

REVIEW

Balancing protection and release of DNA: tools to address a bottleneck of non-viral gene delivery

Christopher L. Grigsby and Kam W. Leong*

Department of Biomedical Engineering, Duke University, 136 Hudson Hall, Box 90281, Durham, NC 27708, USA

Engineering polymeric gene-delivery vectors to release an intact DNA payload at the optimal time and subcellular compartment remains a formidable challenge. An ideal vector would provide total protection of complexed DNA from degradation prior to releasing it efficiently near or within the nucleus of a target cell. While optimization of polymer properties, such as molecular weight and charge density, has proved largely inadequate in addressing this challenge, applying polymeric carriers that respond to temperature, light, pH and redox environment to trigger a switch from a tight, protective complex to a more relaxed interaction favouring release at the appropriate time and place has shown promise. Currently, a paucity of gene carriers able to satisfy the contrary requirements of adequate DNA protection and efficient release contributes to the slow progression of non-viral gene therapy towards clinical translation. This review highlights the promising carrier designs that may achieve an optimal balance of DNA protection and release. It also discusses the imaging techniques and three-dimensional *in vitro* models that can help study these two barriers in the non-viral gene transfer process. Ultimately, efficacious non-viral gene therapy will depend on the combination of intelligent material design, innovative imaging techniques and sophisticated *in vitro* model systems to facilitate the rational design of polymeric gene-delivery vectors.

Keywords: non-viral gene delivery; nanomedicine; biophotonics; responsive delivery; nanoparticle; polyplex

1. INTRODUCTION

Gene therapy has the potential to treat a wide variety of inherited and acquired genetic disorders, including diabetes, cystic fibrosis, cancer and haemophilia (Vile *et al.* 2000; Ferrari *et al.* 2002; Walsh 2003). The theoretical simplicity of the technique, along with the sequencing of the human genome, gave rise to the field amidst considerable excitement in the 1990s (Mulligan 1993). To treat diseases caused by a missing or aberrant protein, DNA encoding the desired gene is introduced to the nucleus of a cell, where it is subsequently processed into a functional protein. In practice, however, this has proved much more difficult than predicted. The lack of safe and effective gene-delivery methods has hindered the clinical translation of gene therapy. Many potential barriers exist along the path from the laboratory bench to the nucleus of a cell. First, the DNA must reach the plasma membrane of a target cell and be taken up. The

path to target cells is non-trivial, as the extracellular environment contains many molecules able to trap and degrade DNA. Once inside the cell, the DNA encounters a harsh enzymatic environment that promotes its degradation. In most cases, the administration of naked DNA results in insufficient amounts of intact coding sequence reaching the nucleus. In an effort to increase the amount of intact DNA reaching the nucleus, various techniques have been used with mixed degrees of success. Poration of the plasma membrane has been accomplished using electrical and mechanical means such as electroporation, ultrasound-based sonoporation and the gene gun. However, for reasons of low efficacy, tissue damage or poor access to deep tissue, these methods pose significant barriers to translation *in vivo*. The bulk of current research is focused on the use of vectors designed to package and protect a DNA payload for delivery into the cell. Vectors commonly used today range from viruses to lipids, peptides and polymers.

The majority of gene therapy clinical trials have relied on viral vectors for gene transduction owing to their high efficiency. However, the high transfection efficiency of viral vectors comes at a cost. Viral vectors pose safety

*Author for correspondence (kam.leong@duke.edu).

One contribution to a Theme Supplement 'NanoBioInterface: crossing borders'.

concerns stemming from their immunogenicity and toxicity (Check 2003; Williams & Baum 2003; Cavazana-Calvo *et al.* 2004; Kaiser 2004). Limitations of cell mitosis for retrovirus, contamination of adenovirus and packaging constraints of adeno-associated virus also lessen their appeal. Non-viral vectors composed of cationic polymers, lipids and peptides able to form ionic complexes with DNA currently achieve lower and transient transgene expression levels, but possess the potential advantages of unrestricted DNA cargo size, ease of synthesis, low immunogenicity and potential for repeated administration. They can also address many pharmaceutical considerations better than viral vectors, such as scale-up, storage stability and quality control. Still, non-viral gene delivery remains prohibitively inefficient for most therapeutic applications. Development of safe and effective non-viral gene carriers is critical to the eventual success of gene therapy.

Polycationic polymers have been extensively investigated as gene-delivery vectors because of their versatility. They are able to interact electrostatically with negatively charged nucleic acids to form stable particles, termed polyplexes, with diameters of the order of nanometres. Physical properties of polymers such as rigidity, hydrophilicity, charge density, biodegradability and molecular weight can be tuned to modulate gene-delivery properties such as DNA binding, colloidal stability of ionic complexes, endosomal escape, vector unpacking, cytotoxicity and transfection efficiency. Also, it is likely that for different target cells and tissues, or different routes of administration, the optimal characteristics of the DNA-polymer nanoparticles would differ. For gene therapy to advance, rational design of gene-delivery vectors able to address the individual rate-limiting steps identified along the gene-delivery pathway is necessary. These steps include cellular localization and binding, internalization, subcellular trafficking, endosomal escape, unpacking and release and nuclear translocation (figure 1). Each must be studied individually and in concert, as changes in the structure of a polymeric vector designed to increase unpacking could adversely influence its behaviour during another step up- or downstream. Without considering each step independently and systematically, advances will remain phenomenological and stochastic. Once each step is understood, the knowledge can be integrated towards the rational design of polymeric gene carriers that address multiple barriers.

There are many excellent reviews covering all the barriers to non-viral gene delivery (Pack *et al.* 2005; Read *et al.* 2005; Wong *et al.* 2007). This review attempts to focus on one particular aspect, namely the conflicting requirement of protection and release of DNA. One crux of the non-viral gene-delivery pathway is the release of intact nucleic acids from the polymer into the cytoplasm or nucleus of a target cell. If release occurs too slowly or not at all, the DNA will not be accessible to the transcriptional machinery and will eventually be lost or diluted out by processes including exocytosis and mitosis. If release occurs too readily, or if the polyplex is not compact enough to resist enzyme penetration, the DNA is susceptible to degradation prior to reaching the nucleus. Various

vector design strategies have been tried to achieve release at the right time and place. So far, the competing functionalities of protection and efficient release have proved difficult to engineer into polymeric gene carriers. Fortunately, recent advances in imaging modalities, microscopy and complex tissue models have begun to help elucidate the barriers encountered by a polyplex on the path to transfection. Both live and fixed cell imaging have advanced such that single particles can be tracked intracellularly in the spatio-temporal domain. Polyplexes can be tagged and tracked to determine their uptake kinetics and subcellular localization at specific time points, as well as their dissociation status. The ability to resolve structure-function relationships at the subcellular, single-particle level provides valuable information for the rational design of the next generation of polymeric gene carriers. This review examines the current rational design strategies to effect appropriate DNA release at the optimal time and place while maintaining adequate DNA protection, as well as the most important imaging techniques and engineering tools that allow for the characterization and evaluation of these gene carriers. Systematic studies using state-of-the-art imaging techniques and models will enable the rational design of polymeric gene carriers that address the tradeoff between protection and efficient release of DNA, helping to close the gap between engineering phenomena and therapeutic success.

2. RELEASE AS A RATE-LIMITING BARRIER

It is intuitive that polyplexes must dissociate in order for the bound DNA to be processed within a target cell. Early computational models of non-viral gene delivery using an integrative systems approach identified the efficient release of DNA from its carrier as a critical step in the transfection process. A first-order mass-action model predicted a biphasic dependence of transgene expression on the rate of vector unpacking, which was validated with *in vitro* transfection data (Varga *et al.* 2001). An optimal intermediate value for the release rate constant was computed when the model was populated with values for polylysine gene carriers taken from the literature. The model predicted a dissociation threshold rate constant of 10^{-3} min^{-1} , above which transfection efficiency is significantly increased. A more recent and comprehensive model arrived at the same conclusion (Dinh *et al.* 2007). Some of the data used to create the model were borrowed from an earlier report that proposed vector unpacking as a potential rate-limiting barrier to non-viral gene delivery. In that study, Schaffer *et al.* (2000) found that fluorescently labelled 180-residue polylysine (MW approx. 28 kDa) remained colocalized with plasmid DNA in the nucleus and perinuclear region of transfected fibroblasts. In contrast, 36- and 19-residue polylysines delivered free plasmid to the nucleus. *In vitro* transcription assays showed that the shorter polycations freed plasmid more readily for transcription, and cell transfection studies showed that the intermediate polymer length resulted in the highest transgene expression levels. The authors explained that

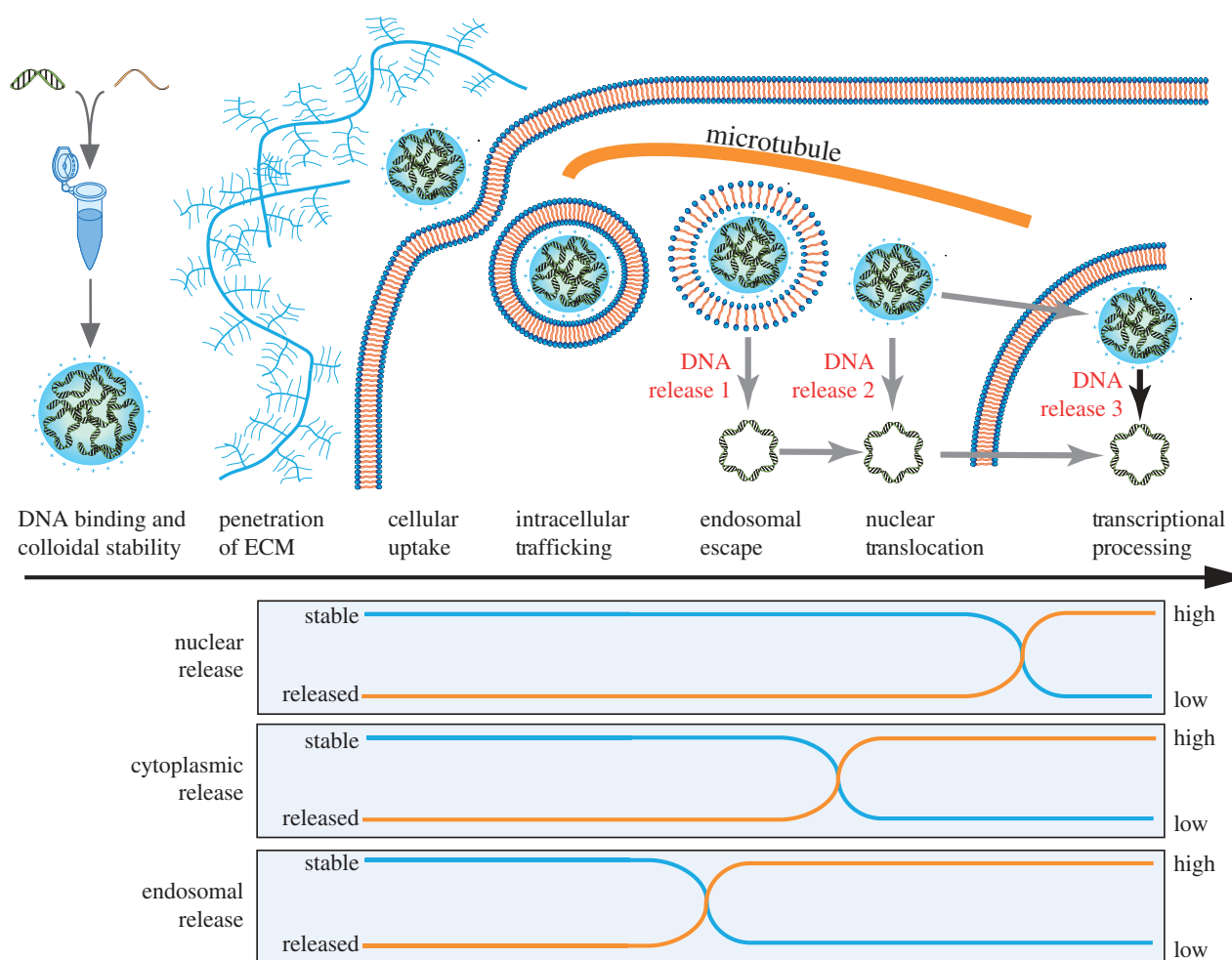


Figure 1. After shuttling a DNA payload into the cell, a carrier must then release it. This happens either (i) in the endocytic vesicle, (ii) in the cytoplasm or (iii) in the nucleus. Ideally, the carrier would switch from a protective element to one favouring release at one of these locations.

dissociation might occur spontaneously owing to thermodynamics or via competitive displacement of DNA by another anionic species. Genomic DNA in the cell nucleus can participate in polyplex destabilization by ion exchange (Schaffer *et al.* 2000), but it remains unknown precisely which dissociative mechanisms dominate for most carrier systems. Nuclear microinjection of polyethyleneimine (PEI)–DNA polyplexes results in high transgene expression levels, showing unpacking can take place in the nucleus (Pollard *et al.* 1998). Again, chromosomal DNA may be implicated or polymerases may mediate DNA release similar to their stripping of histone proteins during DNA replication (Thomas & Klibanov 2003). Another study demonstrated that RNA found in the cytoplasm could also promote DNA release by ion exchange (Huth *et al.* 2006). Regardless of exactly where and by which processes polymer and payload dissociate, it is certain that inefficient release of DNA precludes efficient transfection.

