanoparticles, to combine the advantages of the nucleic acid binding properties of the polymer and the possibility of magnetic guidance of the particles, as well as their use as contrast agents. The initial nanoparticle diameter was 8 nm, and after growth of 5 generations of dendrimer they remained small and individualized. They were able to adsorb AS-ODN, as shown by zeta potential measurements, had minimal cytotoxicity and were rapidly internalized by cells.

Recently, chemical modifications have been made to dendrimers in order to reduce both interactions with plasma proteins and their toxicity. For example, water-soluble carbosilane dendrimers have been used to complex nucleic acids (Shcharbin *et al.*, 2007; 2008). The polymers formed complexes with AS-ODN and reduced their interactions with serum albumin. In another study, dendrimers were coupled to the amino acid lysine by using its α and ε amino groups. Generation 5 dendrimers were able to complex AS-ODN, changing in size from 12 to 73 nm in AFM images. The dendrimers had slightly lower cytotoxicity than PAMAM dendrimers and linear poly(lysine) and showed moderate efficiency for AS-ODN delivery to HeLa-Luc cells (Eom *et al.*, 2007).

Other polymers used to form complexes with nucleic acids are natural polypeptides. Protamine is a cationic, non-toxic, naturally occurring polypeptide, rich in arginine residues that confer positive charge and the ability to bind nucleic acids. Complexes between this small polypeptide as the free base and AS-ODN also form spontaneously on mixing. Junghans *et al.* (2000) called these assemblies 'proticles'. They were formed at protamine to AS-ODN ratios of 0.5–5. At the ratio of 5, the particles reached a size of 150–170 nm after 30 min of co-incubation and rose to about 300 nm after 3 days. Particles with higher protamine content were stable in phosphate buffer and cell culture medium, with very low AS-ODN release. However, particle formation required a minimum AS-ODN length of nine bases. In such conditions, phosphodiester AS-ODN were protected against nuclease degradation (Junghans *et al.*, 2001). Similar particles have been prepared by Dinauer *et al.* (2004). Protamine with a molecular weight of about 6000 was labelled with a fluorescent marker to follow the uptake of the particles by mononuclear cells. Again, protamine to AS-ODN ratios of 0.5–5 yielded particles of 150–180 min diameter, with a zeta potential that varied according to the ratio and become positive at ratios above 1.5. Proticles significantly decreased cellular growth in a cell proliferation assay by using AS-ODN against the c-myc proto-oncogene (Junghans *et al.*, 2000) as well as specific inhibition of tat mediated HIV-1 transactivation. In Vero cells, the internalized AS-ODN were localized in the cytoplasm and in the nucleus of the cells, and particle uptake was shown to be an energy-dependent endocytotic mechanism (Junghans *et al.*, 2001). However, the transfection ability of these complexes is lower than that of PEI or polylysine complexes (Mok *et al.*, 2007) because of weaker links between the guanidino residues and the nucleic acid.

Other disadvantages of protamine–nucleic acid systems are their tendency to aggregate at physiological salt concentrations and poor intracellular release of nucleic acid from the complex, particularly when phosphorothioates are used. In an attempt to solve these two problems, Lochmann *et al.* (2005) added human serum albumin to the preparation. This was mixed with protamine before adding AS-ODN. Nanoparticles of 230–320 nm are formed in this way. These particles showed low cytotoxicity and good penetration into mouse fibroblasts (Weyermann *et al.*, 2005). Recently, an attempt has been made to target these protein/AS-ODN complexes to specific cells by coating them with the apolipoprotein A-1, to promote transcytosis across the blood–brain barrier (Kratzer *et al.*, 2007).

A similar approach was applied to siRNA. The system is which is slightly more complex than those described above, is composed of nucleic acids, protamine and cationic liposomes. The nanoparticles are then modified by PEG-lipid containing a targeting ligand, anisamide for targeting sigma receptor-expressing B16F10 tumours, which were stably transduced with a luciferase gene (Li *et al.*, 2008). Targeting siRNA for silencing luciferase activity was significantly higher than the other formulations, lasted for 4 days and was shown to be due to a specific targeting effect (Li *et al.*, 2008).

