Expert Opinion

Controlled delivery of antisense oligonucleotides: a brief review of current strategies

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Antisense therapy has been investigated extensively over the past two decades, either experimentally for gene functional research or clinically as therapeutic agents owing to the conceptual simplicity, ease of design and low cost. The concept of this therapeutic approach is promising because short antisense oligonucleotides (ASOs) can be delivered into target cells for specific hybridisation with target mRNA, resulting in the inhibition of the expression of pathogenic genes. However, the efficient delivery of the ASO molecules into target cells remains challenging; this bottleneck together with several other technical hurdles need to be overcome before this approach becomes effective and widely adopted. A variety of vectors such as lipids, polymers, peptides and nanoparticles have been explored. This review outlines the recent advances of the non-viral ASO delivery strategies. Several recent scientific studies, including authors' contributions, have been selected to highlight the technical aspects of ASO delivery.

Keywords: antisense, cationic lipids, controlled release, gene delivery, liposomes, microspheres, nanoparticles, oligonucleotides, polymers, surface matrices

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1. Introduction

Antisense oligonucleotides (ASOs) are short single-stranded molecules, each typically < 30 nucleotides in length [1-3]. They have been investigated extensively for some three decades since their first discovery in the late 1970s [4-6]. The molecular mechanism of the antisense technology is conceptually straightforward. ASO molecules bind to specific sequences of mRNA through Watson–Crick base pair hybridisation, resulting in unavailability of the mRNA for interacting with those molecules that are crucial in the cell intermediary metabolism. The alteration of the molecular pathway significantly affects the cell behaviour, including translation, splicing, growth, and so on. The interaction of ASO analogues with mRNA can also activate RNase H that can degrade the duplexes formed by ASO analogues and mRNA [1-3,6-12]. These concepts and phenomena have been used by scientists and physicians to develop many disease treatments, especially in the fields of cancer, AIDS, diabetes and cardiovascular disease therapies [3,13-17]. Several ASOs and aptamers have been used for clinical trials. (For a review of recent status, see [18,19]). The first successful antisense therapy for AIDS has been approved by the FDA [19,20].

With increasing understanding of gene-related diseases, antisense therapy has become promising in clinical medicine for many more diseases [21]. For example, it has also been used recently as an antibiotic therapy [22]. However, the benefits cannot be fully realised before appropriate delivery systems have been constructed [9,23]. For successful delivery of ASO, several obstacles have to be overcome. These include the delivery of the ASO molecules to the cell, binding to the cell

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membrane, internalisation, release of ASO to cytosol, binding to a particular mRNA, and cleavage of the ASO/mRNA duplexes to inhibit protein expression [24,25]. So far, the efficient delivery of ASOs into target cells is still one of the bottlenecks for the successful utilisation of ASOs as therapeutic agents [10,13,17,26]. The naked ASOs are rapidly degraded by DNases after internalisation into the cells, with most of the previous delivery systems being either inefficient or having high toxicities. Hence, the poor delivery efficiency and high toxicity have limited clinical applications [2]. Owing to the unavoidable concerns over safety issues, immunological and mutational hazards and potential infections, viral delivery systems were not ideal and have gradually been substituted by non-viral strategies [13,25,27,28]. With the understanding of the delivery mechanisms underlying non-viral delivery routes, many new methods and chemicals have emerged. Over the past decade or so, the effort to develop more effective formulations with lower side effects has become fruitful.

2. Delivery strategies for ASOs

Many strategies have been developed to improve the stability and efficiency of ASO delivery [8,29]. A variety of chemically modified oligodeoxyribonucleotides (ODNs), such as peptide nucleic acids (PNAs), polymer-conjugated oligonucleotides and those with taggings of phosphorothioate ASO, methylphosphonate ASO and morpholino ASO, have been developed to overcome the instability of normal DNA and RNA oligonucleotides in both research and therapeutic applications [3,6,17,30-32]. Although these modified ASOs have better stability, some of them result in the reduction of biological activity and increased side effects [14,33,34]. More details of common chemical modifications of ASOs have been summarised previously in several reviews [3,8,11,16,18,29,32,35,36]. In recent years, peptides have been increasingly investigated for the purpose of drug delivery vectors. A wide variety of cell-penetrating peptides (CPPs) have been reported to promote the cellular uptake of DNA molecules. (For a review, see [34,37,38]). Designed peptides have also been investigated [39,40]. However, for antisense oligonucleotides, most peptide-based delivery studies were conducted with PNAs in which the ribose-phosphate skeleton had been substituted with a simpler polyamide backbone. This change protects the molecular degradation by nucleases and proteases. On the other hand, it also results in an inability to activate RNase H. (For a review, see [37,41]).

Cationic lipids and polymers are now the most widely used vectors to help ASOs enter cells and increase the circulation half-life [25,29,42]. Functionalised nanoparticles have also been investigated. In some particular applications, such as medical implants, engineered surfaces have been created as substrates for ASO release. Other strategies have also been investigated for enhancing the ASO delivery, such as functionalised carbon nanotubes [43], polysaccharide-based delivery [24,44,45], microinjection, electroporation or particle bombardment. (For a review, see [46]). This review focuses on the recent advances relating to the applications of lipoplexes, polyplexes, particles and surface-based encapsulation strategies.

2.1 Lipoplexes and polyplexes

Various cationic lipids and polymers have shown promising effects in facilitating DNA delivery with a varying degree of success. The basic mechanism for ASO delivery is relatively simple. The cationic vectors readily interact with ASOs to form complexes through electrostatic interaction [47]. The neutralised and/or positively charged complexes facilitate their binding to anionic cell membrane. The vectors protect the ASOs from enzyme degradation after being internalised within the cells and transport the ASOs to the target position. The complexes should also be able to dissociate before the ASOs can undertake their biological functions properly.