3. MODULATING PROTECTION AND RELEASE WITH POLYMER PROPERTIES

Many physical, chemical and structural features of a polymeric gene carrier affect its ability to bind,

condense and protect a DNA payload from enzymatic and non-specific degradation. These same features also play important roles in determining when and where the DNA is released. With many polymeric systems, the abilities to protect and efficiently release DNA are inversely related. One goal of carrier design should be to strike an optimal balance between protection and release to maximize transfection (figure 2).

3.1. Polymer length

Molecular weight or length of the polymer chains is one property known to influence the tradeoff between protection and release. For instance, one study demonstrated that polyplexes based on highly defined low-molecular-weight chitosan oligomers (10- to 50-mers) dissociated more easily than those derived from high-molecular-weight (1000-mer) chitosan chains (Koping-Hoggard *et al.* 2004). Below about 14 monomer units, the polyplexes formed were weak and unstable. The more easily dissociated polyplexes also mediated higher transgene expression levels *in vitro* and *in vivo*. However, the polyplexes needed to be formed with a much higher polymer-to-DNA ratio (N:P charge ratio as high as 60:1, as opposed to 5:1 for the high-molecular-weight chitosan) to bind

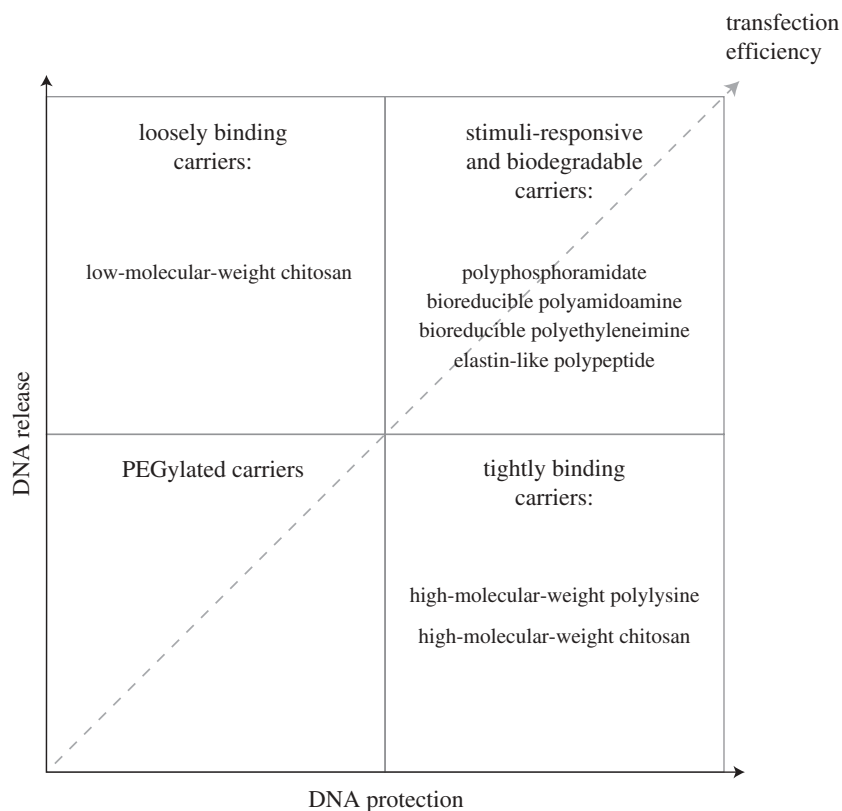


Figure 2. Vectors exhibiting both strong protection of DNA and efficient intracellular release mediate the highest levels of transfection. Vectors capable of only one of these qualities typically do not perform as well. Vectors capable of neither are largely ineffective.

and retain DNA as determined by gel retardation assay. Lower mass ratios resulted in unstable polyplexes, yielding poor transfection results. The decreased ability of the shorter polycations to effectively complex the anionic phosphate groups of DNA can be explained by their lower binding valency combined with the loss of the chain entanglement effect exhibited by the longer polymers. Longer polymer chains more easily entangle free DNA once the initial electrostatic interaction occurs, creating an additional non-ionic, knot-like binding component (Kiang *et al.* 2004). Shorter chains are less able to physically entangle molecules of DNA, and the requirement of additional molecules to match the binding strength of longer chains may be energetically unfavourable to polyplex formation.

As discussed previously, Schaffer *et al.* (2000) reported that polylysine (figure 3) of an intermediate length (36 residues) possesses superior gene-delivery properties than shorter (19 residues) and longer (180 residues) chains. Dissociation rates and transcription levels were higher *in vitro* for the smaller chains, whereas longer polymer chains significantly inhibited RNA synthesis. However, once the chains became too short, transgene expression levels dropped. Premature release and degradation of the plasmid was again the putative culprit. Selecting the optimal chain length of a polymer gene carrier is perhaps the most straightforward and established way to change the release kinetics and DNA protection properties of a polymeric gene carrier, but that alone may not be enough because the

structural and charge characteristics of a polymer are also important.

3.2. Charge density and structural rigidity

There are few published systematic studies investigating the effects of charge density and stiffness of polymeric gene carriers on their DNA-binding affinity and release characteristics. One such study using a series of linear polyamidoamines (PAAs; figure 3) looked at how their physico-chemical properties and colloidal stability were affected by changes in charge density and structural rigidity (Jones *et al.* 2000). The authors reported that the DNA-binding behaviour of the PAAs depended on their molecular structures, showing by gel retardation assays that chains with a single tertiary amino group per monomer bound DNA more weakly than those with two tertiary amino groups. However, when the methylene linker in the diacrylamide segment of the polymer possessing two tertiary amino groups was substituted for a piperazine ring, the DNA-binding affinity was greatly diminished. The authors speculated that this decrease in binding is caused by the resultant increase in rigidity from the ring substitution. The increase in rigidity, despite a higher charge density, resulted in decreased colloidal stability and transfection efficiency. It is difficult to separate the effects of decreased uptake of the larger polyplexes and premature degradation of plasmid when ascribing the blame for the decrease in transfection, but it is likely that both effects contribute.

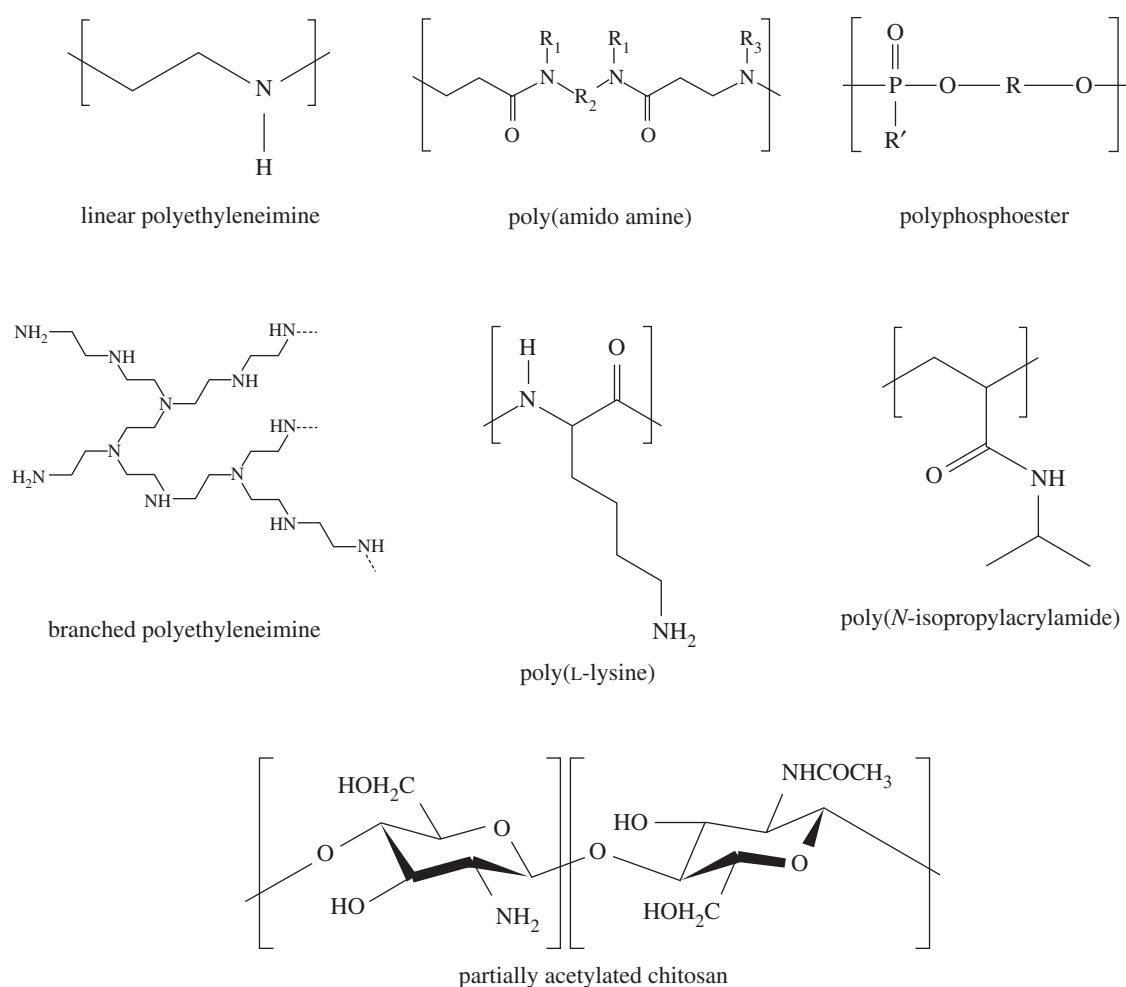


Figure 3. A few of the polymers most commonly used and modified for non-viral gene delivery.

Another series of studies aimed to improve the transfection efficiency of PEI (figure 3) through the optimization of charge density. Forrest *et al.* (2004) acetylated primary and secondary amines of PEI to varying degrees, decreasing the number of positive charges available to bind DNA at neutral pH. They found that acetylation of 43 per cent of the primary amines and 23 per cent of the secondary amines of 25 kDa branched PEI decreased the surface charge and increased the size of polyplexes, while mediating up to 21-fold higher transgene expression compared with unmodified PEI. The authors attribute the increase in transfection, at least partially, to more efficient release of DNA from the acetylated PEI. Gabrielson & Pack (2006) studied 25 kDa branched PEI with even higher degrees of acetylation. They found that acetylation of the primary amines up to 57 per cent increased transfection, while further acetylation resulted in diminished efficacy. This time, the authors' speculation that the decrease in charge density results in more efficient intracellular release of DNA was confirmed via fluorescence imaging techniques. These studies demonstrate that an optimal charge density exists for PEI, and that it alters the polymer–DNA binding and release characteristics.