Natural and synthetic polysaccharides have also been used to complex nucleic acids. In particular, chitosan, a positively charged polysaccharide from the exoskeleton of invertebrates, has often been used alone or in combination with other polymers (Liu and Yao, 2002). To form complexes, the nucleic acid is added to an acidic solution of low molecular weight chitosan and stirred to allow nanoparticle formation. A modified galactosylated chitosan has also been synthesized and used to form particles with AS-ODN designed to be taken up by liver cells (Dong *et al.*, 2008). A detailed study (Liu *et al.*, 2007) has shown the importance of various physicochemical parameters: particularly polymer molecular weight and degree of deacetylation, on particle formation and gene silencing. Indeed, high values of both parameters were necessary to obtain small, stable particles with siRNA (Liu *et al.*, 2007). When incubated with H1299 human lung carcinoma cells, silencing of endogenous green fluorescent protein (GFP) was obtained (Howard *et al.*, 2006) and shown to vary according to the type of complex. Indeed the higher the molecular weight and degree of deacetylation, the more efficient the silencing effect (Liu *et al.*, 2007). Force spectroscopy studies have shown the importance of low pH for strong siRNA–chitosan interactions (Xu *et al.*, 2007). Moreover, it is important to stress that these particles can be freeze-dried without loss of activity (Andersen *et al.*, 2008).

A versatile system for complexing AS-ODN has been developed by using a polymer based on cyclodextrin molecules linked by short sequences of a cationic polymer, which forms particles when mixed with nucleic acid. PEG or PEG bearing a targeting moiety (transferrin) can be added to the system by modifying them with adamantine at one extremity of the PEG, giving an affinity for the central cavity of the cyclodextrin. 'Stealth' and targeted particles can then be prepared by mixing water-soluble components (Bartlett and Davis, 2007). The PEG-based molecules can be added before or after complexing the cyclodextrin-containing polymer with nucleic acid. Functional efficacy of the delivered siRNA was demonstrated through luciferase reporter protein knock-down in Hela cells (Bartlett and Davis, 2007) and *in vivo*, after systemic administration through the inhibition of EWS-Fli-1 gene in a murine model of metastatic Ewing's sarcoma (Hu-Lieskovan *et al.*, 2005). In this study, the targeted formulation displayed the highest efficacy through recognition of transferrin by the transferrin-receptor expressing cells (Hu-Lieskovan *et al.*, 2005). The safety of this system after repeated administration to non-human primates has also been demonstrated (Heidel *et al.*, 2007), and tumour-specific targeting has been shown by *in vivo* imaging in mice bearing subcutaneous Neuro2A tumours. In this model, although both non-targeted and transferrin-targeted siRNA nanoparticles exhibit similar biodistribution and tumour localization by positron emission tomography, transferrin-targeted siRNA nanoparticles reduce tumour luciferase activity by about 50% compared with non-targeted siRNA nanoparticles 1 day after injection (Bartlett *et al.*, 2007). Mathematical model calculations of siRNA-mediated target protein knock-down and tumour growth inhibition were used to provide guidelines for designing more effective siRNA-based treatment regimens by adjustment of the dose and dosing schedule (Bartlett and Davis, 2008).

Micelle-forming copolymers have also been used to complex nucleic acids. For example, PEG-polycation di-block polymers with diamine side-chains can be used as endosome-escaping carriers for siRNA (Kataoka *et al.*, 2005). Several types of smart polymeric micelles have been designed for nucleic acid delivery. These include PEG-poly(aspartic acid) copolymers (Kakizawa *et al.*, 2004) that can exert a 'proton sponge' effect in endosomes, lactosylated PEG that can form complexes with poly(lysine) and AS-ODN and allow targeting to galactose receptors on liver cells (Oishi *et al.*, 2005; 2007) and PEG-poly(methylmethacrylate) block copolymers (Kakizawa *et al.*, 2006) that can be used to produce hybrid organic-inorganic nanoparticles.

