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Review

Gene delivery with synthetic (non viral) carriers

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Abstract

Non-viral gene delivery involving the use of cationic polymer and cationic lipid based carriers still continues to enjoy a high profile due to the safety advantages offered by these systems when compared with viruses. However, there are still problems associated with the use of these agents, notably their comparatively low efficiency and the inability to target gene expression to the area of pathology. On intravenous administration gene expression is found predominantly in the first capillary bed encountered—the lung endothelium. The clinical use of non-viral gene delivery systems in cystic fibrosis or cancer has involved their direct application to the site of pathology due to the targeting difficulties experienced. For gene expression to occur genes must be transported to the interior of the cell nucleus and a number of biological barriers to effective gene delivery have been identified. These may be divided into extracellular such as the targeting barrier mentioned above and intracellular such as the need for endosomal escape after endocytosis and the inefficient trafficking of genes to the nucleus. Targeting ligands have been used with moderate success to overcome the targeting barrier while endosomal escape and nuclear targeting peptides are some of the strategies, which have been employed to overcome the problems of endosomal escape and nuclear trafficking. It is hoped that the next generation of carriers will incorporate mechanisms to overcome these barriers thus improving the efficacy of such materials. © 2001 Published by Elsevier Science B.V.

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1. Introduction

The completion of a working draft of the human genome project (McIlwain, 2000) paved the way for a greater understanding of genetic diseases. It is now theoretically possible to treat diseases of genetic origin by administering healthy copies of mutated (disease) genes or promote a protective immune response by administering genes encoding for specific antigens. Currently, however, the greatest hurdle to the actual realisation of these therapies is the development of non-toxic and efficacious delivery systems (Ander-

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son, 1998). Gene expression results when DNA is transported inside the cell nucleus of the target cell and there is still a need for carriers, which perform this feat safely and efficiently.

It is currently possible to obtain local transient transgene expression when naked plasmid DNA is administered to muscle tissue (Wolff et al., 1990). To obtain a systemic effect with the injection of naked DNA is difficult however as the intravenous injection of naked DNA results in low levels of gene expression in all major organs (Liu et al., 1995). There is thus an acute need for a delivery system in situations where widespread gene expression is desired such as in the treatment of metastatic disease. Over 500 gene therapy trials have been completed to date (Journal of Gene Medicine, 2001) with the majority using the more efficient viral vectors such as retroviruses and adenoviruses and with only about 20% of trials reporting the use of non-viral vectors. Viral vectors are more efficient yet plagued by safety concerns (Verma and Somia, 1997) and within the last 2 years the safety issues surrounding the use of viruses have been brought sharply into focus with the death of an 18-year-old and reasonably fit gene therapy trial patient (Marhsall, 2000). Hence despite their comparatively low efficiency when compared with viruses, non-viral vectors continue to attract a great deal of interest due to their advantageous safety profile. Add to the safety issues outlined above, the recent report on the comparative activity of retroviral and non-viral (liposomal) gene transfer in a mouse model which found no survival advantage with the use of retroviruses (Princen et al., 2000) and the case for intensified research into non-viral gene delivery becomes even more compelling.

Unfortunately despite the plethora of activity in various gene delivery laboratories world wide, there are still no licensed gene medicines available for the treatment of patients. This review charts the progress achieved so far in the quest to develop safe and effective non-viral gene delivery systems and highlights the major difficulties still facing the area.

Non-viral gene delivery refers to the use of naked DNA (Wolff et al., 1990), cationic lipids formulated into liposomes and complexed with DNA (lipoplexes) (Song et al., 1997), cationic polymers complexed with DNA (polyplexes) (Ogris et al., 1999), polymeric vesicles complexed with DNA (Brown et al., 2000) or a combination of both cationic lipids and cationic polymers complexed with DNA (lipopolyplexes) (Kircheis et al., 1999; Guo and Lee, 2000) (Fig. 1). There have also been attempts to combine the benefits of viral and non-viral systems into one delivery vehicle (Curiel et al., 1991).

2. Naked DNA

The application of plasmid DNA to skeletal muscle cells results in gene expression (transfection) (Wolff et al., 1990). This muscle transfection ability has led to naked DNA being administered intramuscularly for vaccination purposes, where DNA encoding for an antigen is administered with the objective of developing a protective immune response to the transgene antigenic product (Smith et al., 1998; Valez-Fiarcloth et al., 1999). Efficient transfection levels have also been obtained on direct application of naked DNA to the liver (Hickman et al., 1994; Zhang et al., 1997), solid tumours (Yang and Huang, 1996), the epidermis (Yu et al., 1999) and hair follicles (Yu et al., 1999). In some instances the direct application of a transgene using gene carriers such as cationic liposomes does not enhance (Meyer et al., 1995; Balasubramanian et al., 1996) and actually hinders gene expression (Yang and Huang, 1996; Yu et al., 1999; Cohen et al., 2000). The fact that gene expression levels are sometimes not enhanced by gene carriers when the genes are applied directly to certain tissues is evidence that there is no single optimum strategy for delivering genes. Gene delivery for gene therapy will have to be optimised on a mode of administration and disease basis. This lack of enhancement of activity by carriers is observed even when DNA tissue clearance is inhibited by the use of cationic liposomes (Meyer et al., 1995). The deleterious effects of cationic lipids such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate (DOTAP) to cells such as erythrocytes (Uchegbu et al., 1998) and macrophages (Filion and Phillips, 1998) may be responsible for this observed lack of activity.

It is clear that the application of naked DNA close to the site of pathology and away from degradative elements such as plasma is thus a viable strategy for gene delivery. However this method is ineffective if DNA dosing to anatomically inaccessible sites (e.g. solid tumours in organs) is desired.