2.1.1 Lipoplexes

Among those vectors used for gene delivery research and clinical applications, lipids are the early non-viral vectors that have been investigated extensively [8,13,28,29]. The first use of lipids for gene delivery was in 1987 [48]. It was developed as a result of the unsatisfactory performance of an existing formulation. In the past two decades, many lipid formulations have been commercialised as gene delivery reagents (e.g., Lipofectin®, Oligofectamine™, Invitrogen) for research purposes or as therapeutic agents for clinical use [8,13,25,27].

Lipid materials can form bilayers, stacked multilayers, various micelles and other aggregates in aqueous solution or in solvents such as ethanol. The types of aggregate formed can be controlled by the types of lipid used as well as the solution conditions. DNA or other drug molecules can be formulated into liposomes/vesicles or bind onto the surfaces of the bilayers or multilayers, depending on the chemical properties of the drugs and the particular applications concerned. Unmodified ASO molecules are negatively charged and can easily be captured by cationic lipids through electrostatic interaction [25]. The complexes formed by lipids and ASOs are called lipoplexes [13]. The binding of lipids with ASOs not only protects ASOs from being degraded by the enzymes inside the cells, but also increases the circulation lifetime in biological fluids and cellular uptake [8,27].

The commonly used lipids in liposome formations include 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), dimyristooxypropyl dimethyl hydroxyethyl ammonium bromide (DMRIE), *N*-[1-(2,3-dioleoxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), cetyl trimethylammonium bromide (CTAB), dioctadecyl dimethylammonium bromide (DODAB), 1,2-bis-(oleoyloxy)-3-(trimethylammonium)propane (DOTAP), *N*-(a-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), 2,3-dioleoyloxy-*N*-[2(sperminecarboxamido) ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA), dioctadecylamidoglycylspermine (DOGS), and so on. (For a review, see [8,13,21,29,49,50]). Most of them are cationically charged,

as the cationic property enables the ASOs to be encapsulated and the anionically charged cell membranes to be perturbed. However, binding of positive lipid/ASO complexes to serum components often results in aggregation, poor transportation and reduced efficiency [51]. Anionic complexes have been studied for DNA delivery proposes; however, their poor efficiency has prevented them from becoming popular [8]. In recent years, extensive research has concentrated on the improvement of lipid formulations. Many chemical additives have been added to lipid formulations to enhance transfection, increase circulation time and moderate toxicity. DOPE is one of the chemicals that has been widely used to fuse with the endosomal membrane, thereby destabilising the endosome and facilitating DNA transport into the cytoplasm [51,52]. Another example is that the presence of natural lipids (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy(polyethylene glycol)-2000 [PEG-DSPE]) in the formulation could maintain a high concentration of complexes for several hours in blood fluids [53]. Recently, Pakunlu *et al.* [54] have demonstrated that PEGylated liposomes can penetrate cancer cells *in vitro* and tumour cells *in vivo*. The ASOs delivered by the PEGylated liposomes effectively inhibited the expression of targeted mRNA and proteins. However, other reports also found that the PEG-conjugated lipids may be detrimental for ASO delivery as they can decrease ASO internalisation and inhibit ASO release from the endosomal/lysosomal compartment [55]. Biomolecules such as antibodies have also been used to increase delivery efficiency and targeting. For example, endothelium-specific antibody was successfully incorporated into lipid vesicles. The antibody efficiently mediated the delivery of ODN to mouse lung endothelial cells *in vitro* and *in vivo* [56]. Apart from incorporating antibodies, many receptor systems have also been introduced. Chiu *et al.* [57] demonstrated that transferrin receptor (TfR)-targeted liposomal formulations had good colloidal stability and transfection efficiency. Zhang *et al.* [58] developed liver-targeting cationic liposomes as a gene carrier for the treatment of hepatitis B virus (HBV) infection. The cationic liposomes (DPPC/DC-Chol) were co-modified with the ligand of the asialoglycoprotein receptor (ASGPR), β-sitosterol-β-D-glucoside (sito-G) and the non-ionic surfactant, Brij 35. The ligand sito-G enhanced ASGPR-mediated endocytosis whereas the non-ionic surfactant Brij 35 facilitated membrane fusion. Thus, the modified formulation resulted in efficient transfection without enhancing cytotoxicity.

Cationic lipids are mostly toxic as they can result in cell shrinkage, reduced number of mitoses, vacuolisation of the cytoplasm and interruption of functioning of some protein kinases [59]. Although they have reasonably good delivery efficiency and reduced toxicity after extensive efforts to improve them (e.g., adding co-lipids or polymers), most current formulations suffer from the use of organic solvents, and are still unsuitable for *in vivo* applications [60]. On the other hand, these lipid systems are by far the best candidates for *in vitro* gene delivery studies. Development of the better formulations still remains an attractive research area.