Adsorption onto preformed nanoparticles

Matrix nanoparticles, called nanospheres (NS), prepared from poly(alkylcyanoacrylates) (PACA) (Fattal *et al.*, 1998) or poly(lactide-co-glycolide) (PLGA) (Singh *et al.*, 2003; Oster *et al.*, 2005) have been used to adsorb AS-ODN, by the intermediary action of cationic surfactant (Figure 4). More recently, block copolymers of poly(isobutylcyanoacrylate) (PIBCA) and chitosan have been used to adsorb siRNA (de Martimprey *et al.*, 2008). Although in these systems the nucleic acid is not truly encapsulated within the polymer, its close association with the particle surface is able to protect it from degradation by nucleases. The PACA NS are produced by an emulsion polymerization process, in which droplets of water-insoluble monomers are emulsified in an aqueous phase (Couvreur *et al.*, 1979). Anionic polymerization takes place in micelles after diffusion of monomer molecules through the water phase and is initiated by the water itself. The pH of the medium determines both the polymerization rate and the adsorption of the drug when the latter is ionizable (Couvreur *et al.*, 1979). Drugs can be combined with NS after dissolution in the polymerization medium either before the introduction of the monomer or after its polymerization.

Because only non-toxic additives are used, no further purification is needed and freshly prepared NS may be freeze-dried with their drug content. Nucleic acids have little affinity for the polymer; therefore their adsorption on the NS surface is obtained by ion pairing with a cationic surfactant, cetyltrimethylammonium bromide (CTAB) (Fattal *et al.*, 1998). The adsorption of the negatively charged AS-ODN onto the positively charged NS can be monitored by measuring the zeta potential (Lambert *et al.*, 1998). Another way of adsorbing nucleic acids onto PACA nanoparticles is to use cationic diethylaminoethyl (DEAE)-dextran as a surfactant during nanoparticle preparation (Schneider *et al.*, 2008).

When AS-ODN were bound to PACA NS by means of cationic surfactant they were protected from nucleases *in vitro* (Chavany *et al.*, 1992; Lambert *et al.*, 1998) and their intracellular uptake was increased (Chavany *et al.*, 1994). A study of their distribution *in vivo* showed accumulation of intact AS-ODN in the liver and in the spleen (Nakada *et al.*, 1996). AS-ODN within this sort of formulation were able to specifically inhibit mutated Ha-ras-mediated cell proliferation and tumorigenicity in nude mice after injection into tumours (Schwab *et al.*, 1994). Other hydrophobic compounds such as cationic lipids were also employed as adjuvants for loading AS-ODN to nanoparticles (Figure 4) (Balland *et al.*, 1996). However, using compounds such as polyamines [dioctadecylamidoglycyl-spermine (DOGS)], the absorption was lower than with CTAB in similar conditions of adsorption (Balland *et al.*, 1996). AS-ODN coupled to cholesterol has also been adsorbed onto poly(isohexylcyanoacrylate) nanoparticles (Godard *et al.*, 1995). However, because this conjugate was negatively charged, it was partially repulsed by the negative charges displayed by the polymer surface leading to a low loading efficiency of AS-ODN in the absence of CTAB.

A novel nanoparticulate system proposed recently by (de Martimprey *et al.*, 2008) consists of a biodegradable core of PIBCA surrounded by a shell of chitosan. The preparation method involves a first step where the polysaccharide is hydrolysed as described by Bertholon *et al.* (2006) and used to initiate redox radical polymerization of the isobutylcyanoacrylate monomer in emulsion in the presence of cerium ions. NS formed spontaneously from the resulting amphiphilic block copolymer. The siRNA complementary sequence to the ret/PTC1 junction oncogene was adsorbed to such NS at a NS : siRNA ratio of 50 leading to 90% siRNA adsorption (de Martimprey *et al.*, 2008). These NS were tested against subcutaneous tumours in mice made up of papillary thyroid carcinoma cells carrying this particular oncogene. The expression of ret/PTC1 was inhibited, and the tumour size was reduced to a greater extent than with free siRNA.