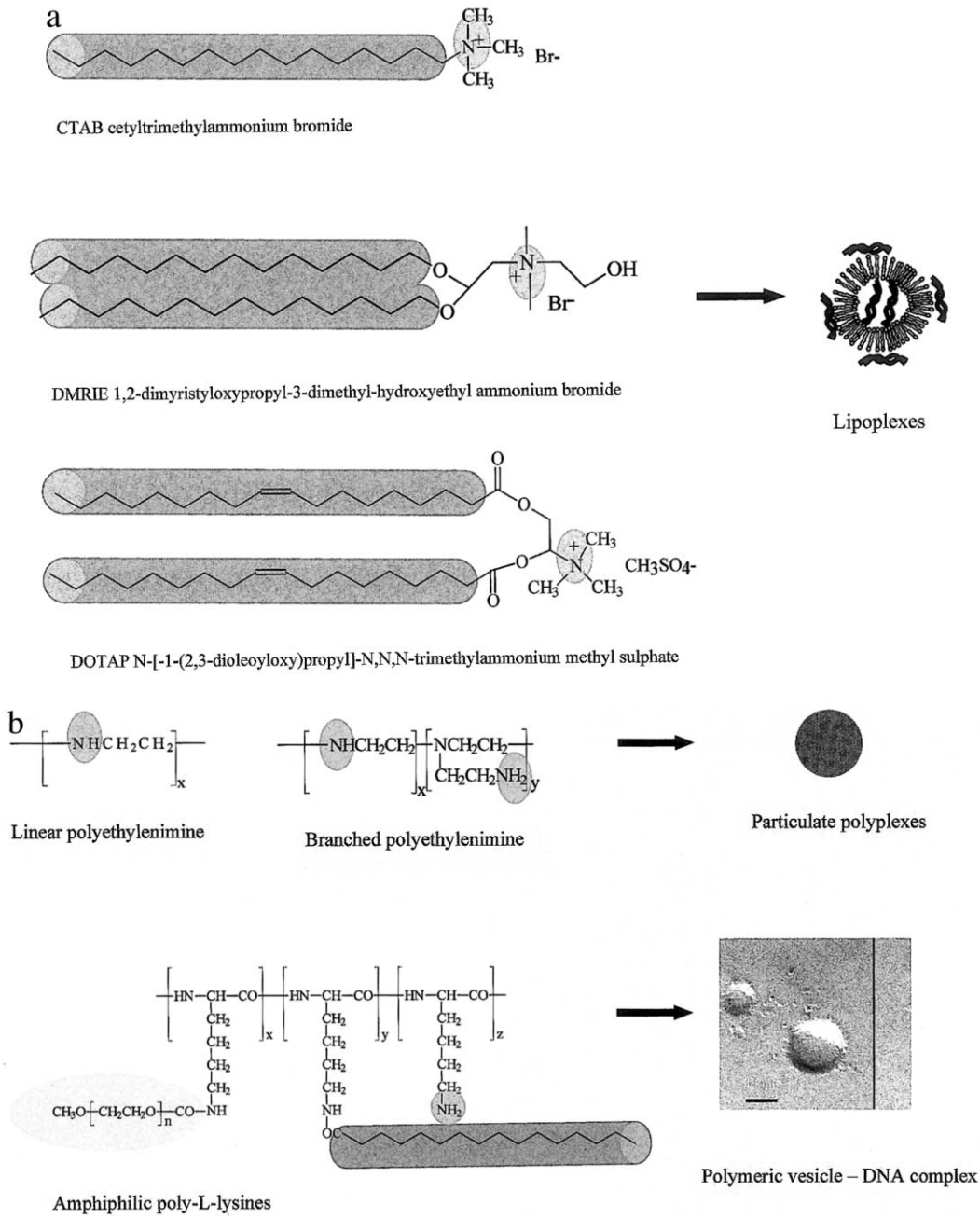

Fig. 1. (a) Examples of cationic lipids used for gene delivery. (b) Examples of cationic polymers used for gene delivery.

3. Cationic lipids

If the administration of genes by the intravenous or oral route or indeed to any remote site is envisaged some form of gene carriage must be employed. Felgner pioneered gene delivery with liposomes formed from lipids with a polar head group (protonated at physiological pH), in 1987 (Felgner et al., 1987). This has since resulted in the commercial production of in vitro gene delivery kits, e.g. lipofectin (*N*-[1-(2,3 - di - olyloxy)propyl] - *N*,*N*,*N* - trimethylammonium chloride, 1,2-dioleoylphosphatidylethanolamine-DOPE, 1:1) and the use of liposomal gene delivery in clinical trials (Porteous et al., 1997; Laitinen et al., 2000). A large number of cationic lipids have been synthesised and studied for gene delivery (Felgner et al., 1994; Remy et al., 1994; Gao and Huang, 1995; Balasubramanian et al., 1996; Budker et al., 1996; Stephan et al., 1996; Lee and Huang, 1997; Rosenzweig et al., 2000; Serikawa et al., 2000; Rosenzweig et al., 2001), some of which are shown in Fig. 1. All cationic lipids possess a hydrophobic group, which may either be one or two fatty acid or alkyl moieties of 12–18 carbons in length or a cholesteryl moiety, in addition to an amine group. The hydrophobic moieties ensure that the cationic lipids assemble into bilayer vesicles on dispersion in aqueous media, effectively shielding the hydrophobic portion of the molecule and exposing the amine head groups to the aqueous medium. The amine group is an absolute necessity for transfection competence as this is the DNA binding moiety interacting electrostatically with DNA and condensing the large anionic molecule into small transportable units—lipoplexes. Structure activity relationship studies have shown that increasing the number of amine groups per molecule (Wheeler et al., 1996) and the distance between the amine groups and the hydrophobic units (Remy et al., 1994) is advantageous to gene delivery. This arrangement of atoms allows an intimate level of DNA binding in the lipoplex (by increasing contact sites) as well as a separation of the bound DNA from the cohesive interaction of the hydrophobic units. Lipoplexes range from 50 nm to just over a micrometer in size (Labatmoleur et al., 1996; Song et al., 1997; Templeton et al., 1997). The influence of lipoplex size on transfection efficacy is contrary to what would be expected and the larger lipoplexes have been reported to improve transfection in vitro (Liu et al., 1997; Templeton et al., 1997; Ross and Hui, 1999; Wells et al., 2000).

A positively charged lipoplex is necessary for cell binding prior to internalisation (da Cruz et al., 2001) by endocytosis. Some neutral lipids such as DOPE (Farhood et al., 1995) and cholesterol (Semple et al., 1996; Hong et al., 1997; Liu et al., 1997) have been incorporated into the cationic lipid bilayer with DOPE said to improve in vitro transfection of some cell lines by facilitating endosomal escape (Farhood et al., 1995). The role of cholesterol however is unclear (Hong et al., 1997; Song et al., 1997).

