Polymers for gene delivery across length scales

A number of human diseases stem from defective genes. One approach to treating such diseases is to replace, or override, the defective genes with normal genes, an approach called 'gene therapy'. However, the introduction of correctly functioning DNA into cells is a non-trivial matter, and cells must be coaxed to internalize, and then use, the DNA in the desired manner. A number of polymer-based synthetic systems, or 'vectors', have been developed to entice cells to use exogenous DNA. These systems work across the nano, micro and macro length scales, and have been under continuous development for two decades, with varying degrees of success. The design criteria for the construction of more-effective delivery vectors at each length scale are continually evolving. This review focuses on the most recent developments in polymer-based vector design at each length scale.

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In the nascent years of gene therapy, synthetic DNA-delivery vectors with greater and greater efficacy were reported with regular frequency. However, as the field has matured over the past two decades, the rate at which significant and clinically relevant advancements are reported has become almost asymptotic, even though the rate of published reports has grown exponentially[1–16].

Polymeric systems for DNA delivery have been extensively studied in part due to their well-defined chemistries and physical characteristics, and their high degree of molecular diversity that can be modified to fine-tune their physicochemical properties. For example, the molecular weight, polydispersity, side-chain composition and side-chain density of a specific polymer can be collectively altered to optimize one or more desired properties, such as delivery efficiency and biocompatibility. However, these advantages are counterbalanced by their low level of gene-transfer efficiency (relative to viral-based delivery systems).

Gene therapy is defined as the transfer of genetic information to specific cells to direct the synthesis of a specific protein. Typically, the 'genetic information', or gene, is encoded within plasmid DNA, although other forms of DNA as well as RNA can also be used. The nucleic acid sequence of the gene directs the cell to synthesize a protein with a specific, and correct, amino acid sequence through the processes of transcription (that is, synthesis of messenger RNA from DNA) and translation (that is, synthesis of protein from messenger RNA).

The goal of gene therapy is application-dependent. For example, in the case of genetic diseases (such as cystic fibrosis), the goal is to attenuate or override the influence of a malfunctioning gene. Gene therapy can also be used to prevent disease, through vaccination, using a gene encoding for the antigenic component of a specific pathogen.

Although the genetics community has identified many genes that can be potentially used for therapeutic purposes, the efforts of the DNA-delivery community have been hindered by a number of as yet unresolved challenges. These challenges can be broken down into three general categories: formulation, organism and cellular. Box 1 outlines these challenges in detail, and provides the design criteria for synthetic materials that are considered, based on the current literature, to be favourable for overcoming them. In brief, a delivery system should be pharmaceutically and economically acceptable (formulation level), provide the gene with acceptable access to the target tissues/cells (organism level) and effectively deliver its genetic payload to the nucleus of the cell (cellular level).

Synthetic gene-delivery platforms typically encompass three length scales — nano, micro and macro — depending on what is the desired

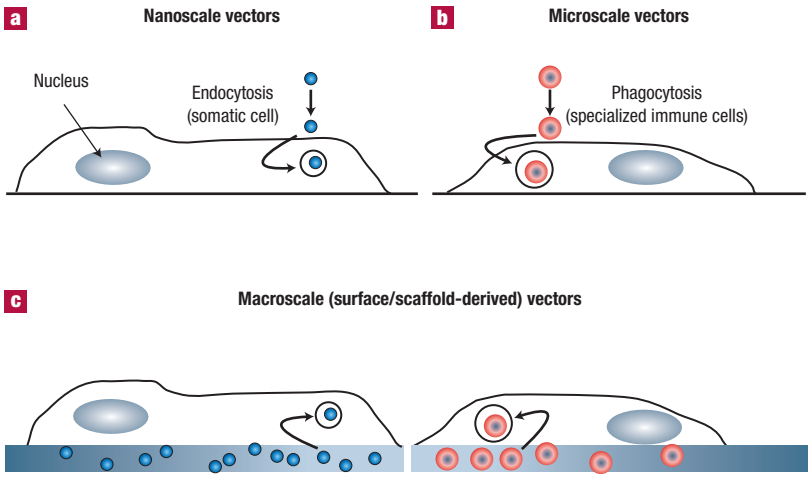


Figure 1 Length scales associated with DNA delivery vectors. **a**, Nanoscale-vectors, defined as systems with hydrodynamic diameters <1,000 nm (but predominantly of the order of <200 nm) are internalized by a number of mechanisms including clathrin-dependent receptor-mediated endocytosis, absorptive/fluid phase pinocytosis and caveolae-mediated endocytosis. **b**, Microscale vectors (1–10 μm) are internalized by phagocytosis, primarily by specialized antigen-presenting cells (for example, macrophages, dendritic cells). **c**, Macroscale systems are defined as surfaces or 3D scaffolds that release nanoscale and or microscale vectors. Macroscale systems generally deliver DNA to the underside of cells growing at their surface. It is important to note that the challenges, barriers, needs and design criteria defined for the nanoscale systems in Box 1 hold true for each length scale.

cell type, anatomical site or clinical application. Nanoscale delivery vectors are generally polycationic polymers or lipids that self-assemble with the polyanionic nucleic acids to form polyelectrolyte complexes that are internalized by endocytic pathways in somatic cell lineages. Microscale delivery systems usually consist of DNA entrapped within a polymer matrix for DNA vaccination or local DNA delivery. Macroscale systems are two-dimensional (2D) or 3D scaffolds designed to deliver DNA to a population of cells proximal to the scaffold surface, for tissue engineering and other applications. A schematic that illustrates DNA delivery at each length scale is provided in Fig. 1, and Table 1 provides a representative list of specific materials previously used as delivery systems according to length scale.