Poly(lactide-co-glycolide) NS containing a range of cationic material: PEI, N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA), 3β [N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), CTAB, have been prepared by a spray-drying technique (Takashima *et al.*, 2007). Chitosan-coated PLGA NS prepared by an emulsion-solvent evaporation technique using poly(vinyl alcohol) as a stabilizer have also been developed as a platform to carry nucleic acids (Nafee *et al.*, 2007). They were shown to increase cell uptake of AS-ODN in A549 lung cells (Nafee *et al.*, 2007).

Nanospheres based on poly(methylmethacrylate) have also been used as a platform to adsorb AS-ODN. In one study, methylmethacrylate and aminoalkylmethacrylate monomers were copolymerized by free radical polymerization to form NS of undisclosed size with positive zeta potential (Zobel *et al.*, 2000). Oligonucleotides were adsorbed by co-incubation for 2 h at 20–25°C, and the whole complex was tested on PC12 cells for the inhibition of ecto-5′-nucleotidase expression. As a result of enhanced intracellular penetration, these NS led to around 75% inhibition of enzyme expression. Using this technical platform, Tondelli *et al.* (1998) included a reactive cationic monomer in the emulsion polymerization medium to produce core-shell NS with a mean diameter of 480 nm, onto which AS-ODN could be adsorbed by an ionic mechanism. They demonstrated a strong inhibition of proliferation in HL60 cells when delivering an AS-ODN targeted to the codons 2–7 of *c-myb* (Tondelli *et al.*, 1998). In later work (Tondelli *et al.*, 2003), PEG was included in the shell to generate 'Stealth' NS.

Encapsulation within nanoparticles

True encapsulation of nucleic acids in polymeric systems encounters the problem that nucleic acids are hydrophilic while the polymers used to form nanoparticles are hydrophobic (Figure 4). As one solution, Lambert *et al.* (2000b) developed a method of producing nanocapsules (NC) with an aqueous core and a PIBCA shell, which could encapsulate hydrophilic molecules such as nucleic acids. In this procedure, an aqueous solution of the drug to be encapsulated is emulsified in an oily phase containing surfactant, after which the isobutylcyanoacrylate monomer is added and polymerizes at the surface of the aqueous droplets (Lambert *et al.*, 1998; Lambert *et al.*, 2000b; 2001). The NC are recovered from the oily phase by centrifugation over an aqueous phase containing a suitable surfactant. This method has proved to be efficient for the encapsulation of both AS-ODN (Lambert *et al.*, 2000b) and siRNA (Toub *et al.*, 2006b). The stability of AS-ODN in serum was improved compared with both the free molecule and molecules adsorbed onto NS. The kinetics of degradation suggested that only released AS-ODN were available to nucleases. The release from NC was biphasic, with a high initial burst followed by a slower, linear release, which was probably a reflection of heterogeneity in the NC population (Lambert *et al.*, 2000b).

Poly(isobutylcyanoacrylate) NC containing a radiolabelled 20-mer model oligonucleotide were able to deliver their cargo to cultured vascular smooth muscle cells, a difficult cell type to transfect (Toub *et al.*, 2005). Using an original technique based on selective solubilization of membranes with different detergents, these authors were able to determine the subcellular distribution of the AS-ODN. While the most of the small amount of free AS-ODN associated with the cells was found to be in the plasma membrane fraction, most of that delivered by NC was found to be internalized. The intracellular fraction of free AS-ODN was mainly in the membrane fraction, whereas with the NC formulation about 50% was found in the nuclear fraction, and less than 40% was in the vesicular fraction. The use of a fluorescently labelled AS-ODN revealed

a similar distribution by confocal microscopy. The amount of AS-ODN in the nucleus increased with time between 2 and 15 h (Toub *et al.*, 2006a).