The use of cationic liposomes to deliver genes increases the level of protein expression obtained on intravenous injection (Liu et al., 1995; Barron et al., 1999a,b). One of the reasons for this is that the complexation of DNA with cationic liposomes prevents DNA degradation in the plasma (Houk et al., 1999; Monck et al., 2000). However these carriers are severely limited in their applicability via the intravenous route as they are rapidly cleared by the plasma and accumulate within the lung tissue (Ishiwata et al., 2000) with protein expression seen primarily in the lung endothelium (Song et al., 1997; Song and Liu, 1998; Barron et al., 1999a,b), the first capillary bed encountered. Protein expression is transient, peaking 4–24 h after dosing and disappearing within a week of dosing (Song et al., 1997). Diverting lipoplexes from the lung can be achieved by incorporating polyethylene glycol (PEG) lipids into the lipoplex, a strategy which increases the circulation time of the lipoplexes and allows protein expression in distal tumours (Anwer et al., 2000a,b). This strategy is reminiscent of that used to divert drug carrying liposomes from the liver and spleen (Blume and Cevc, 1990). In vitro, however a PEG coating decreases uptake and gene transfer (Harvie et al., 2000). An alternative means of increasing transfection to sites out with the lung endothelium is the use of targeting ligands (see Section 9).

Cationic liposomes may also be applied directly to the site of pathology in order to avoid the targeting difficulties encountered when administered intravenously. Access to the alveolar epithelium has been achieved via the intratracheal route of administration, resulting in expression of the α-1-antitrypsin (Canonico et al., 1994), the β-galactosidase reporter (Griesenbach et al., 1998) genes and a reduction in the size of pulmonary tumours on administration of the p53 apoptosis-inducing gene (Zou et al., 2000). However, intratracheal administration is not routinely applicable in the clinic and access to the alveolar epithelium has been successfully sought via the use of aerosols (Stribling et al., 1992). Lipoplexes were effective in preventing degradation of DNA during aerosolisation (Crook et al., 1996).

Successful gene delivery to the eye will have an enormous impact on the treatment of genetic eye diseases. Transfection of the retinal ganglion cells has been observed on instillation of lipoplex eye drops with no inflammation reported (Matsuo et al., 1996).

As cell toxicity has been reported with cationic liposomes (Filion and Phillips, 1998; Uchegbu et al., 1998), research into reducing the toxicity of these carriers is required as once this important goal is achieved, the efficiency of these carriers may improve.

In addition to trying to improve the biological properties of lipidic gene carriers, some studies have focused on improving the stability of lipoplexes employing lyophilisation (Li et al., 2000a,b) with the aid of monosaccharide (Allison et al., 2000), disaccharide (Allison et al., 2000) or PEG lipid (Hong et al., 1997) cryoprotectants.

4. Polymers

As with cationic lipids, polymers bearing groups which are protonated at physiological pH have been employed as gene carriers (Fig. 1). The electrostatic attraction between the cationic charge on the polymer and the negatively charged DNA results in a particulate complex—the polyplex, which is the transfecting unit.

4.1. Poly-L-lysine based polymers

The first polycation to be employed for gene delivery was poly-L-lysine conjugated with asialoorosomucoid for hepatocellular gene targeting (Wu and Wu, 1987). Unlike cationic liposomes much of the early work involving the use of polyplexes utilised ligands to facilitate cellular uptake, e.g. asialoorosomucoid (Wu and Wu, 1987), transferrin (Cotten et al., 1990; Wagner et al., 1990; Curiel et al., 1996), folate (Mislick et al., 1995), monoclonal antibodies (Chen et al., 1994; Schachtschabel et al., 1996; Shimizu et al., 1996) and basic fibroblast growth factor (Sosnowski et al., 1996). The gene transfer activity of poly-L-lysine polyplexes without the use of receptor-mediated strategies is poor (Brown et al., 2000) unless endosomolytic or lysosomotropic agents (e.g. chloroquine) are added (Wadhwa et al., 1997; Pouton et al., 1998). This is an important difference in the biological activity of the amphiphilic cationic lipids and the soluble polymer poly-L-lysine. Cellular uptake of and gene transfer by polyplexes in the presence (Schaffer and Lauffenburger, 1998) or absence (Pouton et al., 1998) of targeting ligands is however still dependant on the presence of a positively charged polyplex (Schaffer and Lauffenburger, 1998), presumably to allow interaction with the negatively charged cell surface and subsequently endosomolytic uptake. Lipidic poly-L-lysines complexed to DNA have been prepared and found to be more efficient in vitro gene delivery agents than cationic liposomes (Zhou et al., 1991; Surovoy et al., 1998), evidence of the advantages offered by the use of more efficient DNA binding amphiphiles. Various other poly-L-lysine copolymers have also been shown to transfer genes into mammalian cells such as those incorporating L-tryptophan (Wadhwa et al., 1997), and graft poly-L-histidine (Benns et al., 2000). The conjugation of histidine to ε-L-lysine residues of poly-L-lysine (Midoux and Monsigny, 1999) resulted in a transfecting polyplex which was more efficient than a poly-L-lysine—chloroquine mixture. This graft copolymer enjoyed an enhancement in activity in the absence of chloroquine because of the additional endosomal buffering capacity offered by histidine

which is protonated below pH 6 (Midoux and Monsigny, 1999). In a similar strategy gluconylated polyhistidine has also been used to transfer genes to mammalian cells and again does not require chloroquine to be active (Pack et al., 2000). Hence the use of histidine residues seems to offer an endosomal escape facility.

A further interesting method of preparing poly-L-lysine based polyplexes involves the replacement of some L-lysine residues with cysteine and tryptophan residues (McKenzie et al., 2000). Cross linking of the cysteine residues in the polyplex increased the gene transfer activity of the polyplex, indicating that DNA release may be triggered by the intracellular reduction of disulphide bonds (McKenzie et al., 2000).

Although poly-L-lysine polyplexes prevent the degradation of DNA by serum nucleases (Chiou et al., 1994) in a similar manner to liposomes (Houk et al., 1999; Monck et al., 2000); on intravenous injection, these polyplexes, are bound by plasma proteins and rapidly cleared from the plasma (Dash et al., 1999) again like cationic liposomes (Ishiwata et al., 2000). Polyplex opsonisation by plasma proteins may be suppressed by coating the polyplexes with a hydrophilic polymer such as hydroxypropyl methacrylic acid, and the cellular uptake of the polyplexes may once again be promoted by the conjugation of targeting ligands such as transferrin (Dash et al., 2000) or fibroplast growth factor (Fisher et al., 2000) to the surface of the coated polyplexes.