2.1.2 *Polyplexes*

Polymers have been investigated as promising candidates for gene delivery vectors due to their low cost and ease of synthesis. Through copolymerisation, a variety of functional groups can be incorporated. DNA delivery using polymeric vectors has been studied extensively in the past two decades. (For a review, see [21,26,47,49,61-64]). The most widely used cationic polymer vectors include poly-L-lysine (PLL), polyethyleneimine (PEI), polyamidoamine dendrimers (such as PAMAM) and chitosan. PEI is a good DNA delivery vehicle because of its pH responsive charge dissociation [65]. It raises the endolysosomal pH by accepting the protons pumped into endolysosomes. The pH mediating effect will alter protein folding within the endolysosome and inactivate functional enzymes, effectively helping PEI/DNA complexes escape from the endosome, resulting in relatively high transfection efficiency [62,65]. PEI thus not only helps internalisation but also improves the bioactivity of AS-ODNs. However, it has been reported that PEI might interfere with the host genes as it was found to be inside the nuclei with ordered structures [26]. Transfection by PLL often encounters a balance between efficiency and toxicity [21]. Their derivatives (such as poly(ethylene glycol)-grafted-poly(L-lysine) [PEG-PLL] and poly(ethylene glycol)-grafted-poly(ethylenimine) [PEG-PEI]) have also been studied and reviewed extensively [29,63,66-72]. PEGylation is one of the most important modifications that increases circulation half-life and reduces toxicity. (For a review, see [42]). Recently, Williams and co-workers [73,74] have used PEG-PEI copolymers as a vector for Exon skipping oligonucleotide delivery. They concluded that PEGylated PEI2K copolymers are efficient carriers for local delivery of oligonucleotides and can be developed further as potential therapeutics. PEI-PEG-folate conjugates and PEI/Pluronic® (BASF Corp.) conjugated block copolymers have also be used to form nano-sized polyelectrolyte complexes to improve AS-ODN delivery [75,76]. Micelle-like PEI/Pluronic/ODN particles were obtained after mixing with ODN solution. The polymers effectively increased the zeta-potential and condensed the diameter of the complexes. Cell transfection and inhibition studies demonstrated that the formulations can facilitate the uptake of ODN in human oral epidermoid carcinoma cells and increased sequence-specific activity of antisense ODN, thereby resulting in the inhibition of the functional activity of P-glycoprotein (P-gp) in multi-drug resistant cells [76].

The polymers used so far for DNA delivery are diverse and vary in size, molecular architecture and chemical nature. There is still a lack of understanding to guide the design of polymer structure to match a given DNA and cell type for optimal performance [47,64]. Evidence has shown that molecular masses, polymer and DNA concentration, ionic

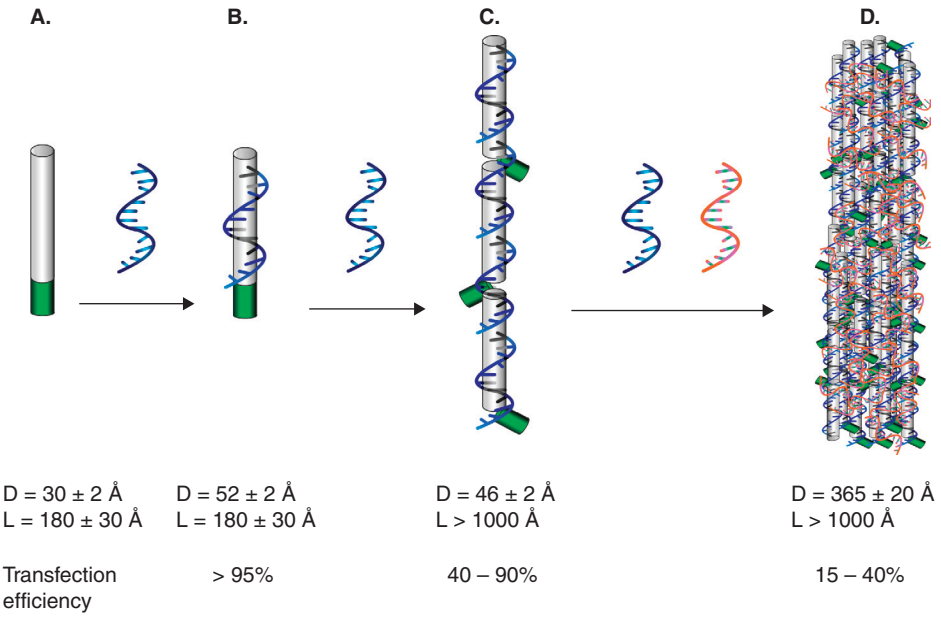

Figure 1. Schematic models to indicate the progressive binding of AS-ODN onto copolymer MPC30-DEA70 to form polyplexes at different N/P ratios (grey cylinders for DEA blocks; green tails for MPC blocks) with their dimensions (diameter D and length L) and transfection efficiencies given under the structures. A. A copolymer cylinder. **B.** A small polyplex at N/P ratios between 10/1 and 5/1. **C.** A small but long polyplex at N/P ratios between 2/1 and 1/1. **D.** A large polyplex at N/P ratios around 0.6/1.
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strength and the methods used to prepare complexes all contribute to the properties of complexes and subsequently affect the efficiency of DNA delivery [62,63]. Hence, details about molecular size, architecture and chemical nature affecting the mechanism and efficiency need to be investigated to gain a better understanding of the systems. One of the most recent examples is the study of the PEI-PEG copolymers with different molecular mass for ASO delivery [77]. The molecular mass of PEG was found to be the determinant of polyplex size, whereas the molecular mass of PEI was found to be significant to the surface charges (zeta-potential) of the complexes. The variation of molecular mass was subsequently found to have varying effects on the stability and transfection efficiency of the complexes formed.