Similar results were obtained for siRNA encapsulated in PIBCA NC with an aqueous core (Toub *et al.*, 2006b). Confocal microscopy studies showed that when a fluorescently labelled naked siRNA was added to the culture medium, only a small amount of fluorescence localized in the extracellular matrix was found. In contrast, when siRNA was encapsulated within NC, punctuate fluorescence was observed within the cells, suggesting an endosomal localization of the siRNA (Toub *et al.*, 2006b).

In vivo, AS-ODN-containing NC were tested on a murine model of Ewing sarcoma-related tumours targeting EWS-Fli-1, a fusion gene resulting from a translocation that is found in 90% of both Ewing sarcoma and primitive neuroectodermal tumour (Lambert *et al.*, 2000a). All treatments were given by the intratumoural route. At a cumulative dose of 14.4 nmol, only AS-ODN encapsulated within NC led to a significant inhibition of tumour growth, while a slight effect was observed with naked AS-ODN at the same dose. These results can be compared with those of Tanaka *et al.* (1997) in the same tumour model, in which a significant reduction of tumour growth was observed only after injection of 500 nmol naked AS-ODN. This suggests that encapsulation in NC renders the AS-ODN 35 times more effective. A number of different nanoparticles formulations have been tested in the EWS-Fli-1 model. The NC containing encapsulated AS-ODN were compared with poly(isohexylcyanoacrylate) NS onto which the same AS-ODN had been adsorbed with the aid of CTAB, as described above (Maksimenko *et al.*, 2003). Furthermore, both a completely phosphorothioate and a chimeric phosphorothioate/phosphodiester AS-ODN were tested. Both nanoparticulate systems led to a down-regulation of EWS-Fli-1 mRNA and significant reduction of the tumour volume from about 66–82%. CTAB may also play a role in endosomal escape and efficient delivery of AS-ODN to the cytoplasm. siRNA within PIBCA NC is also very effective in this *in vivo* experimental model, in contrast to free siRNA (Toub *et al.*, 2006b). In this case, substantial inhibition of tumour growth, up to 80%, was observed, especially with a high cumulative dose.

Nucleic acids can also be encapsulated within NS prepared from preformed polymers. The polymers most frequently employed for this purpose are poly(lactide) and PLGA, and the particulate systems are prepared by water-in-oil-in-water multiple emulsion/solvent evaporation or extraction technique, which allows the nucleic acid to be introduced into the preparation in aqueous solution, minimizing contact with organic solvent (Berton *et al.*, 2001; Delie *et al.*, 2001). Anti-VEGF AS-ODN were successfully encapsulated within PLGA NS prepared by this method and tested for VEGF inhibition in a human retinal pigment epithelial cell line (ARPE-19) (Aukunuru *et al.*, 2003). The AS-ODN inhibited VEGF mRNA and protein secretion when delivered by using NS but not in its free form. This was consistent with the ability of NS to increase AS-ODN cell uptake fourfold (Aukunuru *et al.*, 2003). In a rat carotid model *in vivo*, PLGA NS containing an AS-ODN against the platelet-derived growth factor receptor showed an anti-restenosis effect 14 days after the treatment (Cohen-Sacks *et al.*, 2002). In this case, however, the same effect was observed with naked AS-ODN administered at the same dose. It is noteworthy that in the case of NS, only a small proportion of the loaded AS-ODN would be released over 14 days; consequently, the naked AS-ODN was effective compared with the encapsulated AS-ODN, but at higher available doses (Cohen-Sacks *et al.*, 2002). For siRNA, only one recent report has used PLGA NS containing a GFP gene silencing siRNA with GFP-expressing 293T cells (Yuan *et al.*, 2006). A significant silencing effect was observed after 1 day incubation and was still persistent 1 week later (Yuan *et al.*, 2006).