4.2. Polyethylenimine

Recently both branched (Boussif et al., 1995, 1996) and linear (Ferrari et al., 1997; Chemin et al., 1998) polyethylenimine have been introduced as cationic polymers for gene delivery and unlike poly-L-lysine this polymer shows efficient gene transfer without the need for endosomolytic or lysosomotropic agents or indeed any agents facilitating receptor mediated uptake. PEI is endocytosed by cells and is also believed to facilitate endosomal escape (Klemm et al., 1998; Kichler et al., 2001). As with all the other non-viral gene delivery systems mentioned above a positively charged (Boussif et al., 1995) polyplex is necessary to allow gene transfer to take place. The influence of molecular weight on the activity of PEI is as yet unclear with some reports detailing an increase in gene transfer activity with a decrease in molecular weight (from 100 to 11.9 kDa) (Fischer et al., 1999) and some reports detailing a decrease in activity on decreasing the molecular weight (from 70 to 1.8 kDa) (Godbey et al., 1999). It is likely that an optimum molecular weight exists somewhere between 11.9 and 70 kDa.

PEI is quite an efficient gene transfer agent (Ferrari et al., 1997), however, the addition of targeting ligands to this polymer enhances its activity in some cell lines (Kircheis et al., 1997; Zanta et al., 1997; Erbacher et al., 1999; Li et al., 2000a,b). Recently hydrophobised PEI has been incorporated within DOPE, egg phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes, producing an efficient gene transfer agent although the activity of this soluble amphiphilic polymer was diminished when administered without the liposomal lipids (Yamazaki et al., 2000). Unfortunately, PEI as with some of the cationic lipids (Filion and Phillips, 1998) has also been reported to be toxic to cells (Boussif et al., 1995; Ferrari et al., 1997; Godbey et al., 2001).

PEI polyplexes have been used to achieve gene expression in experimental animals by direct application to various anatomical sites such as rat kidneys by intrarterial injection (Boletta et al., 1997), mouse brains (Boussif et al., 1995; Lemkine et al., 1999), and mouse tumours (Coll et al., 1999; Aoki et al., 2001) by direct injection and rabbit lungs by intratracheal administration (Ferrari et al., 1997, 1999). PEI gene expression also appears to be transient and is undetectable 14 days after administration to the kidney (Boletta et al., 1997). The direct administration of these polyplexes to organs in order to achieve transduction is evidence of the lack of targeting ability of these polyplexes on intravenous administration.

On intravenous injection of PEI polyplexes, transfection occurs primarily in the lung (Goula et al., 1998; Bragonzi et al., 1999) as with cationic liposomes (Song et al., 1997) with some expression being detected unusually in the alveolar epithelium (Goula et al., 1998). Intravenously administered PEI-transgene is reported to cross

the endothelial barrier and become expressed in alveolar epithelial cells within 2 h after administration (Goula et al., 1998). To divert transgene expression from the lung tissue, once again a PEG coating has been used, resulting in increased transfection in the liver (Nguyen et al., 2000). Additionally the combination of a PEG coating and the transferrin targeting ligand, resulted in gene expression in distal tumour sites (Ogris et al., 1999). PEG not only allows gene transport to sites out with the lung but also modulates the toxicity of PEI (Ogris et al., 1999), although in vitro uptake and transfer is diminished by PEG (Choi et al., 2001) as was observed for cationic liposomes (Harvie et al., 2000). Overall the gene expression seen with linear PEI is superior to that seen with cationic liposomes both on intravenous (Bragonzi et al., 1999) and intratracheal (Ferrari et al., 1997) administration.

4.3. Other polymers

A transfecting peptide has been prepared from the N-terminal of the human adenovirus fibre protein which promotes transport of DNA to the nucleus and shows improved transfection rates when compared with the cationic liposomes prepared from DOTAP (Zhang et al., 1999). Poly(dimethylamino)ethylmethacrylate (Lim et al., 2000; Hennink et al., 2001), poly(*N*-ethyl-4-vinylpyridinium) polymers (Kabanov et al., 1993), poly-L-histidine (Pack et al., 2000) polylactide co-glycolide (PLG) (Cohen et al., 2000) and chitosan (Murata et al., 1996, 1997; Erbacher et al., 1998; Roy et al., 1999) have also been used for gene delivery. Both chitosan (Erbacher et al., 1998) and PLG (Cohen et al., 2000) nanoparticles appear to control the release of DNA and prolong its action both in vitro (Erbacher et al., 1998) an in vivo (Cohen et al., 2000). The use of PLG is an unusual example of the efficacy of a polymer which is not protonated and hence not cationic at physiological pH as the PLG particles actually possess a negative surface charge (Cohen et al., 2000). Chitosan has also been used via the oral route to achieve an immune response against a peanut allergen (Roy et al., 1999), the first report of an orally active gene delivery system.

4.4. Dendrimers

A range of polyamidoamine (Bielinska et al., 1995a,b; Kukowska-Latallo et al., 1996; Bielinska et al., 1997; Du et al., 1998; BenMamoun et al., 1999; Bielinska et al., 1999; Hudde et al., 1999; Toth et al., 1999; Bielinska et al., 2000) and phosphorous containing (Loup et al., 1999) dendrimers have been studied as gene delivery systems. Terminal amino groups bind DNA by electrostatic means (Bielinska et al., 1999) and once again positively charged complexes must be formed and are necessary for gene transfer (Shah et al., 2000). An increase in the level of terminal amino groups appears to enhance gene delivery (Toth et al., 1999). Dendrimer-gene complexes are presumably internalised by endocytosis and there are also advantages associated with the star shape of the polymer as DNA appears to interact with the surface primary amines only, leaving the internal tertiary amines available for the neutralisation of the acid pH (Lee et al., 1996) within the endosomal/lysosomal compartment. Following internalisation, the release of polyamidoamine-gene complexes by the endosome has been attributed to the protonation of the internal tertiary nitrogens by endosomal protons which then results in a swelling of the endosome and the release of the DNA to the cytoplasm (Tang et al., 1996). The hydrolytic degradation of polyamidoamine dendrimer amide bonds in water or ethanol (Tang et al., 1996; Hudde et al., 1999) increases transfection efficacy up to 50-fold which the authors attribute to the increased flexibility of the polymer on heat degradation. This increased flexibility is said to be crucial to the swelling of the endosome (Tang et al., 1996). Partially hydrolysed polyamidoamine dendrimers were found to be more effective gene transfer agents than branched PEI in the in vivo transfection of the carotid artery of rabbits (Turunen et al., 1999), but less effective than branched PEI in transfecting the lungs of mice on intratracheal administration (Rudolph et al., 2000).