At each length scale, gene-delivery vectors face a progressive set of impediments at both the organism and cellular levels (Box 1). It is generally accepted that virus-mediated gene delivery is far more efficient than their synthetic counterparts because evolution has selected for viruses with the capability to successfully circumvent these barriers. Most research efforts that focus on enhancing the efficacy of synthetic vectors use a design-based approach to engineer materials with functional characteristics that overcome one or more of the barriers to delivery. Unlike the early years of gene therapy, new delivery reagents are no-longer 'discovered' from random testing of off-the-shelf materials, and a number of new synthetic vectors have been designed through hypothesis-driven research (Table 1).

THE NANOSCALE

Viruses have evolved to deliver DNA payloads efficiently to a wide range of cell types. Viral coats comprise proteins, lipids and carbohydrates organized into well-defined nanostructures. Over the past two decades, synthetic materials, primarily cationic polymers and lipids, have been developed to create 'synthetic artificial viruses' — self-assembled nanoconstructs to deliver genes to cells in culture as well as in higher organisms (that is, humans). Although cationic polymers and lipids are promising synthetic materials for gene delivery, they have limited utility due to intrinsic cytotoxicity and poor delivery efficiency. Conversely, viral-based gene-delivery vehicles are highly efficient, but are of limited use because they can be immunogenic or may integrate into the host genome. For these nanoscale delivery systems, some of the most intense efforts focus on either improving the delivery efficiency of non-viral vehicles, or lessening the immunological concerns of viral vehicles.

Although the hypothesis-driven design of enhanced synthetic vectors has led to more effective gene-transfer reagents, their efficacy remains orders of magnitude below viral vectors. To further improve the efficiency of synthetic vectors, some investigators have adopted combinatorial tactics to approach the problem. Rather than design synthetic vectors in a linear fashion (that is, make one, test one), these investigators are using combinatorial methods to synthesize new delivery reagents in a parallel fashion (that is, make many, test many), effectively increasing the rate at which new vectors can be created and identified.

The initial effort towards identifying new, more-efficient synthetic delivery vectors through combinatorial methods was reported in 1998 by Chiron Corporation[17]. In this seminal report, the investigators used existing solid-phase methods originally developed for peptide synthesis to create a series of substituted glycine 'peptoid oligomers' with varying characteristics such as main-chain length, cationic density and hydrophobicity (Fig. 2a). A total of 67 structures were synthesized from 13 potential sidechains, 24 of which were further investigated for their ability to transfect cells with a model plasmid.

Biophysical characterization of the peptoids revealed that most were able to complex electrostatically with plasmid DNA, protect the DNA from nuclease attack, and condense plasmid DNA into nanoscale structures. Transfection data revealed that a repeating side-chain motif of two phenyl groups followed by a primary amine-terminated side-chain yielded optimal activity. However, substitutions of the aromatic ring, such as with a *p*-hydroxyl group, eliminated transfection efficacy even though they condensed the DNA and protected it from nuclease degradation. These data illustrated that even seemingly minor changes in the carrier structure can significantly alter the transfection efficiency, a characteristic of synthetic gene-delivery systems that makes transfection prediction by limited structure–function information a difficult task.

Table 1 Representative examples of polymer-based DNA delivery reagents at the nano, micro and macroscales. References for each example are not included herein due to space restrictions, but are available from the author upon request.

Length scale and primary use	Representative examples
Nanoscale (<1 μm) Systemic delivery	Polylysine and its derivatives, branched polyethylenimine, linear polyethylenimine, degradable polyethylenimine, polyethylenimine derivatives, PEGylated polyethylenimines, cyclodextrin-based polymers, poly[α-(4-aminobutyl)-L-glycolic acid], imidazole-containing polymers, poly(propylacrylic acid) and derivatives, polyacetals and acetal-containing polymers, polyphosphoesters, polyphosphazenes, poly(2-(dimethylamino)ethyl methacrylate), poly-*N*-(2-hydroxypropyl)methacrylamide, poly(amidoamine)s, pAMAM dendrimers, non-condensing polymers (Pluronics, PINCs), poly(β-amino ester)s, chitosan, engineered DNA, inorganics/ceramics, peptides, multifunctional nanorods, photosensitive dendrimers
Microscale (1–10 μm) DNA vaccines and localized DNA delivery	Poly(lactic-*co*-glycolic acid) microspheres, polyorthoester microspheres, cationic microparticles, poly(β-amino ester)-containing microspheres, polyanhydride microspheres, hyaluronan microspheres
Macroscale (surfaces) Tissue engineering	Hyaluronic acid/collagen hydrogels, crosslinked PEG hydrogels, genetically engineered protein hydrogels, 2D cationic surfaces, 3D hydrophobic scaffolds, inorganic surfaces, polyelectrolyte thin films, thermosensitive hydrogels

A similar approach was used to enhance the efficiency of cationic lipid vectors (Fig. 2b)[18]. This collaborative effort focused on screening cationic lipids for gene delivery. The motivation stemmed from a reported correlation between the biophysical characteristics of lipid/DNA complexes and their transfection efficiency, which is in contrast to the peptoid results that showed the biophysical characteristics of cationic polymers were not necessarily predictive of transfection efficiency[17].

A cationic lipid 'library' was synthesized using a four-step solid-phase strategy yielding a total of 16 unique structures and stereochemical configurations (Fig. 2b). Interestingly, akin to the peptoid results, the transfection efficiency increased with increasing hydrophobicity of the transfection reagent, with the maximum transfection efficiency and lowest cytotoxicity associated with a di-C_{14}-tailed lipid. As with the peptoid library, the underlying mechanism(s) leading to these transfection results was not investigated.

Perhaps the broadest effort to use combinatorial chemistry to improve gene delivery has been with the parallel synthesis of a class of poly(β-amino ester)[19]. The synthetic route was designed to allow vector synthesis in one step by the conjugate addition of primary or secondary diamines to diacrylate monomers (Fig. 2c). From a series of seven diacrylates and 20 primary or secondary diamines, a set of 140 structures was created. Of this library, two mediated transfection comparable to the most efficient existing reagents, branched polyethylenimine and Lipofectamine 2000. Although the overall results of the experiments did not yield structures with transfection efficiencies far exceeding existing reagents, the report did outline a clear strategy that could be used to screen polymer structures efficiently as a function of structure, DNA concentration, DNA/polymer ratio, cell lineage, cell density and so on.