To realise the therapeutic goals of ODNs, not only should effective delivery be achieved, but also a better insight of the mechanism should be provided, including how DNA interacts with vector molecules to form different structured complexes, what kind of complexes show greater efficiency and why. MPC-based block copolymers have been used as vectors for both plasmid DNA and oligonucleotide delivery [47,61,78,79]. For example, the pH-sensitive poly(2-(methacryloyloxy)ethylphosphorylcholine)-*co*-poly(2-(diisopropylamino) ethylmethacrylate) (PMPC–PDPA) diblock copolymer polymersomes have recently been used as non-cytotoxic vectors for encapsulation and delivery of plasmid DNA into human dermal fibroblast cells [78]. The PMPC–PDPA diblock copolymers can efficiently encapsulate DNA and form stable polymersomes at neutral pH. The polymersomes protect the DNA in blood environment, increase the circulation time and facilitate endocytosis. After internalisation the polymersomes dissociate and form polymer/DNA complexes owing to the protonation of tertiary amine groups on the PDPA chains at endocytic pH [78,79]. In previous studies, the authors have investigated complexation mechanisms between a well-defined phosphorylcholine (PC) copolymer and two model DNAs (the small (15 bases) single-strand AS-ODN and large (5.1 kb) double-stranded luciferase plasmid DNA) and correlated complexation with transfection efficiencies [47,61]. The copolymer used was called MPC30-DEA70 (where MPC referred to 2- (meth acryloyloxy)-ethyl phosphorylcholine and DEA referred to 2-(diethylamino)-ethyl methacrylates; 30 and 70 referred to the mean degrees of polymerisation of each block). Small angle neutron scattering (SANS) was used to reveal the structure of the complexes formed between the copolymer and model DNAs. For small AS-ODN, it was found that the AS-ODN molecules bound around the copolymer cylinders at high N/P ratios (10/1 to 5/1, equal to the molecular ratio 2/1 to 1/1) (**Figure 1B**). The complexes then became broader in diameter than the pure copolymer molecules with similar lengths. With the reduced percentage of copolymer molecules (at N/P ratios between 2/1 and 1/1), complexes were interconnected through ODN binding and

bridging (**Figure 1C**). Further reduction in the N/P ratio resulted in the cross-binding (bundling) of the complexes by extra ODN molecules (**Figure 1D**), resulting in a drastic increase in the diameter of the aggregates. These findings were particularly useful in understanding why the complexes had different delivery efficiencies. Cell transfection and growth inhibition were examined using HeLa cells. Complexes formed at high N/P ratios (**Figure 1B**) were small and carried net positive charges, thus having the highest transfection efficiencies (> 95%). The complexes as shown in **Figure 1C** were relatively bigger and almost neutralised, thus having moderated efficiencies (40 – 90%). Very low transfection efficiencies were observed with complexes in **Figure 1D** as their sizes became much bigger and they remained negatively charged. An MTT assay of HeLa cell growth inhibition by copolymer/ODN polyplexes after 24 h incubation demonstrated that the copolymers had low cytotoxicity to HeLa cells over the effective transfection dosage range. It was further found that the complexes formed at high N/P ratios had enhanced cell growth inhibition effect and the complexes outperformed those formed from commercial Oligofectamine in the presence of FBS [47]. The elucidation of the binding mechanism between the copolymer and the ODN molecules thus helped us to gain useful insight into the key factors affecting transfection efficiencies.

2.2 Nanoparticles, microspheres and hydrogels

Nanoparticles [6,80-89], microspheres [14,90-92] and hydrogels [93,94] have shown advantages in gene delivery by their increased stability and controlled-release ability. Many functionalised particle systems have been studied for ASO delivery. One example is gold nanoparticles [84-86]. Oligonucleotide-modified gold nanoparticles are very effective in cellular delivery although they are negatively charged. They are capable of transfecting a variety of cell types with high intracellular stability and low toxicity. Surprisingly, it was also found that the high density of oligonucleotides on the particle surface led to greater uptake efficiency. Further investigation demonstrated that the size of the particles and surface charge increase after exposure to cell culture conditions owing to the adsorption of proteins onto the particles. The authors hypothesised that it was the adsorbed proteins that facilitated the internalisation [84]. Apart from gold nanoparticles, polymer and protein nanoparticles have also been investigated. Chitosan-coated PLGA nanoparticles with tuneable size and charge have recently been introduced for improved ASO delivery [80]. Polyamidoamine (PAMAM) dendrimer-modified magnetic nanoparticles have shown enhancement of ODN delivery efficiency and inhibited growth of human breast cancer and liver cancer cell lines [81]. Nanoscale networks of crosslinked PEG and PEI nanogels can effectively transport and protect the incorporated ODN molecules across the blood–brain barrier. *In vivo* biodistribution studies demonstrated increased accumulation in mouse models [94]. Human serum albumin (HSA) was used to form nanoparticles with native ODN and phosphorothioate-modified oligonucleotides [88,89]. The rapid uptake of ASO-loaded HSA nanoparticles in different breast cancer cells was observed. The nanoparticles showed no cytotoxicity in these cells as well [88]. To develop an even safer particle system and avoid the use of the toxic glutaraldehyde, the surface-modified HSA nanoparticles prepared by heat denaturation were used to deliver ASOs into HER2-positive cancer cells. A significant reduction of Plk1 mRNA and protein expression was observed using the ASO-loaded HSA nanoparticles [89].

Microspheres have been used in the controlled release of ODN molecules for many years [92,95]. Akhtar and Lewis [92] found that biodegradable poly(lactide-*co*-glycolide) (P(LA-GA)) microspheres could improve the delivery of anti-HIV oligonucleotides into macrophage cells in culture. They also found that microsphere size has a significant effect on ODN entrapment efficiencies and *in vitro* release rates. Smaller microspheres released 70% of the entrapped ODN within 4 days whereas the larger ones could last up to 40 days. The microspheres were also found not only to provide sustained delivery of ODN molecules, but also to improve their cellular distribution in brain cells [95]. The combination of nanoparticles, microspheres and hydrogels with other strategies provides promising approaches for local sustained gene delivery [14]. Small particles can either be injected into a specific target area or released from engineered devices for different applications, thereby offering good flexibility. De Rosa *et al.* [14] demonstrated sustained release and improved transfection of ASO into HeLa cells by using PLGA microspheres and PEI as a cationic vector. The PEI solution was added into the PdT16 oligonucleotide solution to obtain PEI/ASO complexes with a high N/P ratio. Complex encapsulated microspheres (**Figure 2A**, **B**) were subsequently prepared by a modified multiple emulsion/solvent evaporation method. The slower ASO release was controlled by an osmotic effect using sodium chloride. The encapsulation of PEI/ASO complexes inside microspheres protected ASO from enzymatic degradation, improved intracellular penetration of ASO, and promoted its localisation in the nucleus (**Figure 2C**). The release profiles lasted > 40 days. Similar work has been reported by Santos *et al.* [90]. Antisense TGFβ2 ODN/PEI complexes and PLGA microspheres were prepared by a similar method. The microspheres showed adequate encapsulation efficiency and release profiles of ODN/PEI complexes and improved the outcome of glaucoma filtration surgery. Polystyrene microspheres have also been utilised for ODN delivery [91]. They were coated with ornithine/histidine-based cationic peptides (O10H6) by self-assembly to obtain cationic microspheres. ODN molecules were then captured through electrostatic interaction. The peptides thus served as bridges between the microspheres and the ODN molecules. Further experiments demonstrated that the polystyrene microspheres not only could enhance the stability of the ODN complexes, but also could facilitate the uptake of the complexes into the dendritic cells.