Chitosan nanoparticles for RNA delivery can also be formed by ionic gelation with tripolyphosphate. Katas and Alpar (2006) compared encapsulation within and adsorption of siRNA onto chitosan particles formed in this way with simple complexation. Nanoparticles entrapping siRNA in the matrix were found to be the best carriers for silencing the transfected luciferase gene when incubated with the CHO K1 and HEK 293 cell lines, possibly because of their high binding capacity and loading efficiency. The interaction between the negatively charged polysaccharide alginate and poly(lysine) has also been exploited to prepare nanoparticles. These systems can be described as 'nanosponges', as AS-ODN added to the preparation after formation of nanoparticles diffuse progressively into them over a period of a few days. Once inside the nanoparticles, the AS-ODN are protected from nucleases (Aynie *et al.*, 1999). However, these particles distribute essentially into the lungs, probably because of an interaction with serum proteins leading to an aggregation and subsequent filtration by lung capillaries.

The fate of most of these systems is endosomal entrapment, and but the effects observed with AS-ODNs and siRNAs make them likely to escape from the endosomes into the cytoplasm. The mechanism by which this occurs is not yet fully understood. According to Torchilin *et al.* (1993), one possible explanation is that detergents such as sorbitan monooleate, used in colloid formulations as a particle stabilizer, may induce endosomal membrane disruption.

Microspheres for controlled delivery of AS-ODN and siRNA

Controlled release systems are mainly employed in regions of the body where the administration of AS-ODN and siRNA cannot be repeated frequently. Their purpose is to assure long-term delivery in the proximity of the target tissue and cells. Most of the studies have been carried out by using PLGA microspheres, which are degradable and allow drug release for weeks or even months. The nucleic acid is usually introduced in the microspheres during the preparation process by a water-in-oil-in-water multiple emulsion/solvent evaporation or extraction technique (Figure 4). Preliminary studies have demonstrated that length and chemistry (phosphodiester or phosphorothioate), AS-ODN loading as well as the size of the microspheres strongly affect encapsulation efficiency and release properties (Lewis *et al.*, 1998). Higher drug loading is generally associated with a larger size (Akhtar and Lewis, 1997) but is independent of ODN length (Lewis *et al.*, 1998).

The *in vitro* release profile of AS-ODN from particles was found to be affected by microsphere size, drug loading, AS-ODN length and polymer molecular weight. Small microspheres (1–2 μm) released most of the entrapped AS-ODN within the first 4 days, compared with 40 days for larger particles (10–20 μm) (Akhtar and Lewis, 1997). In the case of larger particles, a triphasic release was observed: a burst release in the first 48 h, attributed to the rapid release of the AS-ODN present on or close to the microsphere surface; a sustained release phase (about 25 days) in which an AS-ODN diffusion through pores present in the polymer matrix should occur; a final more rapid release phase (up to a complete release at about 60 days) due to the polymer degradation that would accelerate the ODN release rate (Lewis *et al.*, 1998). In the case of smaller particles, the increased surface area to volume ratio results in a greater concentration of AS-ODN at or near to the surface with the consequent more rapid release (Lewis *et al.*, 1998). An increase in AS-ODN loading also results in a higher burst effect (Zhu *et al.*, 2002; Kilic *et al.*, 2005) and a more rapid release rate (Lewis *et al.*, 1998). The release profile is only slightly dependent on nucleic acid length, with a release rate that increases as the AS-ODN molecular weight decreases (Khan *et al.*, 2004). Finally, a high burst effect and a rapid *in vitro* release rate have been attributed to microsphere porosity. Pores on the microsphere surface occur when an osmotically active agent, such as AS-ODN, is added to the internal aqueous phase. The use of an osmotic agent, such as NaCl, into the external aqueous phase allows to reduce microsphere surface porosity, thus reducing the initial ODN burst effect as well as the *in vitro* release rate (Freytag *et al.*, 2000; De Rosa *et al.*, 2003b; Gomes dos Santos *et al.*, 2006a).