To increase the throughput of their outlined approach, a semi-automated process was next reported by the same group[20]. A total of 2,350 poly(β-amino ester) structures, from 94 amine and 25 diacrylate monomers, were synthesized using the same amine/acrylate chemistry developed in the original report. From their screen the authors identified 46 new structures with transfection efficiencies exceeding optimized polyethylenimine. Of particular note is that the best reagents almost always had a common structural component: a hydrophobic residue. Characterization and optimization of the poly(β-amino ester) structures has been executed by more-traditional approaches[21,22], and efforts have expanded into a preclinical evaluation of the library-derived structures[23].

Comparing the results of the above peptoid, lipid and polymer combinatorial manuscripts, it is intriguing that the most effective reagents contained a hydrophobic component, suggesting that a common transfection mechanism may exist. However, to date there have been no in-depth mechanistic investigations into how these reagents function at the cellular and molecular levels.

Another approach towards the combinatorial synthesis of functionalized polymers is through the use of polyacrylate backbones with reactive side chains, such as poly(*N*-methacryloxysuccinimide)[24–27]. In this work, the polymer side chains were substituted

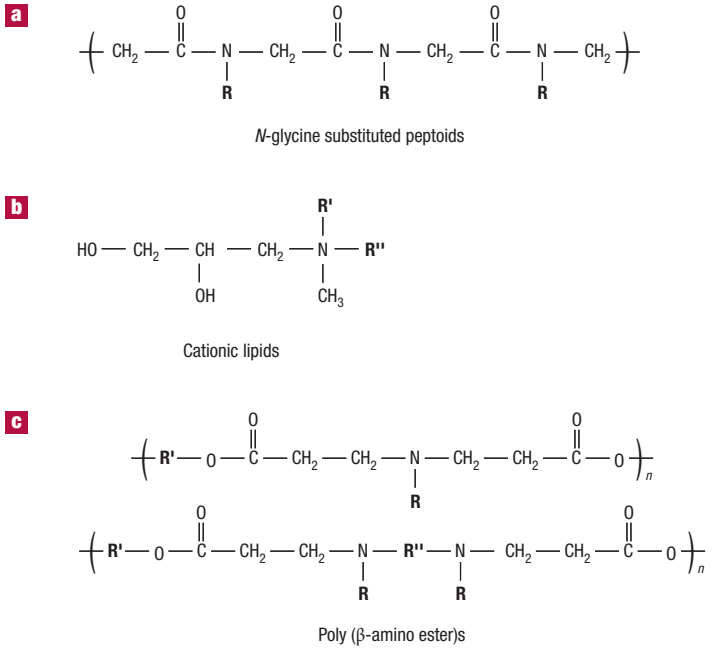


Figure 2 General structures of the first combinatorial libraries synthesized to enhance DNA delivery. For each structural class of library, the R group represents a diverse range of functional groups, which are not practical to delineate herein. The reader is referred to the original manuscripts for the complete library compositions. **a**, *N*-glycine-substituted peptoids[17]. **b**, Cationic lipids[18]. **c**, Poly(β-amino ester)s[19].

by aminolysis to yield polymers with a variety of side chains. Although the use of reactive ester side-chains for the synthesis of functionalized polymers had been pioneered decades earlier[28–30], this most recent series of reports focus on using a combinatorial approach towards the design and synthesis of acrylate-based drug- and DNA-delivery reagents. The primary advantage of their approach is that a set of structures with a diverse array of side chains can be systematically synthesized, permitting an in-depth analysis of structure–function relationships. However, unlike the poly(β-amino ester)s, a limitation of the poly(*N*-methacryloxysuccinimide) approach is that the final products should ideally be purified to remove reaction by-products and excess reactants.

Unlike synthetic vectors, where the challenge is to enhance delivery efficiency, viral vectors are already efficient DNA-delivery vehicles. For viral vectors, a primary challenge is to make them less immunogenic. Just like viruses have evolved to infect cells, immune systems have evolved to prevent infection, leading to the rapid neutralization of viral-based DNA-delivery vectors. By making viruses more 'synthetic', and treating them as synthetic polymeric constructs, it may be possible to attenuate the immune response and improve their safety profiles in humans.

'Stealth' technology, 'PEGylation' or 'pegnology' originated in the 1960s and has been applied to a number of pharmaceutical systems, primarily to enhance the circulation time of proteins, peptides and liposomes[31–33]. Polyethylene glycol (PEG) can reduce the specific (antibody mediated) and non-specific (reticuloendothelial) uptake of proteins, and a number of groups have extended the concept of PEG conjugation to increase the blood circulation half-life of viral vectors, which is typically of the order of a few minutes[34].

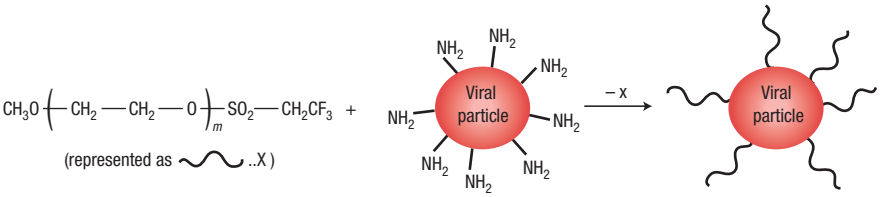
The modification of adenovirus with PEG first appeared in 1998 when adenovirus was non-covalently coated with PEG by electrostatic complexation[35]. Covalent attachment came soon thereafter using a mono-functionalized PEG (tresylmonomethoxypolyethylene glycol) with selective reactivity to the ε-amino group of lysine (Fig. 3a)[36]. A follow-on report that focused on different PEG-conjugation chemistries and formulation issues showed that following intravenous administration to naive, immunocompetent mice, liver infection was greater by six-fold with covalently modified adenovirus preparations than with unmodified adenovirus[37].