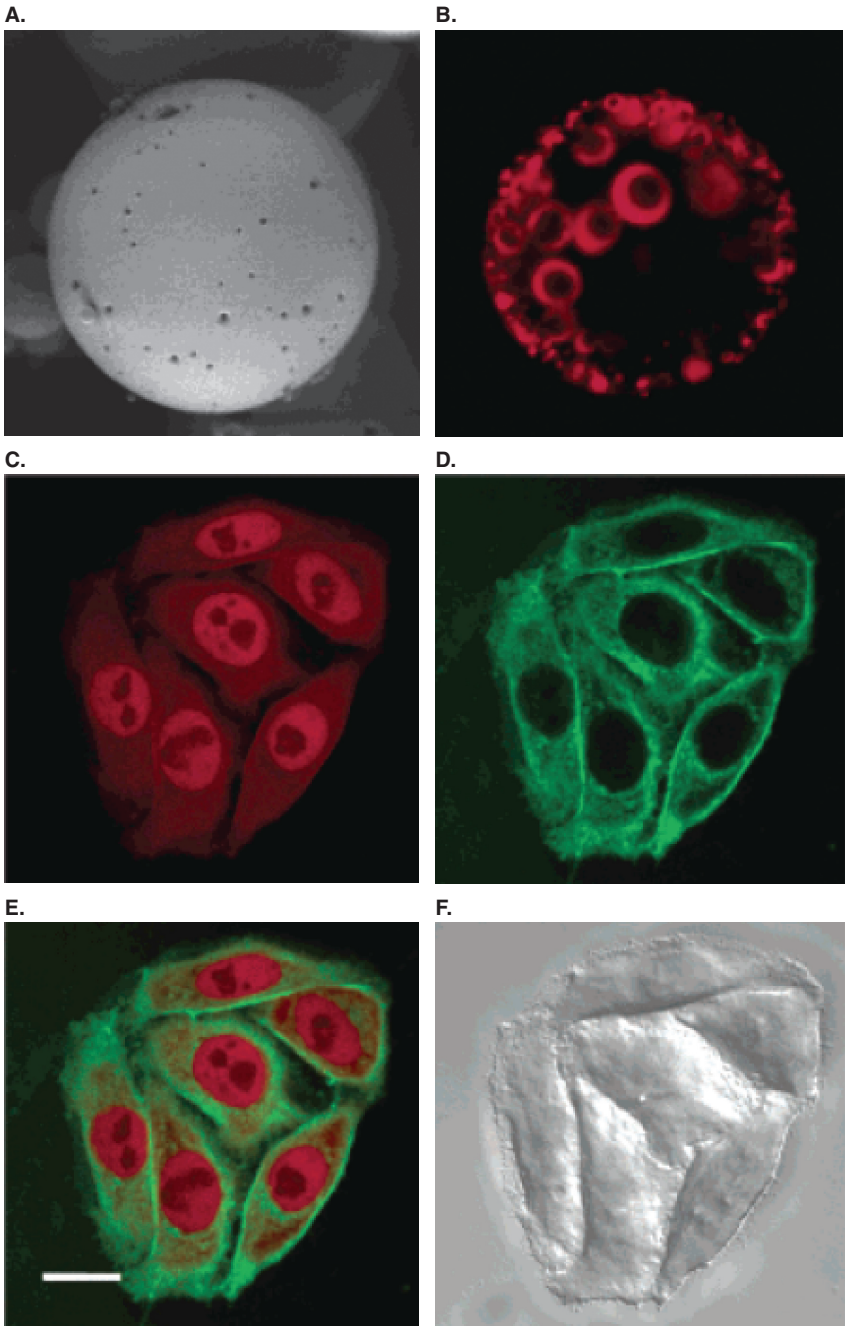

Figure 2. A. Scanning electron microscopy analysis of microspheres encapsulating pdT16/PEI complexes (mean diameter = 28.4 ± 4.0 μm). **B.** Confocal microscopy analysis of microspheres containing pdT16/PEI complexes. **C.** Confocal microscopy of HeLa cells after 24 h of incubation with pdT16/PEI complexes (bar = 20 μm). pdT16 was labelled with rhodamine. **D.** Fluorescence DHPE stain of cell membranes. **E.** Overlapping of the two previous images. **F**. Light microscopy image. All at N/P = 15 and 1% NaCl.
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Table 1. Amount of ASO loading (μg/cm^2) using different layer structures.

Film structure	0% Cationic	5% Cationic	10% Cationic	20% Cationic
400 Å × 1	0.036	0.136	0.426	0.731
400 Å × 5	0.268	0.702	1.604	5.416
2000 Å × 1	0.250	0.938	3.022	5.931
2000 Å × 5	0.580	4.100	13.210	21.815

Reproduced from Zhang Z, Cao X, Zhao X, *et al*. Controlled delivery of antisense oligodeoxynucleotide from multilayered biocompatible phosphorylcholine polymer films. J Control Release 2008;130:69-76.