5. Polymeric vesicles

Modification of the cationic polymers poly-L-lysine and poly-L-ornithine by the covalent attachment of both hydrophobic (palmitoyl) and hydrophilic (methoxy polyethylene glycol) groups produces amphiphilic compounds capable of forming vesicles in the presence of cholesterol (Fig. 1) (Brown et al., 2000). These amino-acid based systems are less toxic and more efficient at delivering DNA to some live mammalian cells in vitro than the unmodified polymers (Brown et al., 2000). They also do not require lysomotropic agents such as chloroquine to be active (Brown et al., 2000) and this is attributed to the amphiphilicity of these materials.

Polymerised vesicles have been prepared by the self-assembly of polymerisable cationic monomers (Wu et al., 2001). Polymerisation of the liposome reduces the toxicity of the lipoplex without adversely affecting the transfection efficacy of these agents (Wu et al., 2001).

6. Lipopolyplexes

These have been prepared by condensing DNA with a polycation such as poly-L-lysine (Lee and Huang, 1996; Guo and Lee, 2000), polyethylenimine (Guo and Lee, 2000), spermidine (Ibanez et al., 1996) or spermine (Shangguan et al., 2000) and entrapping this polyplex within anionic (Lee and Huang, 1996; Guo and Lee, 2000) or neutral (Ibanez et al., 1996) liposomes. This method of packaging DNA is reported to result in a less toxic (Ibanez et al., 1996; Guo and Lee, 2000) and in some cases more efficient (Ibanez et al., 1996) in vitro gene transfer particle which protects DNA to a greater extent from nuclease degradation (Ibanez et al., 1996; Shangguan et al., 2000) when compared with cationic liposomes alone. It is clear that the removal of the cationic liposome mediated toxicity results in a more biocompatible gene transfer agent. In addition, the incorporation of folate targeting ligands into these lipopolylexes improves gene transfer efficiency (Lee and Huang, 1996).

7. Preparation of non-viral gene delivery systems

Non-viral gene delivery systems are usually prepared by simply mixing a solution of plasmid DNA, encoding for the gene of interest with either the carrier cationic liposomes (Templeton et al., 1997), the carrier polymer (Goula et al., 1998) or the carrier polymeric vesicles (Brown et al., 2000). The resulting particulate system, as indicated above, usually bears a positive surface charge (zeta potential) (Ogris et al., 1999) or a sufficient excess of cationic lipid (Song et al., 1997) to confer a positive surface charge. Particle size is usually in the colloidal size range (Templeton et al., 1997; Brown et al., 2000) and the complexes are administered freshly prepared, as the long-term stability of the complexes has not been proven.

8. The therapeutic use of non-viral gene delivery systems

8.1. Anti-cancer gene therapy

Gene therapy for the treatment of cancer is an area which has great potential once the gene delivery problems specific to these therapies have been adequately addressed (Schätzlein, 2001). Gene therapy of cancer could take the form of the administration of a good copy of a mutated tumour suppressor gene, the administration of a gene encoding for an enzyme which activates an anti-cancer prodrug or the administration of a gene which encodes for an antigen designed to generate a protective immune response (Schätzlein, 2001). A range of proof of concept studies have been carried out in animal models each demonstrating that gene therapy of cancer could one day become a clinical reality. The administration of a liposomal plasmid expressing anti-sense RNA aimed at the suppression of the production of K-ras specific p21 protein has been used to reduce the growth of pancreatic tumours in mice models (Aoki et al., 1995). Also the administration of a liposomal IL-2 gene formulation resulted in tumour suppression in severe combined immunodeficient mice (Egilmez et al., 1996) al-

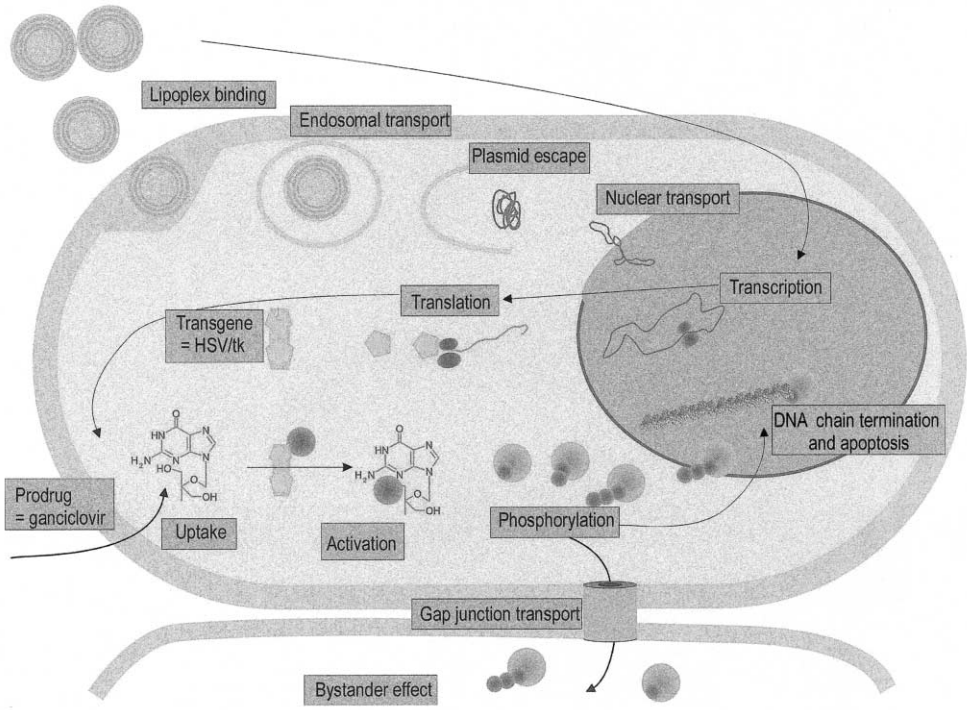

Fig. 2. Schematic representation of the sequence of cellular events occurring after the administration of the herpes simplex thymidine kinase gene for chemotherapeutic activation of the anti-cancer drug ganciclovir.

though the authors conclude that IL-2 independent factors could have resulted in tumour suppression. Other non-viral gene therapy studies with animals which showed good in vivo biological responses include the shrinking of tumours using the gene for the cytokine interleukin 12 (Anwer et al., 2000a,b) or the apoptosis inducing gene p53, which is believed to be defective in certain cancers (Zou et al., 2000).