A recently developed gene carrier is composed of linear PEI, with approximately 20 per cent of the secondary

amines functionalized with hydrolysable methyl ester, charge-shifting side chains (Liu *et al.* 2008). As ester hydrolysis occurs, negative charges are gradually introduced to the polymer, so that the effective positive charge density is decreased, weakening DNA binding and promoting polyplex dissociation. Higher degrees of side-chain addition resulted in polyplexes that dissociated prematurely, but the carrier with 20 per cent substitution mediated eightfold higher transfection levels than unmodified linear PEI. The authors propose that 20 per cent substitution results in the accumulation of negative charges at the proper rate to release DNA intracellularly via competition with other anionic species. Another way of promoting DNA release is the design of biodegradable vectors, such as polyphosphoesters (figure 3) including polyphosphates and polyphosphoramidates (PPAs; Mao & Leong 2005). The rationale of using carriers that can be hydrolysed over time is derived from the desire to promote DNA release. By degrading steadily over time, these carriers can provide sustained release of DNA, improving the bioavailability inside and outside the cell. The release kinetics can be adjusted by varying the polymer-to-DNA ratio, as well as the composition of the backbone and side chains. Polyphosphoesters also offer a high degree of structural versatility, which permits the tailored design of their physico-chemical properties. Their efficacy rivals PEI, but

with much less associated toxicity. However, a steady degradation means that control over the spatio-temporal release of DNA is lacking. It would be advantageous to trigger DNA release at a specific time and place.

3.3. Stimuli-responsive strategies

The ideal gene carrier would protect DNA from nucleases and provide unrestricted access to polymerases. Polymers able to respond to biological or environmental cues that signal the right time and place for DNA deprotection and release are perhaps better suited than their unresponsive counterparts for providing such selective enzymatic access. Polyplexes capable of responding to environmental stimuli have the potential to circumvent barriers to delivery in ways that traditional carriers cannot. Many physical stimuli have been studied, including light, heat, sonication and magnetic fields. Furthermore, some polymers can respond to chemical stimuli such as pH and redox changes. One reason that viruses are such potent gene carriers is their ability to respond to physical and chemical cues in certain subcellular compartments to more efficiently complete the steps of their replication cycle. Many researchers are trying to borrow viral response mechanisms and apply them to polymeric gene-carrier designs.

3.3.1. Redox-responsive polymers. One strategy to facilitate DNA release at the right time and right place is to include bioreducible disulphide linkages in the polymer chain. Disulphide bonds are covalent linkages formed via the oxidation of sulphhydryl (–SH) groups. They are quickly cleaved inside cells, which possess a reducing environment owing to high concentrations of reducing enzymes such as glutathione reductase and sulphhydryl species like glutathione as compared with the extracellular spaces. Consequently, disulphide bonds are relatively stable in plasma and extracellular spaces, but rapidly degrade inside cells. This rapid degradation can lead to the dissociation of a polyplex and DNA release. Disulphide linkages added to polylysine and 25 kDa branched PEI resulted in improved gene-carrier properties such as higher transfection levels and diminished cytotoxicity when compared with their unmodified analogues (Gosselin *et al.* 2001; Oupicky *et al.* 2002).

Lin *et al.* have shown more recently that a minimal number of disulphide linkages in the main chain of PAAs is sufficient to allow efficient intracellular DNA release (Lin *et al.* 2007a,b; Lin & Engbersen 2009). Again, a side effect of the polymer degradation is a marked decrease in cytotoxicity as compared with the polymer analogues lacking disulphide linkages. The ability of the polymer to protect the DNA prior to cleavage of the disulphide bonds is unaffected. Miyata *et al.* (2004) studied the addition of thiol groups to a polylysine backbone with possible modification of its charge density. Using a reagent that substituted positive charges for disulphide linkages, they created a redox sensitivity that led to efficient intracellular DNA release and 50-fold higher transfection efficiency in cells, as compared with the thiolated polylysine with unaltered positive charges. The balance between charge density and disulphide cross-linking can play a crucial role in

the mediation of efficient gene transfer. These reports demonstrate that the inclusion of disulphide bonds into polymer chains can lead to the fast intracellular degradation of polyplexes, efficient DNA release, sufficient protection, improved transfection and decreased cytotoxicity.

3.3.2. pH-sensitive polymers. Polymers sensitive to pH offer another layer of biochemical responsiveness. Decreases in pH are known to occur near tumours, inflammation and in the endocytic vesicles through which polyplexes travel following endocytosis. A triblock copolymer composed of lactosylated poly(ethylene glycol)-*block*-polysilamine-*block*-poly[2-(*N,N*-dimethylamino)ethyl methacrylate] (lac-PEG-PSAO-PAMA) has been developed by Oishi *et al.* (2006) to undergo pH-sensitive conformational changes. When the pH drops from neutral (approx. 7) to slightly acidic (approx. 4) typical of endosomal compartments, the PSAO segments of the 3-layer micelle swell and cause the polyplex to grow in diameter. This conformational change may decrease the binding affinity of the polymer for DNA, allowing intracellular anions to compete with and release the DNA more easily. Prior to release, the tight conformation affords good protection to the bound DNA. The lactose group was incorporated to promote receptor-mediated endocytosis by hepatocytes.

To deliver genes specifically to the acidic interstitium of tumours, PEI–DNA nanoparticles have been further functionalized with a pH-responsive poly(methacryloyl sulphadimethoxine)-*block*-PEG (PSD-*b*-PEG; Sethuraman *et al.* 2006). At a normal physiological pH of 7.4, the nanoparticles mediate very little transfection. The PSD-*b*-PEG layer served to shield the positive surface charges of the PEI-based polyplexes, decreasing both cytotoxicity and the ability of the polyplexes to interact with cell membranes. However, at pH 6.6, the polyplexes shed the PSD-*b*-PEG and exhibited the high transfection and cytotoxicity characteristic of PEI-based systems. Such a high-sensitivity pH-responsive system could be used to shield and protect polyplexes at physiological pH until delivery to an acidic environment, whereupon stripping of the mask makes transfection possible. Despite the fact that the PSD-*b*-PEG used is not easily degraded, the principle shows good promise.

Another strategic alternative to shielding polyplexes with pH-sensitive units and the inclusion of polymer segments that switch conformations upon acidification is the addition of acid-labile linkages within the polymer backbone. Kim *et al.* (2005b) introduced imine linkages into a PEI backbone by cross-linking low-molecular-weight PEI (1.8 kDa) with glutaraldehyde. The half-life of hydrolytic degradation of their polymer decreased two orders of magnitude when the pH was changed from 7.4 (118 h) to 4.5 (1.1 h). A DNase protection assay confirmed that the carrier affords sufficient protection from nuclease activity. The system is designed such that DNA is released upon acidification of the endosomal compartment. The acid-labile PEI polyplexes yielded transgene expression levels similar to 25 kDa branched PEI, but with much lower associated toxicity probably owing to the degradation into smaller chains.

3.3.3. Thermo-sensitive polymers. In contrast to the natural environmental stimuli encountered by the polyplexes discussed above, a stimulus can also be applied externally to trigger DNA release. A targeted increase in temperature is one stimulus frequently exploited. Poly(*N*-isopropylacrylamide) (PNIPAm; figure 3) is a widely used thermo-sensitive polymer that exists in a water-soluble state below its lower critical solution temperature (LCST) of 32°C. Above its LCST, which can be tuned closer to body temperature by varying its monomer makeup, it becomes hydrophobic. This leads to phase transformation to a gel state, or a change in architecture if bound to other molecules. In its hydrophobic state, PNIPAm can condense large DNA molecules and is small enough to enter cells. When cooled below its LCST, PNIPAm reverts to the solution state to release the DNA (Hinrichs *et al.* 1999; Kurisawa *et al.* 2000). PNIPAm has also been grafted with proven gene carriers such as PEI and chitosan to impart responsive properties and improve transfection (Oupicky *et al.* 2003; Sun *et al.* 2005; Bisht *et al.* 2006; Zintchenko *et al.* 2006; Lavigne *et al.* 2007).

Another class of thermo-sensitive polymers used for gene delivery is elastin-like polypeptides (ELPs). These are pentapeptide repeats Val-Pro-Gly-Xaa-Gly, where Xaa specifies any amino acid residue except proline. These polymers exhibit an inverse phase transition with temperature, as well as controllable degradation (Chilkoti *et al.* 2002). In contrast to the conventional thermo-sensitive polymer PNIPAm, polyplexes generated with ELPs allow for hyperthermic DNA release instead of release upon cooling. This can be beneficial in certain cases, as hyperthermia is already an established targeted treatment for some diseases such as cancer. As synthetic polypeptides, ELPs can be produced by recombinant methods to control polydispersity and expressed as fusion proteins if additional domains are desired. Chen *et al.* (2008*b*) used a recombinant diblock copolymer consisting of cationic oligolysine (VGK₈G) and an ELP block with 60 repeat units of VPGXG, where X is Val, Ala and Gly in a 5:2:3 ratio for hyperthermic gene delivery. While these and other novel thermo-responsive polymeric gene carriers, such as the diblock copolymer methoxy PEG-co-poly(ϵ -caprolactone) (Kim *et al.* 2006), are being explored and developed, temperature-sensitive gene-delivery vectors have obvious drawbacks in that a temperature gradient must be applied in a site-specific and non-invasive manner for the technology to be attractive to physicians and patients. The sol-gel transition must also be sharp because the strong temperature-buffering capacity *in vivo* would dampen any thermal gradient. These challenges hinder clinical translation, and currently limit the technology primarily to the realm of *in vitro* experiments.

3.3.4. Light-sensitive polymers. Light is a physical stimulus that can be very convenient, depending on the application and target tissue. It is an appropriate stimulus for targets located near the surface of the body, as near-infrared light between 750 and 1000 nm in wavelength penetrates the skin without damaging cells or tissues. This so-called ‘water window’ is useful

owing to the lack of absorption from water or biological chromophores. Handwerker *et al.* designed a novel photolabile monomer for gene delivery. P25M consists of three domains, a cationic domain of 25 kDa PEI to bind DNA, a polymerizable methacrylamide moiety for cross-linking DNA within the polyplex and a photolabile nitrobenzyl domain for triggered release by 365 nm light (Handwerker *et al.* 2006). Gel retardation demonstrated that P25M condensed DNA at a charge ratio of 3:4, and light scattering showed the polymer forms polyplexes of less than 500 nm diameter. This work demonstrates the feasibility of light-responsive polymeric gene carriers for the spatial, temporal and metered delivery of DNA. Few other light-responsive polymers have been developed for gene-delivery applications, but some have shown promise in the delivery of non-nucleic acid cargo (Jiang *et al.* 2005; Lee *et al.* 2007; Lepage *et al.* 2007).

3.4. Inclusion of enzymes and inhibitors

Polyplex systems need not be limited to simple pairs of polymer and DNA. Other molecules such as proteins, enzymes and chemicals can also be included into the polyplex to enhance DNA protection or facilitate release. In a study of DNA-gelatin nanospheres for gene delivery, Truong *et al.* observed incomplete protection of complexed DNA from nuclease digestion. In an attempt to increase the protection of DNA, they coencapsulated inhibitors of DNase I (Truong-Le *et al.* 1999). The inclusion of sodium iodoacetate and aurintricarboxylic acid each showed minor improvements in DNA protection, but the benefits did not translate to any improvement in transfection levels.