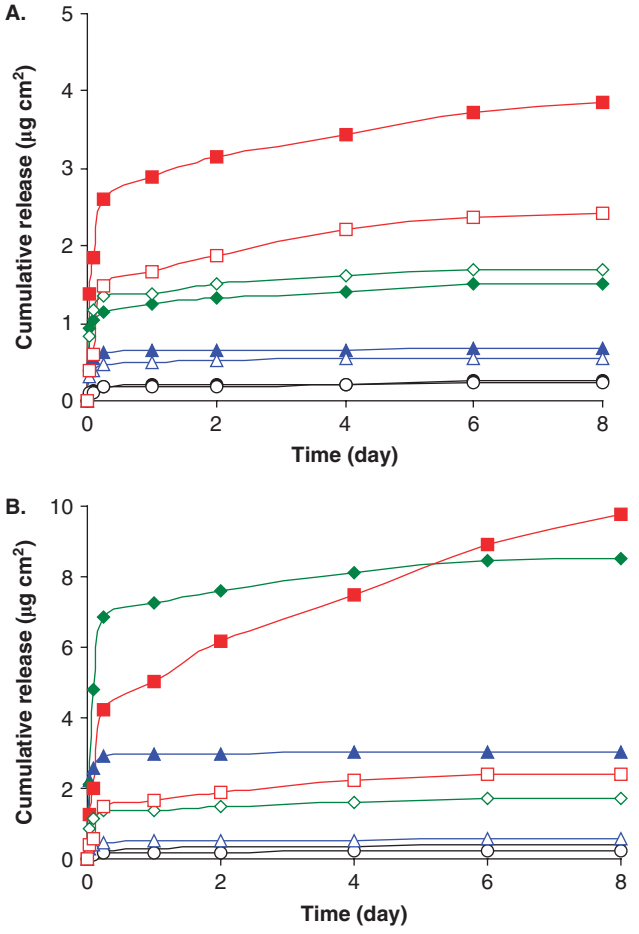

Figure 3. Release profiles of c-myc ASO from different PC/ASO multilayered films eluted with PBS (pH 7.4) at different time points. The curves represent the average cumulative release, as analysed by UV spectrometry. **A.** 400 Å × 5 (filled) versus 2000 Å × 1 (open). **B.** 2000 Å × 1 (open) versus 2000 Å × 5 (filled). Zero per cent cationic (circles), 5% cationic (triangles), 10% cationic (diamonds) and 20% cationic (squares). Unbroken lines are to guide the eye.
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2.3 Surface engineered matrices

The controlled delivery of ASOs to local target tissues remains a challenging technical issue [96]. One successful example is the drug eluting stent. Systemic drug administration has been shown to be inadequate for the delivery of a sufficient amount of drug to the vascular target site, with adverse side effects reported [97]. Alternatively, the controlled release of therapeutic drugs from an engineered stent surface has been regarded as a promising strategy to administer local delivery of drug to targeted cells directly and continuously. Bare metal stents often result in thrombosis and inflammatory reactions, and release toxic metal ions [97]. A polymeric coating on the stent surface not only enhances the biocompatibility of the stents, but also provides a matrix for drug loading and release. As the DNAs are negatively charged, many coating materials with cationic components have been investigated for the application of DNA entrapment through electrostatic interaction.

In previous studies, the authors have devoted efforts to optimising the routes and conditions for controlled loading and release of ASOs from single or multilayered cationically modified phosphorylcholine polymer films [98,99]. PC copolymers performed better with respect to biocompatibility and haemocompatibility than most other types of polymeric material, both *in vitro* and *in vivo* [100-105]. They have been investigated for the controlled release of DNA from surface coatings [106-109], gene transfection [47,61,110], drug delivery [111-114] and stents or other medical devices [102,115,116]. They can form attractive vesicles that can be utilised for controlled loading and release of therapeutic biomolecules. To ensure that the PC copolymers have variable loading capacities and controllable release rates, the PC copolymers were chemically modified. The copolymers used in the authors' work for controlled loading and release study were modified with groups of MPC, lauryl methacrylate (LM), choline methacrylate (CM), 2-hydroxypropyl methacrylate (HPM) and 3-trimethyoxysilylpropyl methacrylate (TMSM). The PC groups provided the copolymers with strong hydrophilicity and biocompatibility. The CM groups were cationic with fractions varying from 0, 5, 10 and 20%. The TMSM was fixed at 5% and worked as a silyl crosslinking agent. HPM helped to adjust the extent of water intake into the hydrogel and the incorporation of LM was used to tune the hydrophobic feature of the copolymers and to promote nanoporous structuring within the polymer network on exposure to an aqueous environment [98,117]. Different film thicknesses (from 8 to 400 nm) were obtained by dip coating using variable copolymer concentrations (from 0.2 to 5 wt%). The ASO was loaded by immersing the film-coated wafer into 0.5 mg/ml ASO solution for 12 h followed by 0.5 h washing and then drying. The thicknesses and masses of the layers before and after loading of ASO were monitored. It was found that the amount of ASO loaded increased with the cationic charge density in the film. There was little incorporation of ASO into the polymer film with 0% cationic