Viruses are reported to be comparatively more efficacious in transferring genes into mammalian cells both in vitro (Brunner et al., 2000) and in vivo (Verma and Somia, 1997). However, a recent study involving the administration of retroviral and liposomal herpes simplex virus thymidine kinase gene to produce the activating enzyme thymidine kinase for the prodrug ganciclovir (Fig. 2) found no increase in survival in the group of mice administered the retroviral when compared with the animals administered the liposomal formulation (Princen et al., 2000). Ganciclovir is only activated on phosphorylation by thymidine kinase (Connors, 1995) and these studies indicate that the therapeutic use of viral and non-viral systems may not mirror the data obtained either in vitro or even in vivo with reporter genes. As far as possible a specific pharmacological response should be measured with experimental gene delivery systems.

Cancer gene therapy trials have been conducted using non-viral (exclusively liposomal and naked DNA so far reported) gene delivery strategies. The intratumoral injection of a liposomal gene encoding for HLA-B7 in order to generate a therapeutic immune response in melanoma patients (Nabel et al., 1994, 1996; Stopeck et al., 1998) resulted in a partial response (Nabel et al., 1996; Stopeck et al., 1998). Naked DNA has been used in cancer gene therapy trials and hepatocellular carcinoma patients have been injected with p53 in the form of naked DNA and have also shown a partial response (Habib et al., 1996).

8.2. Prenatal gene therapy

The prospect of administering genes in pregnancy in order to prevent the postnatal manifestation of genetic disease was brought closer by the report that the administration of cationic liposomes to pregnant mice also resulted in gene expression in the progeny although this was at a low level (Ochiya et al., 1999). These studies could lead to therapies for the correction of genetic defects in embryos and foetuses although societal ethical concerns may prevent research in this area.

8.3. DNA vaccination

DNA vaccines have been administered intramuscularly (Gregoriadis et al., 1997), intradermally (Braun et al., 1999), intranasally (Klavinskis et al., 1999) and orally (Roy et al., 1999). Cellular and humoral immune responses have been detected with intramuscular injection of naked DNA (Smith et al., 1998) and the use of both cationic lipids (Gregoriadis et al., 1997) and cationic microparticles (Singh et al., 2000) has been shown to enhance this immune response. A naked DNA vaccine encoding a mycobacterial antigen when administered via the intramuscular route effectively protects animals against challenge with mycobacterium avium (Valez-Fiarcloth et al., 1999). As well as the intramuscular route, an epidermal route of DNA vaccine administration is being studied (Braun et al., 1999). Using gold particles, antigen-producing DNA is fired into the epidermis resulting in gene expression in the outer layers of the epidermis (Braun et al., 1999). This method of gene delivery is able to protect animals against challenge by bovine herpesvirus-1 (Braun et al., 1999). Oral gene delivery has been reported recently. The oral administration of chitosan-DNA nanoparticles resulted in a protective immune response in a murine peanut allergy model (Roy et al., 1999). Additionally widespread mucosal immunity has been observed in animals administered intranasal naked DNA, a strategy which could prevent disease transmission over mucosal surfaces (Klavinskis et al., 1999). Patient studies have involved asymptomatic HIV patients being administered an experimental HIV DNA vaccine intramuscularly with patients receiving the highest dose showing an immune response (Boyer et al., 1999). The use of DNA vaccines is predicted to be a growth area.

8.4. Gene therapy of cystic fibrosis

A number of currently incurable diseases such as cystic fibrosis are the result of single gene defects. Gene therapy of cystic fibrosis will involve replacement of the mutated cystic fibrosis transmembrane conductance regulator (CFTR) gene, encoding for the chloride membrane transporter which is defective in cystic fibrosis patients (Porteous and Alton, 1993). One of the earliest reports on the efficacy of gene therapy using a non-viral gene delivery system (lipoplex) was the favourable response obtained with the cystic fibrosis mouse model administered the CFTR gene in a liposomal formulation (Alton et al., 1993). A full restoration of the defective chloride transporter was reported in some animals. Cystic fibrosis clinical studies have used cationic liposomes to deliver the CFTR gene to cystic fibrosis patients and some restoration of a functioning chloride channel has been reported (Porteous and Dorin, 1993; Caplen et al., 1995; Gill et al., 1997). The uptake of lipoplexes is however inhibited in the cystic fibrosis lung by mucus and infective sputum (Alton, 2000).

8.5. Other diseases

There are a few other diseases where gene therapy may also have applicability. Arthritis was ameliorated in an arthritic mouse model by the intraperitoneal injection of the interleukin 10 gene, with a decrease in inflammation being observed (Fellowes et al., 2000). Additionally, patients have also been administered the vascular endothelial growth factor complexed with cationic lipids by means of a perfusion-infusion catheter (Laitinen et al., 2000) in an effort to prevent myocardial ischaemia with no adverse effect detected in these preliminary studies although no clinical benefit was also reported (Laitinen et al., 2000).

9. Biological barriers to gene delivery

The ideal gene delivery therapeutic would be an orally active solid dosage form which allowed transport of a large percentage of the dose to the nucleus of the cells within the target tissue. Although oral activity has been documented for a chitosan based formulation (Roy et al., 1999), the above ideal is far from being realised and the current crop of non-viral gene delivery systems are principally most active when administered directly to the site of pathology (Nabel et al., 1996; Stopeck et al., 1998) or as close to the site of pathology as possible (Porteous et al., 1997). The lack of systemic activity on intravenous or even intramuscular delivery and the need to deliver these agents as close to the area of pathology as possible in order to elicit an effect are all evidence of the barriers which have been encountered with gene delivery.