An alternative approach has been reported using the hydrophilic poly[*N*-(2-hydroxypropyl)methacrylamide], or pHPMA. Unlike semitelechelic PEG that links to proteins by a single end-group modification, the pHPMA was designed with multiple reactive side chains to form a hydrophilic 'coat' over the virus particle instead of a PEG-like brush (Fig. 3b)[38]. The guiding principle of their work was to prevent non-specific adenovirus uptake by the liver and perhaps enhance the biodistribution into peripheral tissues. The polymer modification increased the blood circulation time as a function of viral dose, suggesting a saturateable uptake mechanism, and reduced liver infection by several orders of magnitude. However, specific effects on biodistribution were not reported. Additionally, it is important that the virus modification be conducted under relatively dilute conditions to avoid potential crosslinking between disparate virus particles.

Both PEG and pHPMA offer an additional advantage for improving gene delivery with adenovirus. As the hydrophilic polymers mask the native capsid domains that recognize receptors on a number of cell types, the polymers can be used to attach targeting ligands to redirect the viruses to specific cell types (Fig. 3c). Early attempts to alter the tropism of adenovirus, primarily through genetic modifications of the capsid proteins, failed to eradicate the strong natural tropism of the virus[39–41]. By ablating the ability of the virus to infect cells by its route of infection with PEG and/or pHPMA, adenovirus has been redirected to infect a range of different cell types. For example, using a heterobifunctional PEG (tresyl-PEG-maleimide), investigators successfully improved delivery of adenovirus to intraperitoneal tumours using basic fibroblast growth factor (FGF2), and decreased non-specific liver uptake by an order of magnitude[42]. Similar results were reported even earlier with pHMPA conjugated to both FGF2 and VEGF[43]. Similar approaches with different viruses, such as adeno-associated virus and lentivirus, have also recently been reported[44,45].

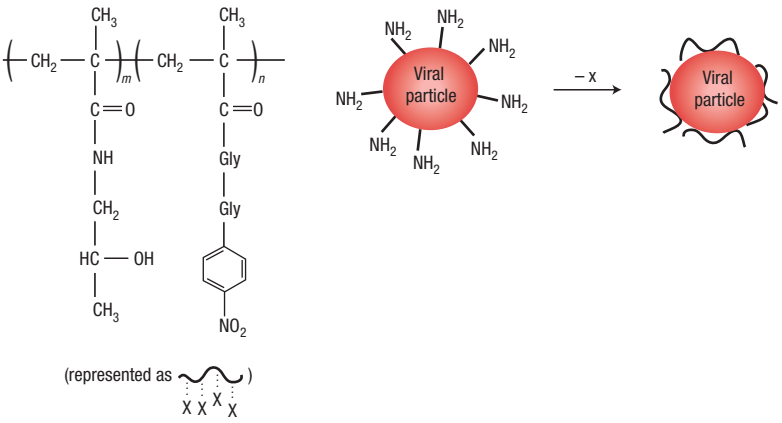
An exciting new way to engineer viruses has emerged that uses directed evolution strategies to

a



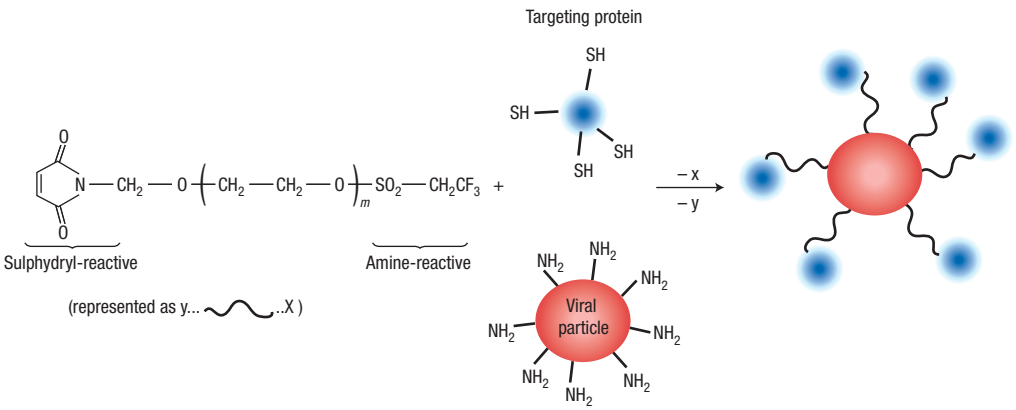
Degree of substitution	Infectivity in presence of neutralizing antibodies	Infectivity in absence of neutralizing antibodies
0	~15,000 RLU	~64,000 RLU
5%	~18,000 RLU	~61,000 RLU
10%	~22,000 RLU	~58,000 RLU
15%	~37,000 RLU	~48,000 RLU
20%	~37,000 RLU	~46,000 RLU

b



Viral particles administered	Infectivity in presence of neutralizing antibodies	Infectivity in absence of neutralizing antibodies
1×10^9	~6×10^4 RLU	~5×10^8 RLU
6×10^{11}	~2×10^7 RLU	~4×10^9 RLU

c



Vector administered	Tumor infectivity (average)	Liver infectivity (average)
Native adenovirus	6,500 RLU mg^{-1} tissue	80,000 RLU mg^{-1} tissue
Adenovirus–PEG conjugate (untargeted)	2,000 RLU mg^{-1} tissue	Not determined
Adenovirus–PEG conjugate (targeted with FGF2)	19,000 RLU mg^{-1} tissue	4,000 RLU mg^{-1} tissue