A pair of more recent studies examined the use of chitosanase to promote the dissociation of chitosan-based polyplexes (Liang *et al.* 2006; Zuo *et al.* 2008). In the first report, the enzyme was pre-delivered to cells prior to transfection. The authors observed that radiolabelled chitosan polyplexes were able to penetrate cells twice as efficiently as commercial lipid transfection reagents, but the resultant transgene expression was far lower. However, upon pre-delivery of chitosanase via osmotic lysis, cells transfected with chitosan polyplexes containing a LacZ reporter gene expressed twice as much β -galactosidase activity as those transfected with lipid reagents. In the subsequent report, the chitosanase gene was cotransfected along with the gene of interest because pre-delivery via osmotic lysis is impractical *in vivo*. The rationale was that once the chitosanase gene was processed, it would start a positive-feedback cascade, leading to the degradation of all the chitosan and efficient release of DNA. In four different cell lines, the authors found that β -galactosidase and green fluorescent protein expression levels were similar between lipid-mediated transfections and chitosan polyplex transfections using the chitosanase construct. Expression levels with an inactive mutant of the chitosanase gene were identical to those with no chitosanase gene at all, barely above the levels achieved by naked DNA. Similar techniques have been used to neutralize the threat of binding and sequestration of nanoparticles by extracellular matrix

(ECM) components. The functionalization of nanoparticles with collagenase resulted in the localized degradation of matrix molecules and a less tortuous path leading to the cell membrane that increased the rate of polyplex transport (Kuhn *et al.* 2006a).

4. IMAGING TECHNIQUES AND ENGINEERING MODELS TO EVALUATE THE PROTECTION AND RELEASE OF DNA FROM POLYPLEXES

Traditionally, fluorescence microscopy has been the method of choice to image molecules involved in gene delivery. Fluorescently labelled carriers and DNA can be followed from the extracellular compartment, through the cell and into the nucleus. However, colocalization of fluorescent signals and imaging at discrete time points can only give so much information about a carrier's function and fate. Recent advancements have vastly improved the precision with which engineers can observe the details of polyplex trafficking and dissociation. Technologies such as time-lapse microscopy, multiple particle tracking, fluorescence correlation spectroscopy (FCS), quantum dot (QD) labelling and fluorescence resonance energy transfer (FRET) now allow much finer details of non-viral transfection to be observed.

4.1. Determination of dissociation status by fluorescence microscopy

While single fluorescent markers affixed to polyplexes allow their subcellular localization to be determined, no information is gained about their integrity or dissociation status. Packaged nucleic acid cargo must be released at the appropriate time and place to ensure processing. One method used to follow the intracellular unpacking fate of polyplexes is colocalization of two different fluorescent markers, one attached to the polymer and one to the DNA (Godbey *et al.* 1999). These approaches are limited by the need for the polymer and DNA to diffuse sufficiently far enough from each other to detect their distinct signals, thereby lacking the sensitivity and spatial resolution to determine when and where the precise onset of dissociation occurs. It is conceivable that the fluorescently labelled molecules could be colocalized without actually being associated. Another fluorescent assay used to study the unpacking of polyplexes and DNA release uses the fluorophore YOYO-1. The fluorescent emission signal of the YOYO dye is three orders of magnitude higher when bound to DNA than when it is unbound in solution. When the YOYO-labelled DNA is complexed with the polymer and the dye molecules are pulled near one another, they interact with one another to cause a self-quenching effect that decreases the emission signal (Zaric *et al.* 2004). Thus, the YOYO signal is high only when bound to free DNA, and can be used to probe the dissociation status of polyplexes. However, this technique is better suited for bulk measurements than single-particle tracking owing to the lack of a clear binary signal indicating binding or release for each polyplex.

4.1.1. Fluorescence resonance energy transfer. Another technique used to study the time-dependent intermolecular interactions of polyplexes is FRET. In FRET, a fluorescent donor label has an emission spectral peak that overlaps the excitation peak of an appropriately chosen acceptor label. When energy is applied at the excitation frequency of the donor, the donor fluorophore emits energy at an appropriate wavelength to excite the acceptor by non-radiative dipole–dipole interactions (Foerster 1948; Jares-Erijman & Jovin 2003). The acceptor then emits at its own emission wavelength (figure 4). The result is that the acceptor will only emit if it is close enough to be excited by a donor. The efficiency of FRET falls off relative to the sixth power of the distance separating the donor–acceptor pair, with appreciable acceptor emission only occurring when the two fluorophores are less than 10 nm apart (Stryer 1978). This property makes FRET pairs ideal to probe the dissociation kinetics of polyplexes with high sensitivity and resolve distances below the diffraction limit of conventional microscopy. Traditionally, FRET pairs consist of commercially available organic fluorophores. Itaka *et al.* employed plasmid DNA doubly labelled with fluorescein and X-rhodamine as a FRET pair to determine the serum stability of poly(L-lysine) (PLL) polyplexes in physiological media. They observed that the fluorescence spectra of the labelled DNA changed drastically upon complexation and condensation with the polymer (Itaka *et al.* 2002, 2004). In a later study, the same group used a fluorescein and Cy5 FRET pair to probe the uptake and release of polyplexes derived from linear (22 kDa) and branched (25 kDa) PEI (Itaka *et al.* 2004). They found that linear PEI promoted fast uptake and unpacking, whereas branched PEI resulted in retarded release of DNA as indicated by the FRET signal. Their results correlated well with measures of transfection efficiency using each carrier. In another report, PEI–DNA polyplexes were labelled with Alexa Fluor 488 and rhodamine as a FRET pair and used to study the effect of substrate stiffness on polyplex uptake and dissociation (Kong *et al.* 2005). FRET is quickly gaining popularity in the field as its utility becomes apparent.

However, traditional organic fluorophores are not without shortcomings, as most of them have narrow excitation and broad emission spectra. If the emission spectrum of the donor overlaps that of the acceptor, cross-talk will occur and contaminate the FRET output signal. Another drawback to the use of organic fluorophores in FRET is their susceptibility to photobleaching, which often renders their use incompatible with time-lapse and real-time polyplex tracking studies. One proposed solution to address the issues with organic fluorophores is the use of semiconducting nanocrystals called QDs as efficient FRET donors (Lee *et al.* 2008; McGrath & Barroso 2008). They are characterized by broad absorption, narrow emission spectra, and high photostability, which help minimize complications typical of conventional FRET such as spectral cross-talk and direct acceptor excitation (McGrath & Barroso 2008). A QD–FRET system allows the tracking of a single polyplex over an extended period of

time in a living cell via confocal microscopy with a high signal-to-noise ratio. Chen *et al.* (2008a) and Ho *et al.* (2006) used such a QD-FRET system to elucidate the intracellular fate of polyplexes derived from three different polymers. Analysis of confocal microscopy images taken in live cells allowed them to construct a three-compartment kinetic model describing the subcellular localization and dissociation status of the three different polyplexes in the cytosol, endocytic vesicles or the nucleus at various times. Their results reveal how unpacking kinetics can correlate with transfection efficiency, a mechanistic insight that could lead to the rational design of better gene carriers.

A recent refinement of this technique is to apply a two-step or relay QD-FRET approach to also monitor DNA degradation at the same time (Chen *et al.* 2009). In this case, plasmid DNA, double-labelled with QD (525 nm emission) and nucleic acid dyes, was complexed with Cy5-labelled polymeric gene carriers. The QD donor drives energy transfer stepwise through the intermediate nucleic acid dye to the final acceptor Cy5. At least three distinct states of DNA condensation and integrity (complexed and intact, unpacked and intact and unpacked and degraded) were distinguished in a single-particle manner and within cells by quantitative ratiometric analysis of energy transfer efficiencies. This novel two-step QD-FRET method allows for more detailed assessment of the onset of DNA release and degradation simultaneously.

4.1.2. Fluorescence correlation spectroscopy. Another means of detecting the dissociation of polyplexes in the cell is FCS, whereby the colocalization of the fluorescent signals is tracked not only spatially, but also temporally in the excitation volume of a confocal microscope (Bacia & Schwille 2003; Remaut *et al.* 2007). The concentration of labelled molecules is kept low, so that each contributes significantly to the detected signal. The detection volume is also very small (approx. femtolitres), so that only a few fluorophores are detected at a given time. Then, the diffusion of fluorescent molecules in and out of the detection volume leads to fluorescent fluctuations that provide information about the molecules. When associated, the tagged molecules move in and out of the fixed excitation volume simultaneously. When dissociated, they move independently. Based on the measured kinetic properties of the labelled molecules, their diffusion coefficients can be calculated to determine if they are bound or free. This approach is convenient and powerful, but does have limitations. It relies on the differences in the diffusive properties of the complexed or dissociated molecules, which are likely to be affected by associations with other intracellular charged species, limiting sensitivity and precision. Lucas *et al.* (2005) employed FCS by transfecting cells with polyplexes consisting of rhodamine-green-labelled nucleotides and either high-molecular-weight poly[2-(dimethylamino)ethyl methacrylate] or low-molecular-weight PLL labelled with Cy5 to determine if the polyplexes dissociated before or after reaching the nucleus.

Image correlation spectroscopy (ICS) is an analogous technique also used to track polyplex behaviour

intracellularly over time. Again, polyplexes are conjugated with fluorophores and excited by a laser under a confocal microscope. However, now the image of the entire cell is used instead of a smaller fixed focal volume. The fluorescent intensities at each pixel are used to calculate autocorrelation functions, gaining insight into the transport and aggregation behaviour of polyplexes once internalized. Using ICS, Kulkarni *et al.* (2005) found that for short intervals (less than 10 s), polyplexes tend to move along distinct paths that perhaps implicate microtubules in polyplex transport. For longer intervals, the motion was much more Brownian, indicative of passive transport.

Sometimes, it is advantageous to employ more than one imaging technique in combination to answer specific questions pertaining to DNA protection and release. One example is the combination of FRET and FCS to probe the stability of DNA delivered intracellularly (Remaut *et al.* 2005). Remaut *et al.* delivered oligonucleotides bearing a rhodamine green and a Cy5 fluorophore on its 3' and 5' termini, respectively. Using the two labels as a FRET pair, the authors performed dual-colour FCS to monitor the red-to-green acceptor-to-donor fluorescence ratio. They were able to study the degradation of the delivered DNA at very low concentrations in cytoplasmic extract and living cells by both methods simultaneously. Degradation of the DNA was indicated by the disappearance of the FRET signal, as well as the red-to-green emission ratio and diffusion times calculated from the autocorrelation curves. They estimated that the cytoplasmic turnover time for an unmodified 40-mer oligonucleotide was between 2 and 10 min, underscoring the necessity of DNA protection until it is delivered to its final destination.