The various barriers to gene delivery have been identified as being both at the extracellular and intracellular level (Schätzlein and Uchegbu, 2001) (Fig. 3) and examples of strategies adopted to overcome these barriers are summarised in Table 1. One of the difficulties faced by the science is the lack of correlation between in vitro and in vivo results (Wells et al., 2000), hence carrier characteristics favouring efficient transfection in vitro may be ineffective in vivo thus making it difficult to identify features which overcome both intracellular and extracellular barriers. Furthermore systematic comparisons of the more widely used non-viral gene delivery systems are not always carried out. A systematic study of both the extracellular and intracellular barriers to gene transfer

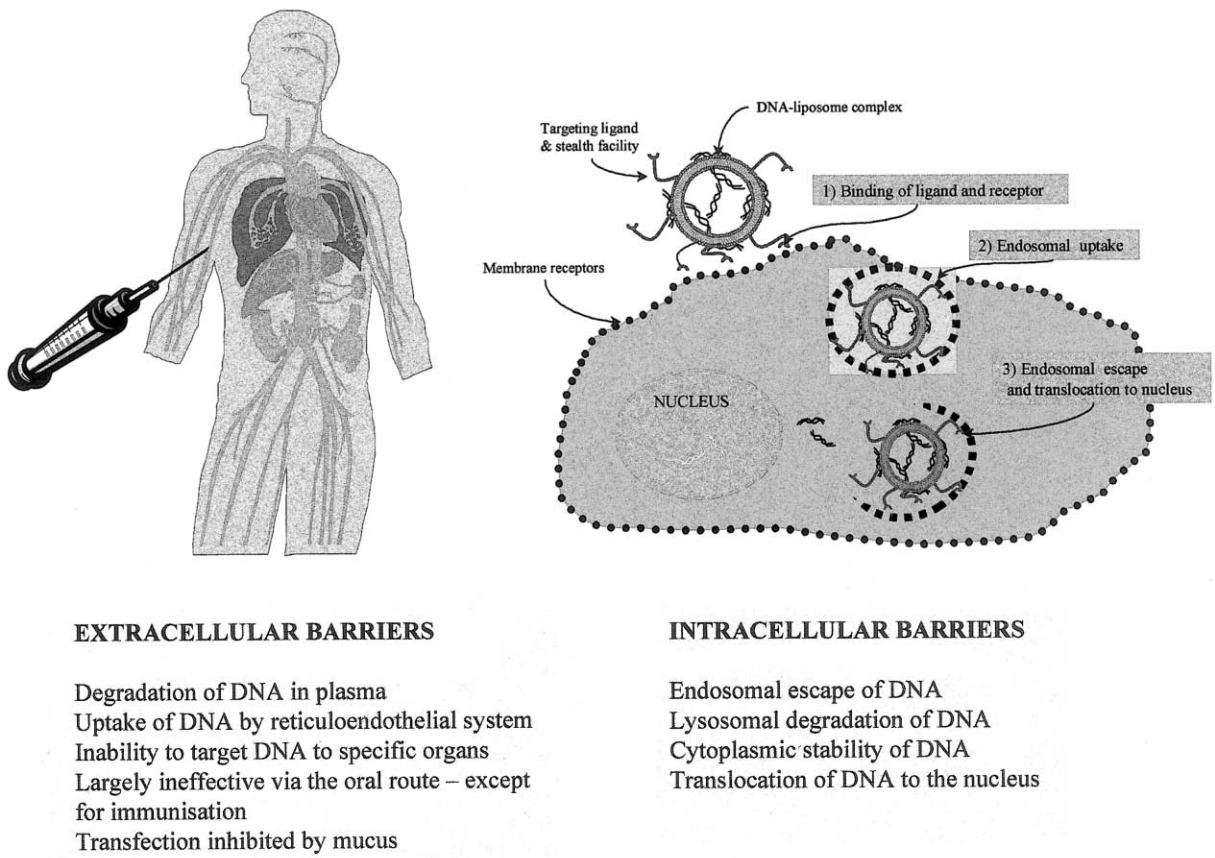

Fig. 3. The barriers to non-viral gene delivery.

Table 1
Methods used to overcome extracellular and intracellular barriers

Identified barrier	Strategy employed	Reference
Degradation of DNA by serum nucleases	Complexation with cationic liposomes	Houk et al., 1999
	Complexation with cationic polymers	Chiou et al., 1994
Targeting of DNA to particular tissue types	Targeting ligands, e.g. asialoorosomucoid	Wu and Wu, 1987
	Transferrin	Ogris et al., 1999
DNA uptake by cells	Electroporation	Wells et al., 2000
	Complexation with cationic liposomes	da Cruz et al., 2001
	Complexation with cationic polymers	Kichler et al., 2001
Endosomal escape	Endosomolytic peptides	Lim et al., 2000
	Polyethylenimine	Klemm et al., 1998
Transport from the cytoplasm to the nucleus	Nuclear localisation peptides	Conary et al., 1996
	Nuclear localisation nucleotide sequences	Vacik et al., 1999

encountered by polyplexes, lipoplexes, lipopolyplexes and polymeric vesicles is urgently needed. With the data currently available, it is difficult to compare the ease with which each of the current systems identified above actually overcomes the individual barriers as the usual endpoint in each study is the level of protein expression. Systemic barriers such as the difficulty in targeting specific organs, tissues or cell types as well as the intracellular barriers such as the crossing of the endosomal and nuclear membrane may all pose different levels of challenge to these various systems. Data on any differential activity in this particular context would aid the development of the next generation of more effective and hence clinically useful gene delivery agents.

9.1. Systemic barriers

The intravenous use of lipoplexes (Liu et al., 1995; Barron et al., 1999a,b) and polyplexes (Goula et al., 1998; Bragonzi et al., 1999) increases the level of protein expression obtained on intravenous delivery when compared with levels obtained with naked DNA as these carriers prevent the plasma degradation of DNA (Chiou et al., 1994; Houk et al., 1999) and promote cellular uptake (Labatmoleur et al., 1996; Kichler et al., 2001). However the limitation of using cationic liposomes (Song et al., 1997; Song and Liu, 1998; Song et al., 1998; Barron et al., 1999a,b) and indeed cationic polymers (Goula et al., 1998; Bragonzi et al., 1999) stems from the fact that transfection has been reported to occur primarily in the lung endothelium, the first capillary bed encountered because of the extensive non-specific interaction of the positively charged complexes with elements in the vascular compartment. Reducing the non-specific interactions of liposomes (Anwer et al., 2000a,b) and polymers (Ogris et al., 1999) by incorporating PEG into the lipoplex or polyplex, thus tends to divert the liposomes from the lung and allow transfection of distal solid tumours.