Figure 3 Virus/polymer conjugates to reduce virus immunogenicity, improve biodistribution profile, and alter tropism. For all figures, RLU stands for relative light units, which is proportional to the level of infectivity. Also, unreacted amines and thiol groups in the final product are not shown. **a**, Virus modified with monovalent PEG-tresylate, reactive to the primary epsilon amines of lysine, afforded particles with the ability to evade antibody neutralization. Data adapted from ref. 36. **b**, Virus modified with a multivalent copolymer of poly(*N*-(2-hydroxypropyl) methacrylamide), or pHMPA. The reactive *p*-nitrophenol terminated sidechain (comonomer fraction y) is reactive to the primary epsilon amines of lysine. Gly represents a glycine residue. Coating the virus particle with pHPMA reduced non-specific liver uptake, potentially improving the virus' biodistribution profile *in vivo*. Data adapted from ref. 38. **c**, Virus modified with heterobifunctional PEG to attach a targeting moiety (blue sphere) to the modified virus. Addition of a targeting protein was shown to improve viral uptake by tumours, and reduction in uptake by liver. Data adapted from ref. 42.

BOX 1

A number of challenges and barriers face the successful delivery of therapeutic DNA to target cells in the body. Physicochemical, economic and sterilization challenges complicate formulation; the complex environment of the human body hinders its successful transport to the target cell population; and endocytic pathway barriers hinder its successful transport to the nucleus of the cell (the site of action). Each known and major barrier is listed in Fig. B1, using nanoscale DNA-delivery systems as representative examples. Each barrier exists independent of length scale. L = lysosome. A number of clever systems have been devised to overcome these barriers, the general design criteria of which are given in Tables B1 and B2.

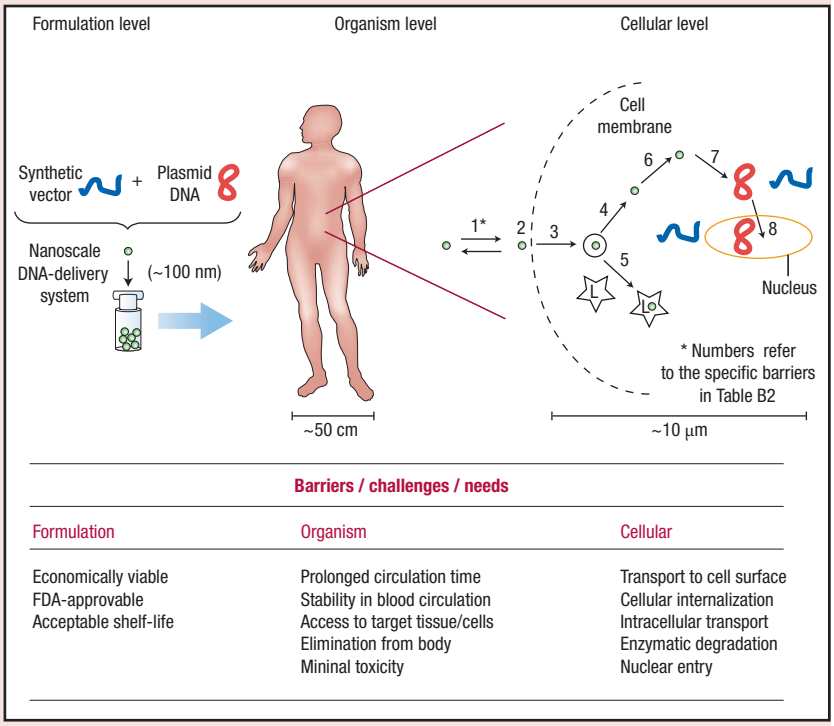


Figure B1 Barriers to DNA delivery.

Table B1

Organism Level			
Barrier/challenge/need	**Rationale**	**Example approaches**	**Materials design criteria**
Prolonged circulation time	Maximize total flux past target cell type	PEG conjugates to minimize interaction with serum proteins	Hydrophilic
			Uncharged
Stability within blood circulation	Maintenance of designed functionality	Crosslinking to maximize overall stability	Stable crosslinks within bloodstream, but reversible upon entry into target cell
Access to target tissue/cells	Transport from capillary lumen to extracellular space to reach target cell surface	Vaso-active protein conjugates (for example, vascular endothelial growth factor)	Retention of protein activity post conjugation
		Targeting restricted to 'leaky' vessel tissues (for example, tumour, liver, spleen).	Small diameter delivery system (for example, <100 nm)
Elimination from body	Minimal build-up of delivery vector over time	Control over molecular weight	Filterable through kidneys
		Engineered biodegradation sites	Biodegradable
Minimal toxicity and immunogenicity	Safety over treatment duration and beyond that required for FDA-approval	Minimize cation density	Non-cytotoxic
		Avoid protein-based materials/conjugates	Non-immunogenic

BOX 1 CONTINUED

Table B2

Cellular Level			
Barrier number (from Fig. B1)	Barrier/challenge/need	Example approaches	Materials design criteria
1, 2 and 3	Transport to cell surface, association with cell membrane, internalization	Receptor/ligand interaction (for example, antibody/polymer conjugates, recombinant protein–polymer fusions, carbohydrate conjugates) Non-specific interaction with cell surface (for example, positive zeta potential, lipid conjugates)	Cell-type specificity, low cross reactivity, if desired Promiscuous attachment, high cross reactivity, if desired (for example, positive zeta potential, lipid conjugation) Endocytic pathway trigger (for example, clathrin-dependent, clathrin-independent, caveolin-dependent)
4 and 5	Escape endosomal vesicle and avoid transport to lysosome	Buffering capacity between pH ~7.2 and ~5.0 Fusogenic peptide conjugate	Ability to disrupt endosomal membrane and/or fusion of endosome with lysosome
6	Transport through cytosol to perinuclear space with minimal degradation	'Higher' molecular weight to maintain complex stability within cytosol	Thermodynamic and kinetic stability of complex within cytosol Minimize DNA degradation within cytosol
7	Separation of complex to allow nuclear translocation	Hydrolytically or reductively degradable polymers to reduce molecular weight	'Triggered' degradation of polymer to reduce thermodynamic and kinetic stability of complex. Release of intact DNA at or near nuclear envelope
8	Nuclear entry	Nuclear localization sequence conjugates Mitosis	Facilitate nuclear uptake of DNA using virus-derived signals Facilitate nuclear uptake during mitosis when the nuclear envelope is dissolved.

selectively engineer desired properties into viral vectors. Unlike PEGylation, where a synthetic polymer is used to hide immunogenic proteins on a virus, some investigators have explored the use of DNA shuffling technology to engineer immunogenicity out of viral vectors while maintaining their ability to deliver DNA to cells[46,47].