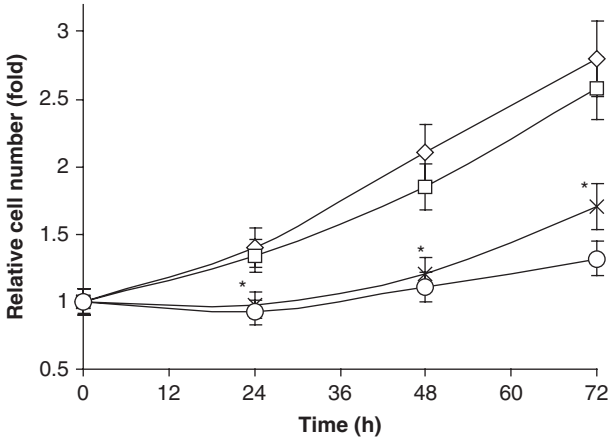

Figure 4. Growth curve of HeLa cells transfected with c-myc ASOs eluted from multilayered films (2000 Å × 5) at 2 – 6 h with Oligofectamine™ as transfection vector. Five per cent FBS stimulant (diamonds), vehicle control (squares), 20% cationic (crosses) and positive control (circles).
*Compared with vehicle control,
$p < 0.05$.
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charge. On the other hand, the extent of ASO loaded in the other three cationic PC copolymer films was approximately proportional to the molar fraction of the charge groups incorporated in the copolymers. Another general feature was that the amount of the loaded ASO increased with film thickness. The smooth zero-order release of ASO from the films was observed with a small burst in the first half hour. The release lasted up to 8 days and the slope of the release profiles increased with cationic charge density. The molecular integrity of the ASO molecules eluted from the films was confirmed by gel electrophoresis. The transfection of ASOs released from different PC copolymer films to the vascular smooth muscle cells (VSMCs) showed efficiency comparable to that of the control ODN samples under identical solution concentrations [98].

Following the success of the single layer ASO loading and release, multilayered polymer and ASO films were constructed by an alternative dipping method [99]. The amount of ASO loaded by the films increased with charge density, film thickness and the number of layers (**Table 1**).

The ASO release profiles of 400 Å × 5 (layers) films are mostly similar to the 2000 Å × 1 (layer) system. A fast release occurred in the first few hours followed by a slow release process up to 8 days. However, at 20% cationic charge density, the ASO released from the 400 Å × 5 film was more than that from the 2000 Å × 1 film [99]. A comparison between the single 2000 Å × 1 films and the multilayered 2000 Å × 5 films showed that increasing the number of sublayers leads to increased ASO loading at a given cationic charge density (**Table 1**). The release study demonstrated that the multilayered films could release significantly more ASO molecules with high slope profiles (**Figure 3**). An *in vitro* study of cell growth inhibition by the released ASO molecules (**Figure 4**) clearly confirmed that the molecules retained full bioactivity [99].

In addition to the *in vitro* study, the cationically modified PC copolymers have been used for *in vivo* research in a pig model by Chan *et al*. [118]. The Bio*divYsio* drug delivery stents were coated with a PC-incorporated copolymer containing 20% cationic charges as described above [98,99,119]. The coated stents were dipped into the ASO-c-myc (partially end-labelled with ^{32}P-ATP) solution for 1 h at room temperature, after which they were dried and deployed into pig coronary arteries. Local cellular uptake of fluorescently tagged ASO-c-myc was observed in both endothelial and medial vascular smooth muscle cells (**Figure 5**). The uptake of ^{32}P-labelled ASO-c-myc delivered from 20% cationic PC stents into porcine coronary artery was 0.28 mg at 1 h, 0.17 mg at 6 h, 0.25 mg at 24 h and 0.29 mg at 48 h [118]. Western blotting results demonstrated that stents loaded with ASO-c-myc inhibited c-myc protein expression at 6 h compared with control stents in 3 of the 4 separate pig experiments. The ASO-c-myc-loaded stents caused a significant increase in vessel lumen area, and reduction in neointimal area and thickness compared with control stents.

3. Conclusions

As non-viral delivery strategies allow advances in nanotechnology and molecular science to be directly adopted, they are powerful tools for the delivery of ASOs and represent the mainstream of future development. Enormous research progress has been made in the field of non-viral delivery of antisense oligonucleotides over the past two decades. Further advances in the selection of molecular vectors, nanoparticles and polymeric matrices would serve to reduce the existing constraints such as biocompatibility, cytotoxicity and cyto-selectivity, bringing more systems closer to real medical applications. This review highlights only the recent representative scientific studies on ASO delivery. Reviews on other related specific topics are widely available, with some of them cited in this work.

4. Expert opinion

With rapid advances in non-viral gene delivery over the past two decades, many vectors and formulations have been developed for controlled release of ASOs under different conditions. Substantial improvements have already been made to increase efficiency and reduce toxicity, but most of the systems developed so far are only competent for *in vitro* research purposes, with clinic applications remaining limited. It is the authors' view that there is a lack of understanding of the detailed delivery mechanisms for different systems. This lack of mechanistic insight has led to a lack of reliable guidance to vector design. Most of the studies have