4.1.3. Magnetic resonance imaging. Magnetic resonance imaging (MRI) is a recent, non-fluorescence alternative means of studying polyplex unpacking that has good potential for use *in vivo*, where non-invasive imaging is very important in studying the biodistribution and pharmacokinetics of gene-delivery vectors. One group has functionalized PEI with ultrasmall, superparamagnetic iron oxide (USPIO-PEI). The resultant polyplexes can condense DNA into nanoparticles and protect it from enzymatic degradation, while retaining the high transfection efficiency typical of PEI (Park *et al.* 2008). Furthermore, the T_2 relaxation time of water enhanced by USPIO can be used to determine the dissociation state of the gene carrier. Biological materials have low inherent contrasts, but can be enhanced with the use of contrast agents such as USPIO (Bjornerud & Johansson 2004). The USPIO nanoparticles were covalently linked to PEI, and subsequent condensation with DNA resulted in interparticle electrostatic interactions. The T_2 relaxation time of the polyplexes is increased when in the condensed state as a result of these interactions. When polyplex dissociation occurs, the T_2 relaxation time decreases. Thus, MRI can be used to ascertain when and where DNA is being released *in vivo*. MRI is a promising technique in gene-delivery applications because it enables high-resolution, non-invasive

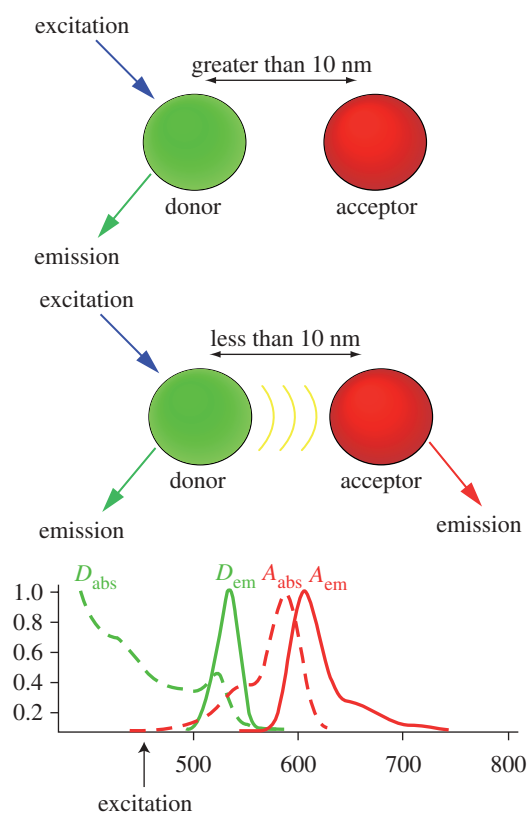


Figure 4. When a FRET donor and acceptor are less than 10 nm apart, energy transfer takes place. Otherwise, the acceptor is not excited and does not emit. When a quantum dot acceptor is excited, it emits at a wavelength that overlaps the acceptor absorbance spectra, but not the acceptor emission spectra.

monitoring *in vivo* (Perez *et al.* 2002). More examples of non-fluorescence imaging techniques to study polyplex dissociation will continue to emerge in the future.

4.2. Unpacking in extracellular space

The intracellular barriers to non-viral gene delivery are better understood than those encountered by polyplexes prior to reaching the cell surface. It is unfortunate that the consideration and characterization of these extracellular barriers are often neglected. Exposure of polyplexes to serum or proteoglycans often decreases their transfection efficiency by causing aggregation or premature DNA release (Ruponen *et al.* 2001, 2003; Burke & Pun 2008). Just as charged species inside the cell, such as chromosomal DNA and cytoplasmic RNA, can cause the release of DNA via competitive ion exchange, charged molecules found in the extracellular compartment and on the cell surface can also influence the release process. Aggregation outside the cell results in both premature unpacking and the formation of larger conglomerates of nanoparticles that hinder uptake. PEGylation, the addition of PEG units, is a common modification made to polymers to increase their salt and serum stability. However, studies have shown that the addition of PEG may actually result in a less stable ionic complex outside the cell and decreased transfection efficiencies (Oupicky *et al.* 1999; Mullen *et al.* 2000; Fischer *et al.* 2004; Mishra

et al. 2004; Merdan *et al.* 2005; Burke & Pun 2008). The hydrophilic PEG chains may induce swelling of the polyplexes and can lead to either premature release of DNA in the extracellular compartment or increased access to nucleases that can degrade DNA before it reaches the cell.

DNA delivered via polyplex is very susceptible to sequestration and degradation by a variety of processes before it ever reaches a target cell. To overcome these challenges, accurate *in vitro* models must be developed to recapitulate the extracellular barriers found *in vivo* for rational carrier design. This is a formidable challenge, as the relevant barriers vary greatly with the route of administration. One route may require the balance of protection versus release to be skewed far towards one extreme, while another demands the reverse. For example, it is known that injection of naked DNA results in substantial transgene expression in skeletal muscle (Wolff *et al.* 1990; Wolff & Budker 2005). However, DNA delivered orally needs to survive the acid and enzymes found in the stomach and gut (Roy *et al.* 1999). It follows that carriers for intramuscular injections should be labile and unpack rapidly, whereas carriers for oral delivery must form tight complexes with DNA to provide maximum protection until they reach their target. Other routes of administration require more intermediate properties. The different demands typical of different routes of delivery complicate the considerations in designing systems to model extracellular barriers.

The study and evaluation of most new gene carriers currently takes place primarily in monolayer cell culture systems. Such two-dimensional environments generally fail to accurately mimic the extracellular environment polyplexes will encounter *in vivo*. When nanoparticles are added to the culture dish, they interact with the cell monolayer from the apical side. In two-dimensional culture systems, the amount of ECM secreted by cells is lower on the apical side than on the basolateral surfaces (Gruber & Hanley 2000; Mao & Schwarzbauer 2005). So, polyplexes delivered to the culture medium have a relatively unimpeded path, where the reality of the situation *in vivo* includes a far more tortuous path through the small pores of the ECM. Furthermore, the molecular constituents of the ECM are neither neutral nor inert. For example, proteoglycans are negatively charged under physiological conditions. They can interact with polyplexes possessing positive zeta potentials, sequestering or disrupting them prior to their ever reaching a target cell (Ruponen *et al.* 1999; Burke & Pun 2008). As a result, some groups are looking at three-dimensional tissue culture systems that more accurately portray the conditions found *in vivo* in terms of the structure, volume and composition of the extracellular domain. More accurate models could result in more reliable assessments of gene-carrier efficiencies *in vitro*, bridging the gap in success that often exists between cell culture experiments and animal studies.

The sophistication of these three-dimensional models ranges from simple hydrogels of cross-linked ECM molecules to multi-cellular spheroids, multi-layer cell cultures and *ex vivo* tissue models (figure 5; Goodman

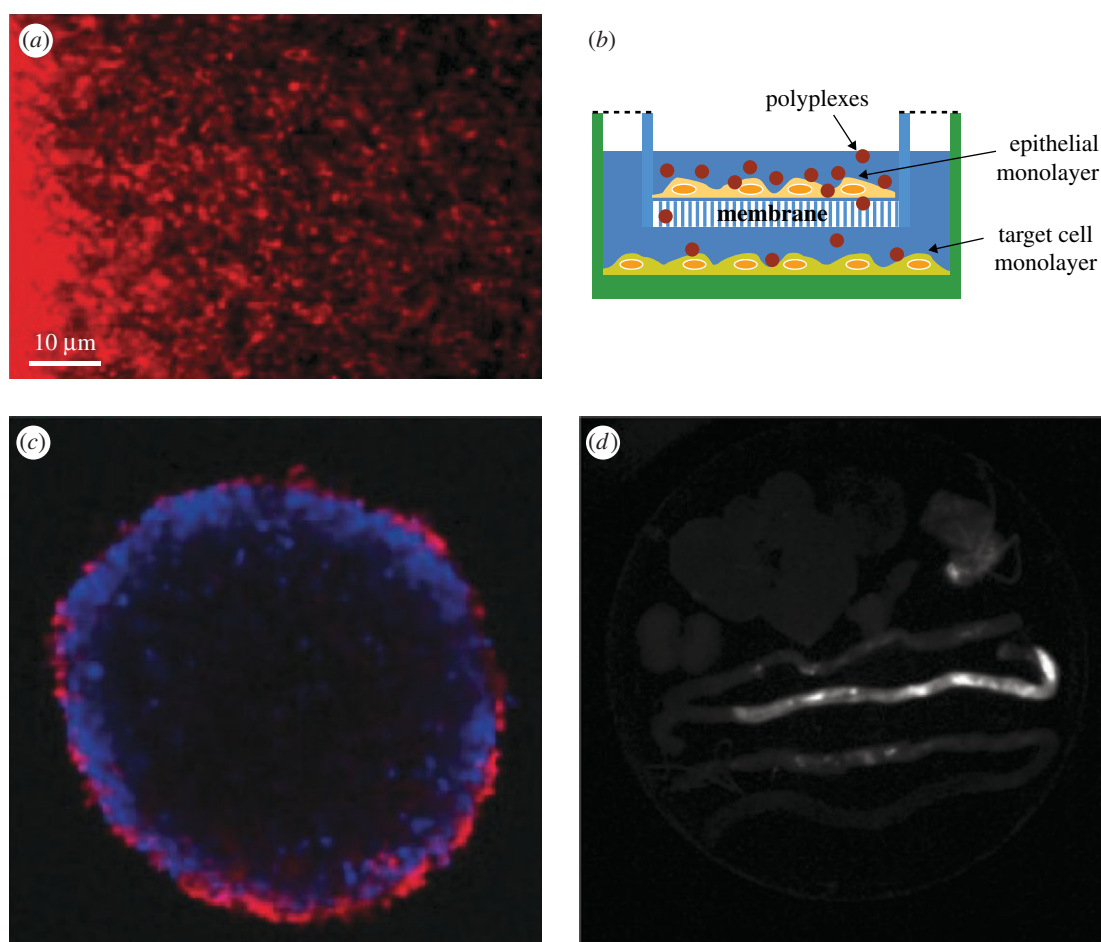


Figure 5. Four common types of three-dimensional models used to evaluate gene carriers. (a) Polymeric micelles (red) penetrate rightward into a hydrogel (Kim *et al.* 2005a). (b) Multi-layer transwell cell culture system used to study transport across cell layers. (c) Multi-cellular spheroid used to study the uptake and distribution of labelled PEI polyplexes (red) (Mellor *et al.* 2006). (d) Quantum dot-labelled chitosan–DNA polyplexes (white) distributed in rat gut and liver (unpublished data).

et al. 2008). A model as basic as a hydrogel of cross-linked ECM molecules, such as collagen or Matrigel, can offer a great deal of insight into the transport properties and dynamic stability of polyplexes in the extracellular domain. The effects of polymer modification or functionalization on polyplex behaviour outside the cell can be studied, as well as the shape and size restrictions imposed on polyplexes by the ECM pore sizes (Kim *et al.* 2005a; Kuhn *et al.* 2006b). Hydrogel models have been used to demonstrate that aggregation and premature dissociation of polyplexes by charged ECM components does indeed take place (Derouchey *et al.* 2008). If desired, cells can be added to the hydrogel to look beyond transport, at the effect of ECM on uptake and transfection. More sophisticated hydrogel systems can be perfused to more accurately mimic the situation *in vivo* where fluids flow and influence nanoparticle transport (Ng & Pun 2008). Hydrogels also allow real-time nanoparticle imaging *in situ* without any significant processing required.

A more complex three-dimensional model of spherical cell clusters that secrete their own ECM is called the multi-cellular spheroid (Nederman & Twentyman 1984; Mellor *et al.* 2006; Han *et al.* 2007, 2009). As perhaps the smallest approximation of native tissues,

multi-cellular spheroids are a convenient means of probing polyplex transport and stability in the intact spheroid, and subsequently in the constituent cells upon enzymatic disruption (Goodman *et al.* 2007). They can be grown to reach various sizes, up to a few millimetres in diameter. They produce ECM in a three-dimensional manner similar to native biological tissues, with a composition more like native tissues than hydrogels made of only a few components (Nederman *et al.* 1984). Small spheroids consist entirely of healthy cells that can be used to study polyplex distribution and transport in normal tissues. Larger spheroids more closely resemble tumour tissue, with distinct proliferating, quiescent and necrotic regions. Multi-cellular spheroids, as with hydrogels, can be used to study the effects of polyplex size, shape, charge and functionalization on transport and transfection. Data collection is facile, often taking place in the form of confocal microscopy, immunohistochemistry or flow cytometry of the dissociated spheroid cells. The use of multi-cellular spheroids is becoming more widespread as the need to evaluate gene carriers in three-dimensional systems becomes more apparent.

Multi-layer cell cultures are another useful tool for studying the transport and stability of polyplexes.