Considerable work is still needed towards developing synthetic nanoscale delivery systems with efficiencies approaching those of viruses. Although the combinatorial approaches described above have led to the classification of a few structure/function relationships that correlate to gene delivery, there is a significant gap in our general understanding of how these synthetic systems work to deliver DNA to the nucleus of the cell. One reason for this knowledge gap is that there are multiple parameters that must be collectively optimized, but the number of different material structure compositions that need to be analysed to fully optimize these parameters is restrictively large (that is, millions). The prospect for the optimization of viral vectors is very different. Unlike combinatorial materials chemistry, which requires each structural

variation to be physically separated from the others in the library, genomic combinatorial libraries representing millions of structural variations can be made in a single solution. This technical advantage allows the screening of many more variations than allowed in materials chemistry. The disadvantage, however, is that the components of the libraries are primarily limited to amino acid structures, which could ultimately hinder the engineering of non-immunogenic viral vectors.

THE MICROSCALE

Microscale delivery vectors focus primarily on targeting the delivery of DNA vaccines (also called genetic vaccines) to antigen-presenting cells (APCs) of the immune system. APCs are specialized cells, (that is, macrophages and dendritic cells) that internalize and digest foreign particulates, such as bacteria, and 'present' their antigenic components to the rest of the immune system to trigger an immune response. Traditional vaccines, generally attenuated viruses and bacteria as well as antigenic protein subunits, contain these antigenic components, whereas the genes of a DNA vaccine encode for them, prompting both a humoral and cellular immune response[48]. Transfection of cells with the DNA vaccine leads to the intracellular synthesis of antigenic components within the cell, prompting an immune response, leading to subsequent immune protection. Unfortunately, the potency of DNA vaccines is generally low, particularly in humans, prompting the need for delivery vectors to enhance the immune response.

The initial materials used for microscale DNA-delivery systems were off-the-shelf, and originally developed for controlled delivery of small molecules, proteins and peptides. The most prominent of these materials are the polyesters based on lactic and glycolic acid (PLGA)[49,50]. PLGA is an FDA-approved material that degrades by hydrolysis on implantation in the body (Fig. 4a). Drugs embedded within the PLGA matrix are released as the polymer matrix erodes, and the release rate is governed by the rate of polymer degradation. The initial premise for DNA vaccines was to embed the DNA within microspheres 1 to 10 micrometres in diameter, which are preferentially internalized by APCs but not other cells[51]. In other words, microspheres in this size range 'target' cells of the immune system by simple size exclusion (that is, small enough to be internalized by APCs but too large to be internalized efficiently by other cell types). Once within the cells, the PLGA matrix erodes, releasing the DNA. Although the PLGA-based systems have shown promise in both animal models and in human clinical trials[52,53], PLGA was not designed for DNA vaccine delivery, suggesting that systems expressly designed to deliver DNA vaccines may further enhance the immune response. Specifically, PLGA hydrolysis leads to a low pH within the microsphere that can lead to DNA degradation[54], the release of DNA from PLGA microsphere can be incomplete[55], and the rate of release can be slower than needed to prompt a robust immune response[56,57]. In addition, many of the techniques used to encapsulate DNA into the PLGA microspheres create high shear stress, potentially leading to physically induced DNA degradation[58].

One approach towards enhancing the immune response to DNA vaccines focused on absorbing the DNA to the particle surface (Fig. 4b)[59]. In this work, cationic lipids were adsorbed to the surface of the PLGA microparticles, providing a coulombic driving force for subsequent DNA adsorption. By adsorbing the DNA to the particulate surface, the investigators minimized the shear-induced DNA degradation associated with microencapsulation. In addition, as the DNA was on the particle surface, the amount of DNA available for immediate release into the target cell was increased. The ability of these innovative particle-adsorbed delivery systems to boost the immune response relative to naked and microencapsulated DNA vaccines has been reported in a number of model systems[60].

Another approach to address the concern that DNA entrapped within a microparticle may not be completely released within the target cell in an appropriate timeframe, is to create new materials from design criteria focused on DNA delivery to APCs (Fig. 4c). At least two new materials have been designed to trigger the rapid intracellular release of DNA. The idea is that if the release rate was significantly enhanced within the cell, the level of antigen expression, and hence the immune response, might be enhanced as well. On internalization by APCs, microparticles are sequestered within vesicles called phagosomes that undergo acidification to pH ~5.0. This drop in pH provides a mechanism through which new materials can be designed to trigger DNA release. Two polymer-based systems that use pH to trigger the intracellular release of DNA, one based on poly(orthoesters) and the other based on poly(β-amino ester)s, have been reported.

Polyorthoesters were originally developed[61] as biomaterials in the 1970s. They are generally stable at pH > 7, but degrade rapidly[62] at reduced pH. These characteristics provided the basis for their recent investigation as materials with the ability to time the rate of DNA release in accordance to the kinetics most favourable to prompt a strong immune response. In animal models of human cancer, the rate of tumour growth was strongly influenced by the rate at which the microspheres released a DNA vaccine against a tumour antigen (Fig. 4d,e)[63]. By delaying the DNA vaccine release, the authors speculate that the activated APC is allowed the necessary time to mature and migrate to lymph nodes to stimulate other immune cells (that is, T cells) in a more optimal timeframe.