Besides single-stranded AS-ODN, double-stranded ODN have also been successfully encapsulated within PLGA microspheres (De Rosa *et al.*, 2005). In particular, microspheres encapsulating a decoy ODN against the transcription factor κB (NF-κB) showed a negligible burst effect and a very gradual release profile, with the decoy ODN completely released after about 40 days (De Rosa *et al.*, 2005). A double-stranded siRNA was also encapsulated within PLGA microspheres (Khan *et al.*, 2004). For the same formulation, the *in vitro* release of siRNA was found to be much slower and with a very limited burst effect, compared with that obtained with a single-stranded AS-ODN. The difference between the release profiles was ascribed to the different chemical nature of the two nucleic acids, while only a modest effect was attributed to the different molecular weight (Khan *et al.*, 2004). Increase in AS-ODN encapsulation efficiency within PLGA or poly(lactide) microspheres, as well as optimization of their *in vitro* release properties, has been achieved by using cationic additives able to complex the nucleic acid within the matrix. The *in vitro* release rate of AS-ODN from PLGA microspheres was slowed by complexing with zinc acetate (Putney *et al.*, 1999). A limited burst effect together with a linear release was observed (70% of the loaded ODN released over 9 days).

Most of the studies dealing with controlled release systems have been conducted in animal experimental models. However a few of them have been focused on the *in vitro* fate of AS-ODN when delivered by large particles that cannot to be taken up by cells. Very surprisingly, the release rate can play an important role in cellular uptake. In recent studies, it was demonstrated that, at a fixed concentration, AS-ODN cell uptake is enhanced by slow release, compared with AS-ODN administered in naked form (De Rosa *et al.*, 2002; 2003b; 2005). Confocal microscopy studies showed a significant higher uptake of a single-stranded AS-ODN when slowly released form microspheres of about 30 μm, compared with naked AS-ODN (De Rosa *et al.*, 2002). Encapsulation in 25 μm PLGA microspheres also increased the cellular uptake of double-stranded decoy ODN against NF-κB (De Rosa *et al.*, 2005). This was accompanied by an increased inhibition of NF-κB activation (80-fold higher than with naked decoy ODN), as suggested by decreased NO production, inducible nitric oxide synthase and TNF, tumour necrosis factor-α (TNF-α) expression as well as reduced NF-κB/DNA binding (De Rosa *et al.*, 2005). All these results could be explained by the fact that AS-ODN entry into cells occurs principally by a pinocytotic process (Lebedeva *et al.*, 2000) that is saturated at high concentrations of naked AS-ODN, exposing a high dose fraction to degradation by exonucleases. When encapsulated within PLGA microspheres, AS-ODN is efficiently protected from the enzymic activity; then, when the AS-ODN is released gradually from microspheres, pinocytosis would not be expected to be saturated and a higher percentage of ODN can enter into cells.

In vivo, PLGA microspheres loaded with AS-ODN against rat tenascin mRNA were tested on smooth muscle cell proliferation and migration (Cleek *et al.*, 1997). The study demonstrated a dose-dependent growth inhibition with the AS-ODN-loaded microspheres. Different AS-ODN distribution in neostriatum of rat brain was observed by comparing a fluorescently labelled AS-ODN administered in naked or in microencapsulated form (Khan *et al.*, 2000). In particular, in the case of naked AS-ODN, a punctuate fluorescence was found in the cell cytosol at 24 h post injection, while a weak signal was observed at 48 h. In contrast, when administered in microencapsulated form, fluorescently labelled AS-ODN were still clearly visible in the neuronal tissue (Khan *et al.*, 2000). Biodistribution of tritium-labelled AS-ODN, administered in naked form or encapsulated into microspheres, was investigated after subcutaneous administration to Balb-c mice (Khan *et al.*, 2004). In the case of naked AS-ODN, the majority of the radioactivity associated to the nucleic acid was eliminated within 24 h. The use of AS-ODN entrapped within microspheres resulted in high levels of radioactivity at the injection site and a lower level throughout the body, 24 h after inoculation. After 7 days, the level of radioactivity remained high at the administration site and was considerably lower in the liver, kidney and spleen. Microautoradiography analysis of liver and kidney showed a similar biodistribution for naked and microencapsulated AS-ODN (Khan *et al.*, 2004). The *in vivo* potential of PLGA microspheres as a delivery system for a decoy ODN to NF-κB has been recently tested in a rat model of chronic inflammation. Subcutaneous injection of PLGA microspheres releasing decoy ODN inhibited λ-carrageenan-induced leukocyte infiltration and formation of granulation tissue for up to 15 days, whereas naked AS-ODN at the same administered dose showed a similar effect only from 1 to 5 days (G. De Rosa, pers. comm.).