A further method, which has been used to achieve gene targeting, is the attachment of targeting ligands to lipoplexes and polyplexes. As such a variety of targeting ligands have been used to increase uptake in specific cell types, including galactose (Remy et al., 1995; Kawakami et al., 1998, 2000; Nishikawa et al., 2000) or asialorosomucoid (Wu and Wu, 1987; Kao et al., 1996; Singh et al., 2001) moieties for increased uptake by hepatocytes, mannose moieties for targeting the liver macrophages (Kawakami et al., 1998), folate (Lee and Huang, 1996; Guo and Lee, 1999) or transferrin (Ogris et al., 1999; Simoes et al., 1999) ligands for uptake by cells expressing the folate or transferrin receptor and cytoskeleton specific ligands for targeting injured cells from within which the cytoskeleton is exposed on cell injury (Khaw et al., 2000).

Another important barrier recently identified illustrates the importance of studying gene therapy in the context of a specific disease. Gene

transfer to the lung epithelium is severely limited by purulent infective sputum, a normal feature of the cystic fibrosis lung and also by normal mucus (Alton, 2000).

9.2. Cellular barriers

It is quite clear that the delivery of genes by the direct application to cells varies with cell type. Non-viral gene delivery systems are taken up by endocytosis (Farhood et al., 1995; Zabner et al., 1995; Klemm et al., 1998; Kichler et al., 2001) and efforts have focused on effecting the release of these particles from the endosome before the gene is destroyed within this organelle. Uptake may be enhanced by the use of targeting ligands which facilitate receptor-mediated uptake as detailed above and by physical techniques such as ultrasound (Anwer et al., 2000a,b), ionising radiation (Jain and Gewirtz, 1999) and electroporation (Wells et al., 2000). Endosomal escape is said to be facilitated by lipids such as DOPE in some cell lines (Farhood et al., 1995) and also by PEI (Klemm et al., 1998; Kichler et al., 2001).

To improve the gene transfer across both the extracellular and intracellular barrier, a combination of both endosomal disrupting peptides and receptor mediated uptake ligands have been used in lipoplexes and polyplexes. As such the endosome disrupting peptide GALA as well as the targeting ligands transferrin (Simoes et al., 1998, 1999) have been used in cationic liposomal formulations, both the endosomolytic transmembrane domain of diphtheria toxin (Fisher and Wilson, 1997) and asialoorosomucoid have been used with a poly-L-lysine gene delivery system and both galactose and the endosomolytic peptide KALA have been used with poly(2-(dimethylamino)ethyl methacrylate polymers (Lim et al., 2000). All these strategies have enjoyed moderate success. A further variation on the same theme has been the employment of a protein construct with cell targeting ability (in the form of an antibody), an endosomal escape facility (in the form of exotoxin A) and a DNA binding domain (Fominaya and Wels, 1996). All parts of this construct were found to be essential for transfection to occur (Fominaya and Wels, 1996). Finally the conjugation of imidazole units (endosomal escape moieties) to poly-L-lysine improved the transfection activity of this polymer in a dose dependant manner (Putnam et al., 2001).

The use of targeting ligands for receptor mediated uptake and an endosomolytic moiety for endosomal escape has resulted in improvements in gene transfer although the constructs are rather complex entities. The transfer of the gene from the endosome to the cytoplasm once effected then results in the gene encountering a further barrier the nucleolar membrane that is regarded as one of the most significant intracellular barriers to efficient transfection (Zabner et al., 1995; Labatmoleur et al., 1996).

Significant cytoplasmic degradation of un-complexed plasmid DNA can occur within the cytoplasm in hours and could severely limit the total amount of plasmid DNA that can actually be transported into the nucleus (Lechardeur et al., 1999). Transport/access of plasmid DNA to the nucleus occurs during cell division, when the nuclear envelope breaks down, or independently of cell cycling, through pores in the nuclear membrane. While efficient transfection has been shown to depend on mitosis (Brunner et al., 2000) there is also evidence that the nuclear pores act as a size-exclusion barrier. Small DNA fragments (oligonucleotides or plasmids) enter the nucleus by passive diffusion while larger fragments are transported through the nuclear pore complex in an energy-dependent manner (Kreiss et al., 1999; Ludtke et al., 1999). Nuclear pore transport can potentially be improved through the attachment of nuclear localisation signal peptides which redirect intracellular protein transport to the nucleus (Conary et al., 1996; Zanta et al., 1999), or the inclusion in the plasmid of nucleotide sequences with affinity for cellular proteins such as transcription factors; these then mediate the actual nuclear transport (Vacik et al., 1999).

To circumvent the nuclear barrier a cytoplasmic expression system has been developed (Mizuguchi et al., 1997), in which the transgene is administered with T7 RNA polymerase and incorporates a T7 promoter sequence (Mizuguchi et al., 1997). The nucleolar barrier makes the use of RNA expression systems particularly attractive, once

issues surrounding the bulk production and stabilisation of the RNA expression systems have been adequately addressed.

10. Conclusions

The potential market for gene therapeutics must be estimated at billions as opposed to millions of United States dollars. Most of the candidate diseases are currently incurable and some such as cancers are widespread. Non-viral gene delivery has been attempted with liposomal and polymer based systems but unsolved problems remain with these systems. These systems are vulnerable in the plasma, sequestered by particular cell types and on arrival at the cell must be endocytosed, escape from the endosome and eventually deliver DNA to the nucleus. Improvements in all these aspects of gene transport are required if efficient systems are to emerge from the current effort. The cell toxicity associated with the efficient polymeric and liposomal systems hamper their widespread use but principally the main issues surrounding these systems concern their efficiency. The transient gene expression obtained with these systems will be less of a problem if repeat dosing is possible with safe well-tolerated systems.

Results of clinical trials demonstrate that the in vitro and in vivo animal model data can be translated into real clinical benefit and interestingly no major clinical toxicities have been reported with the non-viral delivery systems. It is unlikely that a gene delivery system will emerge which has universal applicability and the first licensed gene therapeutics will utilise a gene delivery system which has been tailored to give high levels of gene expression when administered to treat a specific disease. It is thus more cost effective to concentrate efforts on achieving high levels of therapeutic gene expression in specific well-characterised animal models rather than concentrate excessive effort on work with the ubiquitous reporter genes. The way forward is via multidisciplinary consortia comprising disease experts, chemists and pharmacologists all working together on a specific disease basis. The future of medical technology remains to be revolutionised by these therapies.

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