Poly(β-amino ester)s (PBAEs) were originally synthesized as nanoscale DNA-delivery vehicles[64,65]. However, in their unprotonated state, they are sufficiently hydrophobic to allow their co-formulation with PLGA-based microspheres[66,67]. In an animal model for cancer, the PLGA/PBAE composite microparticles slowed tumour growth to an extent significantly greater than PLGA microparticles alone. From the composition of the microparticle formulation, it is likely that on

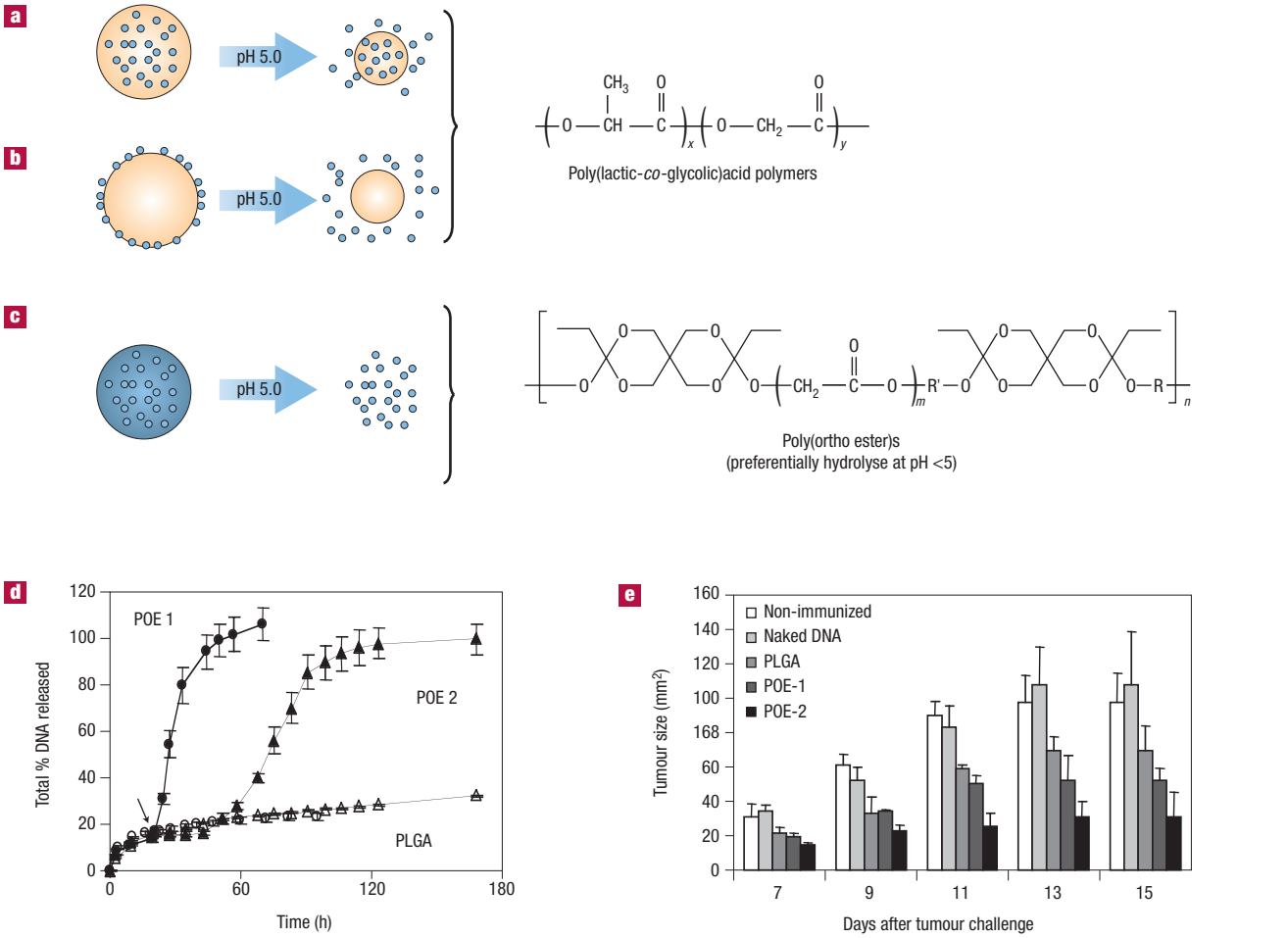


Figure 4 Degradation of polymeric microspheres as a function of pH. Microspheres <10 micrometres in diameter are preferentially internalized by APCs, providing a mechanism by which to target vaccines to the immune system. Following internalization by APCs, the spheres are sequestered within acidic vesicles, providing a mechanism through which to modulate the release of encapsulated DNA intracelluarly. **a**, Poly(lactic-*co*-glycolic)acid (PLGA) microspheres degrade relatively independent of the extracellular and acidic vesicle pH and release DNA as a function of polymer degradation. **b**, DNA adsorbed to the surface of PLGA-based microspheres release DNA more quantitatively than encapsulated DNA, but relatively independent of pH. **c**, Poly(orthoesters) degrade more rapidly at pH ~5.0, allowing triggered release of the DNA in the acidic environment of the phagosome. **d**, The pH-sensitive release of plasmid DNA from microspheres comprised of poly(orthoesters) (POE-1 and POE-2) described in ref. 63 and the pH-independent release of plasmid DNA from PLGA-based microspheres. The arrow shows the time at which the pH was changed from 7.4 to 5.0. The error bars represent the standard deviations over three samples. **e**, The influence that different DNA release kinetics can have on the efficacy of an anticancer DNA-therapeutic (adapted from ref. 63). The error bars represent the standard error from the average over five samples.

protonation in the APC, the PBAE electrostatically complexes with the plasmid DNA to form nanostructures within the cell, possibly retarding the intracellular degradation of the plasmid DNA and leading to a greater number of plasmids available to transfect the cell.