Our group has designed a 'Trojan' delivery system that combines the advantages of microspheres (sustained release) and of PEI/AS-ODN nanoparticulate complexes: that is an enhanced ODN intracellular penetration (Figure 4) (De Rosa *et al.*, 2002; 2003a,b; Gomes dos Santos *et al.*, 2006a). The rationale behind these systems is to provide controlled release of nucleic acids in a form that can penetrate cells. Thus, AS-ODN/PEI complexes have been encapsulated within porous PLGA (De Rosa *et al.*, 2002; 2003a,b; Gomes dos Santos *et al.*, 2006a). The simultaneous release of AS-ODN and PEI resulted in a further improvement of ODN cell uptake with accumulation into the nucleus (De Rosa *et al.*, 2002; 2003b). Several different types of AS-ODN were entrapped with the microspheres, and depending on the ODN properties and on the experimental conditions in which ODN and PEI are mixed, soluble (De Rosa *et al.*, 2002) or insoluble complexes can be encapsulated (De Rosa *et al.*, 2003a; Gomes dos Santos *et al.*, 2006a). AS-ODN complexation with PEI improved its encapsulation efficiency (De Rosa *et al.*, 2002; 2003a; Gomes dos Santos *et al.*, 2006a), probably by an electrostatic interaction between the cationic ODN/PEI complex and the anionic PLGA (De Rosa *et al.*, 2002). The release rate was shown to be dependent on the porosity of the microspheres; the higher the porosity, the faster the release and the higher the burst effect. Using an analytical solution of Fick's second law of diffusion, it was shown that the early phase of phosphorothioate AS-ODN and phosphorothioate AS-ODN/PEI complex release was primarily controlled by pure diffusion, irrespective of the type of microspheres (Gomes dos Santos *et al.*, 2006a). This system was tested *in vivo* in an experimental model involving an AS-ODN against the cytokine TGF-β2 intended to promote wound healing after filtration surgery in a model of glaucoma in the rabbit (Gomes dos Santos *et al.*, 2006a). Twenty-four hours after the subconjunctival injection of microspheres containing AS-ODN/PEI complexes, the AS-ODN was observed to be in the conjunctival stroma. Six days after the injection, the AS-ODN was located in conjunctival cells, with accumulation within the nucleus. No conjunctival hyperaemia was observed for 6 weeks after the injection. Treatment of rabbits with PLGA microspheres allowing slow release of anti TGF-β2 AS-ODN prolonged bleb survival for 28 days (50% of the treated eyes) following trabeculectomy. The effect was significantly higher in the case of PLGA microspheres releasing AS-ODN/PEI complexes, with a bleb survival of 42 days in 100% of the treated eyes (Gomes dos Santos *et al.*, 2006a).

Conclusion

This review highlights the large diversity of particulate systems that have been designed for the delivery of AS-ODN and siRNA. There are still many difficulties to be overcome before any of them can proceed into clinical trials. The development of 'smart' nanotechnologies able to control the interactions with biological fluids and to be recognized by target cells should be pursued. The research on alternative routes of administration and on local controlled delivery systems should also be extended.

Conflict of interest

None.

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