Using transwell-type culture plates, one layer of cells can be cultured on a semipermeable membrane insert. Beneath the membrane is a layer of culture medium covering a second layer of cells. Multi-layer systems are especially useful for modelling scenarios in which polyplexes must pass through a layer of cells prior to reaching their target cells. Such situations are encountered in extravasation through the vascular endothelium after systemic administration of polyplexes, as well as in the escape from the gut through the intestinal epithelium in oral gene delivery. Similar barriers are found in the airway epithelium encountered by polyplexes delivered to the respiratory system. Multi-layer transwell culture models have been developed to study each of these situations (Artursson 1990; Artursson & Karlsson 1991; Molestina *et al.* 1999; Meng *et al.* 2004). Additionally, each layer in the multi-layer system is not confined to a single cell type. Cocultures can be seeded on either layer to more accurately mimic the situation *in vivo*. For example, the intestinal epithelium contains mucus-secreting goblet cells and M-cells of the immune system that continually shuttle particulate contents of the intestinal lumen to their basolateral side via transcytosis. The presence of mucus and transcytotic activity could significantly alter the transport characteristics of polyplexes involved in oral gene delivery, so the inclusion of these cell types in coculture multi-layer transwell models could provide valuable information (Kerneis *et al.* 1997, 2000; Hilgendorf *et al.* 2000).

The final category of common three-dimensional models is *ex vivo* tissue cultures. By using tissue directly from an animal or patient, preservation of the native ECM structure and composition, cell organization and cell phenotypes is possible. Such models have a higher likelihood of predicting *in vivo* efficacy accurately. *Ex vivo* cultures of a wide range of tissue types have been successful: liver, cartilage, airway epithelium (Gersting *et al.* 2004), lung (Lang *et al.* 2007) and intestine (Torche *et al.* 2000). Imaging, histology and most other standard assays are feasible with *ex vivo* tissue cultures. The drawbacks of using tissue explants for the evaluation of gene carriers include the cost and ethics associated with the use of primary tissues, as well as the relative difficulty of maintaining the architecture and phenotype of entire tissues compared with more basic cell layers and spheroids. However, they still may provide the most accurate information about transfection capabilities short of testing in animal models.

5. CELL-SPECIFIC DESIGN REQUIREMENT

A final consideration is the potential differences in the desired protection and release characteristics of polyplexes when introduced to different cell types. The transfection efficiency of a gene carrier can vary by orders of magnitude depending on the cell type being transfected. One can envisage that metabolically active cells favour a tilt towards protection, whereas slowly proliferating cells might favour more rapid unpacking. The proliferation rate of target cells can play a major role in whether or not polyplexes gain

access to the nucleus where some unpacking has been shown to take place. During each iteration of the cell cycle, the nuclear envelope breaks down and reforms. While the nuclear envelope becomes discontinuous during mitosis, polyplexes or DNA may diffuse into what will become the nucleus of the daughter cells and eventually be transcribed. In a non-dividing cell, they may have been unable to penetrate the nucleus. This is often the case with primary cells and cells of the immune system that proliferate more slowly than transformed cell lines, accounting for the lower transfectability of the former.

Furthermore, cells internalize polyplexes through a variety of uptake pathways, including clathrin-mediated endocytosis, calveolin-mediated endocytosis, clathrin- and calveolin-independent endocytosis, macropinocytosis and phagocytosis (Conner & Schmid 2003; Rejman *et al.* 2004). The route ultimately taken probably depends on the target cell type, as well as polyplex size and vector physico-chemical properties. To maximize efficacy, the anticipated internalization pathway should be considered during the vector design process. Another difference between cell types is the amount and composition of ECM they secrete. Secretion of a large volume of ECM with small pore sizes will certainly diminish the ability of polyplexes to reach and transfect cells. Other cell types pose unique challenges, such as macrophages. Macrophages are a key component in inflammatory reactions and the foreign body response. They also function as antigen-presenting cells and participate in B and T lymphocyte development. Thus, they are desirable targets for gene delivery. Unfortunately, macrophages are very active in phagocytosis and degradation of particulate matter they consume (Ganta *et al.* 2008). Polyplexes internalized by macrophages rarely escape the phagocytic/endocytic pathway and are usually degraded. However, these same challenges sometimes offer unique opportunities to hijack cellular processes to enhance gene delivery. Acid-labile carriers are being developed to respond to the acidic environments typical of macrophage phagosomes (Murthy *et al.* 2003). This is one example of the special rational design considerations that will be necessary to efficiently transfect troublesome cell types.

6. CONCLUSIONS

Computational models and experimental evidence have shown that both inadequate protection and inefficient release of DNA from polymeric vectors are serious obstructions to successful gene delivery. A thorough understanding of the properties and processes that contribute to the protection and release of DNA in polyplex-mediated gene-delivery systems is indispensable to the rational design of future vectors. Phenomenological comparisons of vectors based on readouts of reporter genes offer only a glimpse into the complex systems involved in transfection of cells and transport through tissues. The rate-limiting barriers are undoubtedly multi-factorial, so a systematic engineering approach is the only way to gain an understanding of

why, how, where and when one carrier is superior to another.

Researchers have now begun to study and isolate each step, and each molecule, of the non-viral gene-delivery process. The accumulation of knowledge from both the life sciences and physical sciences has made such an interdisciplinary approach to the problem possible. When changing polymer properties such as the molecular weight and charge density is insufficient to provide optimal protection and release, some polymers can be made to respond to environmental or applied stimuli. Changes in temperature, light, pH and redox potential can be harnessed to trigger a switch from a protective complex to one favouring release. Ternary polyplexes can be formed by the inclusion of enzymes or genes meant to help relax the ECM to facilitate extracellular transport or degrade the polymeric carrier intracellularly to allow DNA release.

Fortunately, the developmental pace for imaging technologies and engineering models is accelerating. Fluorescence imaging techniques have advanced from the colocalization of fluorescently labelled DNA and polymer to much more sophisticated technologies such as QD-mediated FRET, FCS and non-fluorescence techniques like MRI. Furthermore, these techniques are often combined with three-dimensional models that can recapitulate native tissues with high fidelity. It is at the interface between materials science, imaging and biology that non-viral gene delivery will make progress towards meaningful clinical translation. While the task remains daunting, successful non-viral gene therapy is crucial to realizing the potential of genetic medicine. As future therapeutics will increasingly rely on nucleic acids such as DNA, antisense oligonucleotides, therapeutic RNA and siRNA, the rewards for this line of investigation will continue to multiply.

We thank Yihua Loo for her contribution of the QD-labelled polyplex biodistribution image. Support by NIH (HL89764) is also acknowledged.

REFERENCES

- Artursson, P. 1990 Epithelial transport of drugs in cell culture. I: a model for studying the passive diffusion of drugs over intestinal absorptive (caco-2) cells. *J. Pharm. Sci.* **79**, 476–482. (doi:10.1002/jps.2600790604)
- Artursson, P. & Karlsson, J. 1991 Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (caco-2) cells. *Biochem. Biophys. Res. Commun.* **175**, 880–885. (doi:10.1016/0006-291X(91)91647-U)
- Bacia, K. & Schwillie, P. 2003 A dynamic view of cellular processes by *in vivo* fluorescence auto- and cross-correlation spectroscopy. *Methods* **29**, 74–85. (doi:10.1016/S1046-2023(02)00291-8)
- Bisht, H. S., Manickam, D. S., You, Y. Z. & Oupicky, D. 2006 Temperature-controlled properties of DNA complexes with poly(ethylenimine)-graft-poly(*N*-isopropylacrylamide). *Biomacromolecules* **7**, 1169–1178. (doi:10.1021/bm0509927)
- Bjornerud, A. & Johansson, L. 2004 The utility of superparamagnetic contrast agents in MRI: theoretical consideration and applications in the cardiovascular system. *NMR Biomed.* **17**, 465–477. (doi:10.1002/nbm.904)
- Burke, R. S. & Pun, S. H. 2008 Extracellular barriers to *in vivo* PEI and PEGylated PEI polyplex-mediated gene delivery to the liver. *Bioconjug. Chem.* **19**, 693–704. (doi:10.1021/bc700388u)
- Cavazzana-Calvo, M., Thrasher, A. & Mavilio, F. 2004 The future of gene therapy. *Nature* **427**, 779–781. (doi:10.1038/427779a)
- Check, E. 2003 Cancer fears cast doubts on future of gene therapy. *Nature* **421**, 678. (doi:10.1038/421678b)
- Chen, H. H., Ho, Y. P., Jiang, X., Mao, H. Q., Wang, T. H. & Leong, K. W. 2008a Quantitative comparison of intracellular unpacking kinetics of polyplexes by a model constructed from quantum dot-FRET. *Mol. Ther.* **16**, 324–332. (doi:10.1038/sj.mt.6300392)
- Chen, T. H. H., Bae, Y. & Furgeson, D. Y. 2008b Intelligent biosynthetic nanobiomaterials (IBNS) for hyperthermic gene delivery. *Pharm. Res.* **25**, 683–691. (doi:10.1007/s11095-007-9382-5)
- Chen, H. H., Ho, Y.-P., Jiang, X., Mao, H.-Q., Wang, T.-H. & Leong, K. W. 2009 Simultaneous non-invasive analysis of DNA condensation and stability by two-step QD-FRET. *Nano Today* **4**, 125–134. (doi:10.1016/j.nantod.2009.02.008)
- Chilkoti, A., Dreher, M. R. & Meyer, D. E. 2002 Design of thermally responsive, recombinant polypeptide carriers for targeted drug delivery. *Adv. Drug Deliv. Rev.* **54**, 1093–1111. (doi:10.1016/S0169-409X(02)00060-1)
- Conner, S. D. & Schmid, S. L. 2003 Regulated portals of entry into the cell. *Nature* **422**, 37–44. (doi:10.1038/nature01451)
- Derouchey, J., Schmidt, C., Walker, G. F., Koch, C., Plank, C., Wagner, E. & Radler, J. O. 2008 Monomolecular assembly of siRNA and poly(ethylene glycol)-peptide copolymers. *Biomacromolecules* **9**, 724–732. (doi:10.1021/bm7011482)
- Dinh, A. T., Pangarkar, C., Theofanous, T. & Mitragotri, S. 2007 Understanding intracellular transport processes pertinent to synthetic gene delivery via stochastic simulations and sensitivity analyses. *Biophys. J.* **92**, 831–846. (doi:10.1529/biophysj.106.095521)
- Ferrari, S., Geddes, D. M. & Alton, E. W. 2002 Barriers to and new approaches for gene therapy and gene delivery in cystic fibrosis. *Adv. Drug Deliv. Rev.* **54**, 1373–1393. (doi:10.1016/S0169-409X(02)00145-X)
- Fischer, D., Osburg, B., Petersen, H., Kissel, T. & Bickel, U. 2004 Effect of poly(ethylene imine) molecular weight and PEGylation on organ distribution and pharmacokinetics of polyplexes with oligodeoxynucleotides in mice. *Drug Metab. Dispos.* **32**, 983–992.
- Foerster, T. 1948 Intermolecular energy transference and fluorescence. *Ann. Phys.* **2**, 55–75.
- Forrest, M. L., Meister, G. E., Koerber, J. T. & Pack, D. W. 2004 Partial acetylation of polyethylenimine enhances *in vitro* gene delivery. *Pharm. Res.* **21**, 365–371. (doi:10.1023/B:PHAM.0000016251.42392.1e)
- Gabrielson, N. P. & Pack, D. W. 2006 Acetylation of polyethylenimine enhances gene delivery via weakened polymer/DNA interactions. *Biomacromolecules* **7**, 2427–2435. (doi:10.1021/bm060300u)
- Ganta, S., Devalapally, H., Shahiwala, A. & Amiji, M. 2008 A review of stimuli-responsive nanocarriers for drug and gene delivery. *J. Control. Release* **126**, 187–204. (doi:10.1016/j.jconrel.2007.12.017)
- Gersting, S. W., Schillinger, U., Lausier, J., Nicklaus, P., Rudolph, C., Plank, C., Reinhardt, D. & Rosenecker, J. 2004 Gene delivery to respiratory epithelial cells by magnetofection. *J. Gene Med.* **6**, 913–922. (doi:10.1002/jgm.569)
- Godbey, W. T., Wu, K. K. & Mikos, A. G. 1999 Tracking the intracellular path of poly(ethylenimine)/DNA complexes