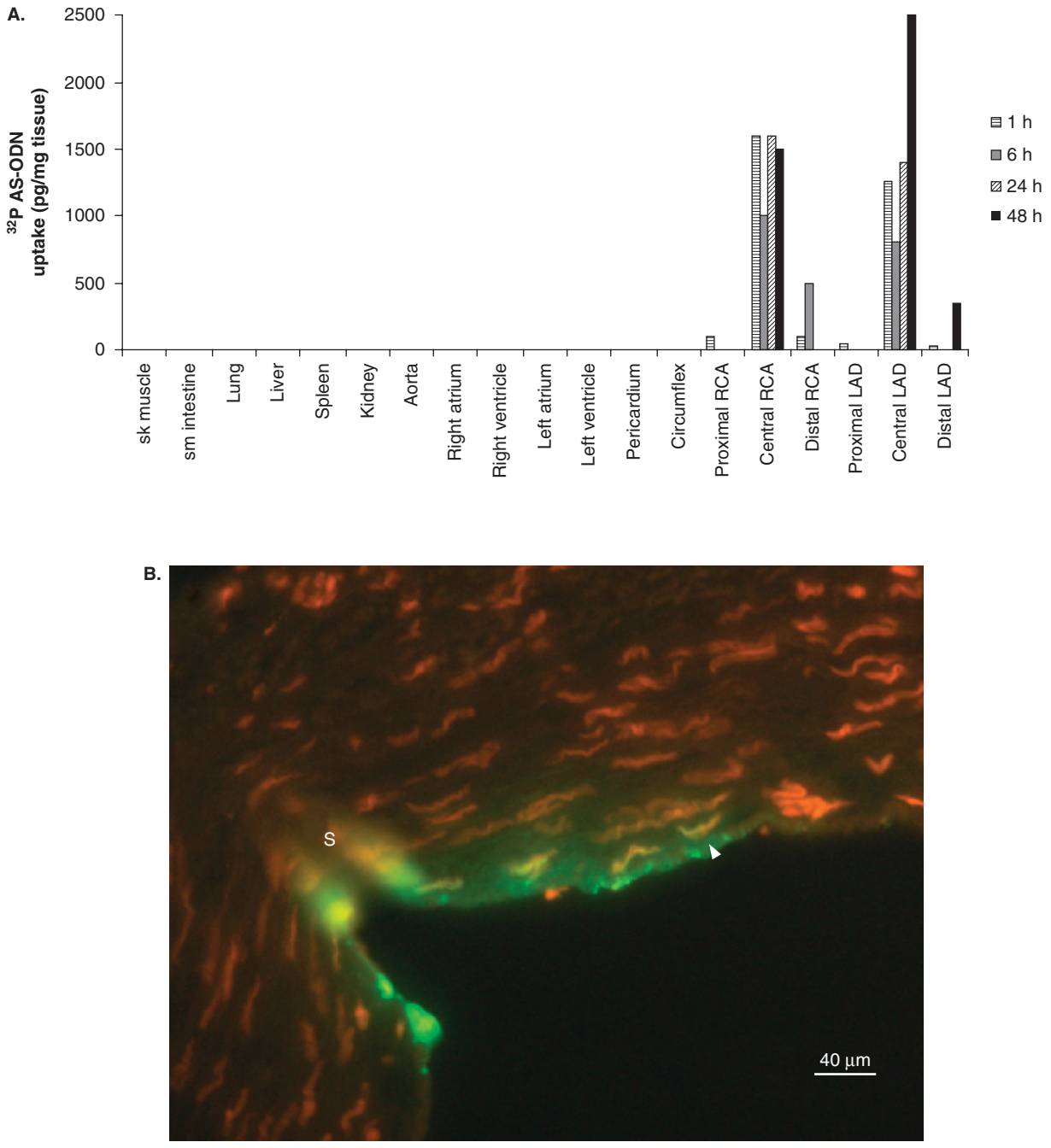

Figure 5. Detection of ^{32}P-and fluoresecently labelled ASO-c-myc delivery into pig coronary artery by means of cationically modified PC-coated stents *in vivo*. A. Quantification of local and systemic delivery of ^{32}P-labelled ASO-c-myc (pg/mg tissue) delivery to porcine coronary artery *in vivo*. **B.** Localisation of fluoresecently labelled ASO-c-myc (green/yellow) delivery to porcine coronary artery *in vivo*. Nuclear uptake of ASO can be seen in the region where the stent struts would have been (S). Section counterstained with ethidium bromide (orange). Original magnification × 40.

Reproduced from Chan KH, et al. Vascular delivery of c-myc antisense from cationically modified phosphorylcholine coated stents. Biomaterials 2007;28:1218-24

focused mainly on the characterisation of the vector/DNA complexes using techniques such as dynamic light scattering, gel electrophoresis, atomic force microscopy, scanning electron microscopy, fluorescent and confocal microscopy. These techniques can reveal only the size distribution, zeta-potential and morphology of the complexes formed before they are presented to cell cultures or animal models. More detailed information such as the specific interactions between vector and DNA, the dynamic processes of attachment of the complexes to cell membrane, the subsequent processes of complex internalisation, trafficking pathways across cytoplasm, and further steps leading to the internalisation to nucleus, is difficult to gain owing to experimental difficulties. Furthermore, information such as when and where the complexes dissociate from ASOs is also lacking. The development of new techniques such as particle tracking and quantum dot labelling will enhance the technical strength of fluorescence microscopy and help to gain more detailed information of gene trafficking across different subcellular compartments inside a given cell. Only when these detailed observations are available will the dynamic and structural implications of vectors to cells be better understood, and modifications of existing formulations will then be made under direct and more reliable guidance so that cell-specific tuning of delivery efficiency, bioactivity, cytotoxicity and cell selectivity can be substantially improved.

Although different DNA molecules have different biological functions, from a biophysical point of view, they have similar physicochemical properties (except those resulting from chemical modification, or artificial design), such as charge density, GC (guanine and cytosine) percentage and hydrophobicity. However, some physicochemical properties such as molecular mass or number of bases, single or double strands have significant effects on complex formation and controlled release. Hence, specific formulations need to be developed for different ranges of DNAs. Some commercial lipid formulations as transfection reagents are specially formulated for small oligonucleotides whereas others are for large plasmids. Subdivision of the formulations according to the different DNA types and/or ranges could improve the efficiency and reduce toxicity. Technological developments of complex fabrication and interfacial loading/entrapment will also have positive contributions.

New materials such as self-assembly peptide hydrogels have already been investigated for drug delivery [120,121]. They can be artificially designed to have different size, charge characteristics and physiological properties with inherently good biocompatibility and feasibility. They are thus potentially promising for gene delivery. The complementarity of vectors and hydrogel matrices in the form of either particles or interfacial films/layers could achieve prolonged controlled release of DNA molecules.

Declaration of interest

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