- for gene delivery. *Proc. Natl Acad. Sci. USA* **96**, 5177–5181. (doi:10.1073/pnas.96.9.5177)
- Goodman, T. T., Olive, P. L. & Pun, S. H. 2007 Increased nanoparticle penetration in collagenase-treated multicellular spheroids. *Int. J. Nanomed.* **2**, 265–274.
- Goodman, T. T., Ng, C. P. & Pun, S. H. 2008 3-D tissue culture systems for the evaluation and optimization of nanoparticle-based drug carriers. *Bioconjug. Chem.* **19**, 1951–1959. (doi:10.1021/bc800233a)
- Gosselin, M. A., Guo, W. J. & Lee, R. J. 2001 Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjug. Chem.* **12**, 989–994. (doi:10.1021/bc0100455)
- Gruber, H. E. & Hanley Jr, E. N. 2000 Human disc cells in monolayer vs 3D culture: cell shape, division and matrix formation. *BMC Musculoskelet. Disord.* **1**, 1. (doi:10.1186/1471-2474-1-1)
- Han, M., Bae, Y., Nishiyama, N., Miyata, K., Oba, M. & Kataoka, K. 2007 Transfection study using multicellular tumor spheroids for screening non-viral polymeric gene vectors with low cytotoxicity and high transfection efficiencies. *J. Control. Release* **121**, 38–48. (doi:10.1016/j.jconrel.2007.05.012)
- Han, M., Oba, M., Nishiyama, N., Kano, M. R., Kizaka-Kondoh, S. & Kataoka, K. 2009 Enhanced percolation and gene expression in tumor hypoxia by PEGylated polyplex micelles. *Mol. Ther.* **17**, 1404–1410. (doi:10.1038/mt.2009.119)
- Handwerker, R. G., Kim, M.-S. & Diamond, S. L. 2006 Crosslinked PEI polyplexes for light-triggered DNA release. *Mol. Ther.* **13**, S415–S416. (doi:10.1016/j.ymthe.2006.08.1184)
- Hilgendorf, C., Spahn-Langguth, H., Regardh, C. G., Lipka, E., Amidon, G. L. & Langguth, P. 2000 Caco-2 versus Caco-2/HT29-MTX co-cultured cell lines: permeabilities via diffusion, inside- and outside-directed carrier-mediated transport. *J. Pharm. Sci.* **89**, 63–75. (doi:10.1002/(SICI)1520-6017(200001)89:1<63::AID-JPS7>3.0.CO;2-6)
- Hinrichs, W. L. J., Schuurmans-Nieuwenbroek, N. M. E., Van De Wetering, P. & Hennink, W. E. 1999 Thermosensitive polymers as carriers for DNA delivery. *J. Control. Release* **60**, 249–259. (doi:10.1016/S0168-3659(99)00075-9)
- Ho, Y. P., Chen, H. H., Leong, K. W. & Wang, T. H. 2006 Evaluating the intracellular stability and unpacking of DNA nanocomplexes by quantum dots-FRET. *J. Control. Release* **116**, 83–89. (doi:10.1016/j.jconrel.2006.09.005)
- Huth, S., Hoffmann, F., Von Gersdorff, K., Laner, A., Reinhardt, D., Rosenecker, J. & Rudolph, C. 2006 Interaction of polyamine gene vectors with RNA leads to the dissociation of plasmid DNA-carrier complexes. *J. Gene Med.* **8**, 1416–1424. (doi:10.1002/jgm.975)
- Itaka, K., Harada, A., Nakamura, K., Kawaguchi, H. & Kataoka, K. 2002 Evaluation by fluorescence resonance energy transfer of the stability of nonviral gene delivery vectors under physiological conditions. *Biomacromolecules* **3**, 841–845. (doi:10.1021/bm025527d)
- Itaka, K., Harada, A., Yamasaki, Y., Nakamura, K., Kawaguchi, H. & Kataoka, K. 2004 *In situ* single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine. *J. Gene Med.* **6**, 76–84. (doi:10.1002/jgm.470)
- Jares-Erijman, E. A. & Jovin, T. M. 2003 FRET imaging. *Nat. Biotechnol.* **21**, 1387–1395. (doi:10.1038/nbt896)
- Jiang, J. Q., Tong, X. & Zhao, Y. 2005 A new design for light-breakable polymer micelles. *J. Am. Chem. Soc.* **127**, 8290–8291. (doi:10.1021/ja0521019)
- Jones, N. A., Hill, I. R. C., Stolnik, S., Bignotti, F., Davis, S. S. & Garnett, M. C. 2000 Polymer chemical structure is a key determinant of physicochemical and colloidal properties of polymer–DNA complexes for gene delivery. *Biochim. Biophys. Acta Gene Struct. Expr.* **1517**, 1–18. (doi:10.1016/S0167-781(00)00220-7)
- Kaiser, J. 2004 Gene therapy. Side effects sideline hemophilia trial. *Science* **304**, 1423–1425. (doi:10.1126/science.304.5676.1423b)
- Kerneis, S., Bogdanova, A., Kraehenbuhl, J. P. & Pringault, E. 1997 Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. *Science* **277**, 949–952. (doi:10.1126/science.277.5328.949)
- Kerneis, S., Caliot, E., Stubbe, H., Bogdanova, A., Kraehenbuhl, J. & Pringault, E. 2000 Molecular studies of the intestinal mucosal barrier physiopathology using cocultures of epithelial and immune cells: a technical update. *Microbes Infect.* **2**, 1119–1124. (doi:10.1016/S1286-4579(00)01266-1)
- Kiang, T., Wen, J., Lim, H. W. & Leong, K. W. 2004 The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials* **25**, 5293–5301. (doi:10.1016/j.biomaterials.2003.12.036)
- Kim, M. S., Hyun, H., Seo, K. S., Cho, Y. H., Lee, J. W., Lee, C. R., Khang, G. & Lee, H. B. 2006 Preparation and characterization of mPEG-PCL diblock copolymers with thermo-responsive sol–gel–sol phase transition. *J. Polym. Sci. A Polym. Chem.* **44**, 5413–5423. (doi:10.1002/pola.21659)
- Kim, Y., Dalhaimer, P., Christian, D. A. & Discher, D. E. 2005a Polymeric worm micelles as nano-carriers for drug delivery. *Nanotechnology* **16**, S484–S491. (doi:10.1088/0957-4484/16/7/024)
- Kim, Y. H., Park, J. H., Lee, M., Park, T. G. & Kim, S. W. 2005b Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. *J. Control. Release* **103**, 209–219. (doi:10.1016/j.jconrel.2004.11.008)
- Kong, H. J., Liu, J. D., Riddle, K., Matsumoto, T., Leach, K. & Mooney, D. J. 2005 Non-viral gene delivery regulated by stiffness of cell adhesion substrates. *Nat. Mater.* **4**, 460–464. (doi:10.1038/nmat1392)
- Koping-Hoggard, M., Varum, K. M., Issa, M., Danielsen, S., Christensen, B. E., Stokke, B. T. & Artursson, P. 2004 Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. *Gene Ther.* **11**, 1441–1452. (doi:10.1038/sj.gt.3302312)
- Kuhn, S. J., Finch, S. K., Hallahan, D. E. & Giorgio, T. D. 2006a Proteolytic surface functionalization enhances *in vitro* magnetic nanoparticle mobility through extracellular matrix. *Nano Lett.* **6**, 306–312. (doi:10.1021/nl052241g)
- Kuhn, S. J., Hallahan, D. E. & Giorgio, T. D. 2006b Characterization of superparamagnetic nanoparticle interactions with extracellular matrix in an *in vitro* system. *Ann. Biomed. Eng.* **34**, 51–58. (doi:10.1007/s10439-005-9004-5)
- Kulkarni, R. P., Wu, D. D., Davis, M. E. & Fraser, S. E. 2005 Quantitating intracellular transport of polyplexes by spatio-temporal image correlation spectroscopy. *Proc. Natl Acad. Sci. USA* **102**, 7523–7528. (doi:10.1073/pnas.0501950102)
- Kurisawa, M., Yokoyama, M. & Okano, T. 2000 Gene expression control by temperature with thermo-responsive polymeric gene carriers. *J. Control. Release* **69**, 127–137. (doi:10.1016/S0168-3659(00)00297-2)
- Lang, D. S., Droemann, D., Schultz, H., Branscheid, D., Martin, C., Ressmeyer, A. R., Zabel, P., Vollmer, E. & Goldmann, T. 2007 A novel human *ex vivo* model for the

- analysis of molecular events during lung cancer chemotherapy. *Respir. Res.* **8**, 43. (doi:10.1186/1465-9921-8-43)
- Lavigne, M. D., Pennadam, S. S., Ellis, J., Alexander, C. & Gorecki, D. C. 2007 Enhanced gene expression through temperature profile-induced variations in molecular architecture of thermoresponsive polymer vectors. *J. Gene Med.* **9**, 44–54. (doi:10.1002/jgm.992)
- Lee, H. I., Wu, W., Oh, J. K., Mueller, L., Sherwood, G., Peteanu, L., Kowalewski, T. & Matyjaszewski, K. 2007 Light-induced reversible formation of polymeric micelles. *Angew. Chem. Int. Ed.* **46**, 2453–2457. (doi:10.1002/anie.200604278)
- Lee, J. I., Ha, K. S. & Yoo, H. S. 2008 Quantum-dot-assisted fluorescence resonance energy transfer approach for intracellular trafficking of chitosan/DNA complex. *Acta Biomater.* **4**, 791–798. (doi:10.1016/j.actbio.2008.01.010)
- Lepage, M., Jiang, J. Q., Babin, J., Qi, B., Tremblay, L. & Zhao, Y. 2007 MRI observation of the light-induced release of a contrast agent from photo-controllable polymer micelles. *Phys. Med. Biol.* **52**, N249–N255. (doi:10.1088/0031-9155/52/10/N04)
- Liang, D. C., Liu, W. G., Zuo, A. J., Sun, S. J., Cheng, N., Guo, G., Zhang, J. Y. & De Yao, K. 2006 Pre-deliver chitosanase to cells: a novel strategy to improve gene expression by endocellular degradation-induced vector unpacking. *Int. J. Pharm.* **314**, 63–71. (doi:10.1016/j.ijpharm.2006.01.044)
- Lin, C. & Engbersen, J. F. 2009 The role of the disulfide group in disulfide-based polymeric gene carriers. *Expert Opin. Drug Deliv.* **6**, 421–439. (doi:10.1517/17425240902878010)
- Lin, C., Zhong, Z., Lok, M. C., Jiang, X., Hennink, W. E., Feijen, J. & Engbersen, J. F. 2007a Novel bioreducible poly(amido amine)s for highly efficient gene delivery. *Bioconjug. Chem.* **18**, 138–145. (doi:10.1021/bc0602001)
- Lin, C., Zhong, Z., Lok, M. C., Jiang, X., Hennink, W. E., Feijen, J. & Engbersen, J. F. 2007b Random and block copolymers of bioreducible poly(amido amine)s with high- and low-basicity amino groups: study of DNA condensation and buffer capacity on gene transfection. *J. Control. Release* **123**, 67–75. (doi:10.1016/j.jconrel.2007.07.006)
- Liu, X., Yang, J. W. & Lynn, D. M. 2008 Addition of ‘Charge-shifting’ side chains to linear poly(ethyleneimine) enhances cell transfection efficiency. *Biomacromolecules* **9**, 2063–2071. (doi:10.1021/bm800291v)
- Lucas, B., Remaut, K., Sanders, N. N., Braeckmans, K., De Smedt, S. C. & Demeester, J. 2005 Studying the intracellular dissociation of polymer–oligonucleotide complexes by dual color fluorescence fluctuation spectroscopy and confocal imaging. *Biochemistry* **44**, 9905–9912. (doi:10.1021/bi0476883)
- Mao, H. Q. & Leong, K. W. 2005 Design of polyphosphoester–DNA nanoparticles for non-viral gene delivery. *Adv. Genet.* **53**, 275–306. (doi:10.1016/S0065-2660(05)53011-6)
- Mao, Y. & Schwarzbauer, J. E. 2005 Stimulatory effects of a three-dimensional microenvironment on cell-mediated fibronectin fibrillogenesis. *J. Cell Sci.* **118**, 4427–4436. (doi:10.1242/jcs.02566)
- McGrath, N. & Barroso, M. 2008 Quantum dots as fluorescence resonance energy transfer donors in cells. *J. Biomed. Opt.* **13**, 031210. (doi:10.1117/1.2939417)
- Mellor, H. R., Davies, L. A., Caspar, H., Pringle, C. R., Hyde, S. C., Gill, D. R. & Callaghan, R. 2006 Optimising non-viral gene delivery in a tumour spheroid model. *J. Gene Med.* **8**, 1160–1170. (doi:10.1002/jgm.947)
- Meng, Q. H., Robinson, D., Jenkins, R. G., Mcanulty, R. J. & Hart, S. L. 2004 Efficient transfection of non-proliferating human airway epithelial cells with a synthetic vector system. *J. Gene Med.* **6**, 210–221. (doi:10.1002/jgm.483)
- Merdan, T., Kunath, K., Petersen, H., Bakowsky, U., Voigt, K. H., Kopecek, J. & Kissel, T. 2005 PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under *in vivo* conditions in a dose-dependent manner after intravenous injection into mice. *Bioconjug. Chem.* **16**, 785–792. (doi:10.1021/bc049743q)
- Mishra, S., Webster, P. & Davis, M. E. 2004 PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur. J. Cell Biol.* **83**, 97–111. (doi:10.1078/0171-9335-00363)
- Miyata, K., Kakizawa, Y., Nishiyama, N., Harada, A., Yamasaki, Y., Koyama, H. & Kataoka, K. 2004 Block cationic polyplexes with regulated densities of charge and disulfide cross-linking directed to enhance gene expression. *J. Am. Chem. Soc.* **126**, 2355–2361. (doi:10.1021/ja0379666)
- Molestina, R. E., Miller, R. D., Ramirez, J. A. & Summersgill, J. T. 1999 Infection of human endothelial cells with *Chlamydia pneumoniae* stimulates transendothelial migration of neutrophils and monocytes. *Infect. Immun.* **67**, 1323–1330.
- Mullen, P. M. *et al.* 2000 Strength of conjugate binding to plasmid DNA affects degradation rate and expression level *in vivo*. *Biochim. Biophys. Acta* **1523**, 103–110. (doi:10.1016/S0304-4165(00)00104-5)
- Mulligan, R. C. 1993 The basic science of gene therapy. *Science* **260**, 926–932. (doi:10.1126/science.8493530)
- Murthy, N., Campbell, J., Fausto, N., Hoffman, A. S. & Stayton, P. S. 2003 Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. *J. Control. Release* **89**, 365–374. (doi:10.1016/S0168-3659(03)00099-3)
- Nederman, T. & Twentyman, P. 1984 Spheroids for studies of drug effects. *Recent Results Cancer Res.* **95**, 84–102.
- Nederman, T., Norling, B., Glimelius, B., Carlsson, J. & Brunk, U. 1984 Demonstration of an extracellular matrix in multicellular tumor spheroids. *Cancer Res.* **44**, 3090–3097.
- Ng, C. P. & Pun, S. H. 2008 A perfusable 3D cell-matrix tissue culture chamber for *in situ* evaluation of nanoparticle vehicle penetration and transport. *Biotechnol. Bioeng.* **99**, 1490–1501. (doi:10.1002/bit.21698)
- Oishi, M., Kataoka, K. & Nagasaki, Y. 2006 pH-responsive three-layered PEGylated polyplex micelle based on a lactosylated ABC triblock copolymer as a targetable and endosome-disruptive nonviral gene vector. *Bioconjug. Chem.* **17**, 677–688. (doi:10.1021/bc050364m)
- Oupicky, D., Konak, C., Dash, P. R., Seymour, L. W. & Ulbrich, K. 1999 Effect of albumin and polyanion on the structure of DNA complexes with polycation containing hydrophilic nonionic block. *Bioconjug. Chem.* **10**, 764–772. (doi:10.1021/bc990007+)
- Oupicky, D., Parker, A. L. & Seymour, L. W. 2002 Laterally stabilized complexes of DNA with linear reducible polycations: strategy for triggered intracellular activation of DNA delivery vectors. *J. Am. Chem. Soc.* **124**, 8–9. (doi:10.1021/ja016440n)
- Oupicky, D., Reschel, T., Konak, C. & Oupicka, L. 2003 Temperature-controlled behavior of self-assembly gene delivery vectors based on complexes of DNA with poly(L-lysine)-graft-poly(*N*-isopropylacrylamide). *Macromolecules* **36**, 6863–6872. (doi:10.1021/ma034039a)
- Pack, D. W., Hoffman, A. S., Pun, S. & Stayton, P. S. 2005 Design and development of polymers for gene delivery. *Nat. Rev. Drug Discov.* **4**, 581–593. (doi:10.1038/nrd1775)

- Park, I. K., Ng, C. P., Wang, J., Chu, B., Yuan, C., Zhang, S. & Pun, S. H. 2008 Determination of nanoparticle vehicle unpacking by MR imaging of a T_2 magnetic relaxation switch. *Biomaterials* **29**, 724–732. (doi:10.1016/j.biomaterials.2007.10.018)
- Perez, J. M., Josephson, L., O'Loughlin, T., Hogemann, D. & Weissleder, R. 2002 Magnetic relaxation switches capable of sensing molecular interactions. *Nat. Biotechnol.* **20**, 816–820. (doi:10.1038/nbt720)
- Pollard, H., Remy, J. S., Loussouarn, G., Demolombe, S., Behr, J. P. & Escande, D. 1998 Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J. Biol. Chem.* **273**, 7507–7511. (doi:10.1074/jbc.273.13.7507)
- Read, M. L., Logan, A. & Seymour, L. W. 2005 Barriers to gene delivery using synthetic vectors. *Adv. Genet.* **53**, 19–46. (doi:10.1016/S0065-2660(05)53002-5)
- Rejman, J., Oberle, V., Zuhorn, I. S. & Hoekstra, D. 2004 Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem. J.* **377**, 159–169. (doi:10.1042/BJ20031253)
- Remaut, K., Lucas, B., Braeckmans, K., Sanders, N. N., De Smedt, S. C. & Demeester, J. 2005 FRET–FCS as a tool to evaluate the stability of oligonucleotide drugs after intracellular delivery. *J. Control. Release* **103**, 259–271. (doi:10.1016/j.jconrel.2004.11.019)
- Remaut, K., Lucas, B., Raemdonck, K., Braeckmans, K., Demeester, J. & De Smedt, S. C. 2007 Can we better understand the intracellular behavior of DNA nanoparticles by fluorescence correlation spectroscopy? *J. Control. Release* **121**, 49–63. (doi:10.1016/j.jconrel.2007.04.008)
- Roy, K., Mao, H. Q., Huang, S. K. & Leong, K. W. 1999 Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* **5**, 387–391. (doi:10.1038/7385)
- Ruponen, M., Yla-Herttuala, S. & Urtti, A. 1999 Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochim. Biophys. Acta* **1415**, 331–341. (doi:10.1016/S0005-2736(98)00199-0)
- Ruponen, M., Ronkko, S., Honkakoski, P., Pelkonen, J., Tammi, M. & Urtti, A. 2001 Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes. *J. Biol. Chem.* **276**, 33 875–33 880. (doi:10.1074/jbc.M011553200)
- Ruponen, M., Honkakoski, P., Ronkko, S., Pelkonen, J., Tammi, M. & Urtti, A. 2003 Extracellular and intracellular barriers in non-viral gene delivery. *J. Control. Release* **93**, 213–217. (doi:10.1016/j.jconrel.2003.08.004)
- Schaffer, D. V., Fidelman, N. A., Dan, N. & Lauffenburger, D. A. 2000 Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. *Biotechnol. Bioeng.* **67**, 598–606. (doi:10.1002/(SICI)1097-0290(20000305)67:5<598::AID-BIT10>3.0.CO;2-G)
- Sethuraman, V. A., Na, K. & Bae, Y. H. 2006 pH-responsive sulfonamide/PEI system for tumor specific gene delivery: an *in vitro* study. *Biomacromolecules* **7**, 64–70. (doi:10.1021/bm0503571)
- Stryer, L. 1978 Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* **47**, 819–846. (doi:10.1146/annurev.bi.47.070178.004131)
- Sun, S. J. *et al.* 2005 A thermoresponsive chitosan–NIPAAm/vinyl laurate copolymer vector for gene transfection. *Bioconjug. Chem.* **16**, 972–980. (doi:10.1021/bc0500701)
- Thomas, M. & Klibanov, A. M. 2003 Non-viral gene therapy: polycation-mediated DNA delivery. *Appl. Microbiol. Biotechnol.* **62**, 27–34. (doi:10.1007/s00253-003-1321-8)
- Torche, A. M., Jouan, H., Le Corre, P., Albina, E., Primault, R., Jestin, A. & Le Verge, R. 2000 *Ex vivo* and *in situ* PLGA microspheres uptake by pig ileal Peyer's patch segment. *Int. J. Pharm.* **201**, 15–27. (doi:10.1016/S0378-5173(00)00364-1)
- Truong-Le, V. L., Walsh, S. M., Schweibert, E., Mao, H. Q., Guggino, W. B., August, J. T. & Leong, K. W. 1999 Gene transfer by DNA-gelatin nanospheres. *Arch. Biochem. Biophys.* **361**, 47–56. (doi:10.1006/abbi.1998.0975)
- Varga, C. M., Hong, K. & Lauffenburger, D. A. 2001 Quantitative analysis of synthetic gene delivery vector design properties. *Mol. Ther.* **4**, 438–446. (doi:10.1006/mthe.2001.0475)
- Vile, R. G., Russell, S. J. & Lemoine, N. R. 2000 Cancer gene therapy: hard lessons and new courses. *Gene Ther.* **7**, 2–8. (doi:10.1038/sj.gt.3301084)
- Walsh, C. E. 2003 Gene therapy progress and prospects: gene therapy for the hemophilias. *Gene Ther.* **10**, 999–1003. (doi:10.1038/sj.gt.3302024)
- Williams, D. A. & Baum, C. 2003 Medicine. Gene therapy—new challenges ahead. *Science* **302**, 400–401. (doi:10.1126/science.1091258)
- Wolff, J. A. & Budker, V. 2005 The mechanism of naked DNA uptake and expression. *Adv. Genet.* **54**, 1–20. (doi:10.1016/S0065-2660(05)54001-X)
- Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A. & Felgner, P. L. 1990 Direct gene transfer into mouse muscle *in vivo*. *Science* **247**, 1465–1468. (doi:10.1126/science.1690918)
- Wong, S. Y., Pelet, J. M. & Putnam, D. 2007 Polymer systems for gene delivery—past, present, and future. *Prog. Polym. Sci.* **32**, 799–837. (doi:10.1016/j.progpolymsci.2007.05.007)
- Zaric, V., Weltin, D., Erbacher, P., Remy, J. S., Behr, J. P. & Stephan, D. 2004 Effective polyethylenimine-mediated gene transfer into human endothelial cells. *J. Gene Med.* **6**, 176–184. (doi:10.1002/jgm.484)
- Zintchenko, A., Ogris, M. & Wagner, E. 2006 Temperature dependent gene expression induced by PNIPAM-based copolymers: potential of hyperthermia in gene transfer. *Bioconjug. Chem.* **17**, 766–772. (doi:10.1021/bc050292z)
- Zuo, A., Sun, P., Liang, D., Liu, W., Zhao, R., Guo, G., Cheng, N., Zhang, J. & Yao, K. 2008 Improved transfection efficiency of cs/DNA complex by co-transfected chitosanase gene. *Int. J. Pharm.* **352**, 302–308. (doi:10.1016/j.ijpharm.2007.10.042)