It is clear that the efficiency of the microscale delivery systems for DNA vaccination is far from optimal. From an economics standpoint, vaccines are currently not a 'high margin' sector, and the potential viability of a DNA vaccine increases as the manufacturing costs decrease. The commercial manufacture of microspheres is a non-trivial, specialized and relatively high-cost process. Additionally, the amount of DNA that must be delivered to achieve an acceptable immune response is high, and further improvement of the delivery efficiency will be necessary to help bring DNA vaccines to the marketplace.

Microscale systems also have the potential to deliver genes at a controlled rate for systemic uses. In contrast to the DNA vaccine delivery systems, whose diameters are generally around 5 μm, systems for controlled systemic delivery should be much larger to avoid internalization by APCs and to remain in the extracellular space. However, once the DNA is released from the delivery system, it needs to be transported to its site of action (probably through the bloodstream) and be internalized by the target cells. At this point the same considerations that are important for the nanoscale systems described above come into play. Some investigators have combined

the microscale and nanoscale systems, but have yet to demonstrate effective systemic controlled release[68,69]. Further development of the nanoscale systems will directly impact the potential use of the microscale systems for systemic purposes.

THE MACROSCALE

Gene delivery at the macroscale originated with the onset of the tissue-engineering field, the practice of tissue replacement using rationally designed polymer/cell hybrid architectures, and has also been examined for localized gene delivery[70,71]. Perhaps the most prominent approach for engineering tissues is to seed cells onto biodegradable polymer scaffolds that act as mechanical supports for initial cell growth, but begin to degrade as the cells synthesize cell matrix. One important component associated with engineered tissues is the maintenance of cell viability and proliferation, which is generally accomplished by the addition of growth factors. However, the delivery of protein-based growth factors to engineered cells is difficult and specialized methods are often necessary[72].

The first papers to address this issue rationalized that cells grown on a polymer scaffold could be transfected with growth-factor-encoding plasmid that was released from the scaffold as it degraded[73]. The transfected cells could then synthesize their own growth factors, leading to enhanced viability and proliferation. Plasmid DNA was incorporated into porous scaffolds fabricated from poly(lactic-*co*-glycolic acid) polyesters that varied in their molecular weight and lactic/glycolic acid ratios (to modulate the rate of polymer degradation). The mechanical properties of the scaffold can also influence the efficiency of gene transfer to seeded cells[74].

DNA transfer to cells in culture can also be improved by DNA delivery from the cell growth substrate. Whereas engineered tissues are generally constructed from 3D scaffolds, cells in culture are generally grown on flat, 2D surfaces and are useful for fundamental cell-biology studies. In essence, these systems control the rate at which plasmid DNA, either alone or formulated as a nanoscale delivery system, is delivered to the underside of cells grown at a solid or semi-solid interface. The DNA, along with the chosen delivery reagent, can be non-specifically adsorbed to the surface, tethered by a receptor–ligand interaction, or sequestered within a matrix.

One of the original reports focused on the development of microarrays to analyse the functions of specific gene-expression products[75]. New genes are discovered with increasing frequency, but it is difficult to determine their function without significant effort. To circumvent this problem, the complementary-DNAs of ~200 genes were individually mixed with gelatin and arrayed onto a glass microscope slide with each spot representing one gene. A lipid transfection reagent was added to each spot, the slide seeded with the cells to be transfected, and results obtained by immunofluorescence. The gelatin performs a number of functions in this system. For example, as a hydrogel the rate of DNA release from the gelatin is inversely proportional to the pore size or crosslinking density. If the gelatin were too concentrated, little DNA would be released and vice versa. Gelatin has also been investigated as a non-viral DNA-delivery reagent (at the nanoscale), supporting a potential secondary delivery mechanism of DNA delivery to the basal side of the cells[76,77].

Building on the matrix-delivery concept from a materials design perspective, a new approach using layer-by-layer fabrication to form multilayered polyelectrolyte thin films to incorporate and subsequently release DNA from a surface has been reported[78]. Using a PBAE as a model polycation, sequential deposition of plasmid DNA and the polycation led to thin films containing DNA/polymer bilayers approximately 20 nm thick. These thin films maintained approximately zero-order release of the DNA over 30 hours. Interestingly, although no shear stresses were incurred during fabrication of the thin films, the integrity of the released plasmid DNA was less than that incorporated into the thin films. Regardless, the DNA released from the films was transcriptionally active as shown by its ability to mediate expression in transfected cell lines.

Another recent 2D approach focused on using mineralized surfaces to entrap, concentrate and release plasmid DNA in close proximity to the cell surface[79]. Inspired by natural biomineralization processes, these investigators created tunable inorganic surfaces that supported cell growth, and prompted transfection levels in multiple cell lines equal to an optimized cell culture media transfection reagent.

Other investigators have developed hybrid approaches that explicitly span the macro- and nanoscales. In one system, plasmid DNA was tethered to a surface, either through a receptor–ligand interaction or non-specific electrostatic adsorption, to affect DNA delivery to the basal side of cells seeded to the surface[80,81]. Biotin–avidin (specifically the non-glycosylated form, neutravidin) binding was used to tether polylysine/DNA or polyethylenimine/DNA complexes to a surface. A range of polylysine lengths and biotin contents were used to ascertain the influence of polycation/DNA binding affinity, and the complex's adherence to the surface on transfection efficiency. For polylysine, increasing the fraction of the polylysine–biotin conjugate in the polymer/DNA complex decreased transfection efficiency, whereas increasing the total number of biotin residues per polylysine chain increased transfection efficiency. For polyethylenimine/DNA complexes, transfection efficiency was not influenced by the number of biotin groups within the complex. The investigators suggest these results stem from different degrees of non-specific absorption of the polylysine and polyethylenimine to the surfaces.

Three-dimensional hybrid scaffolds for tissue engineering have also been developed using polycation/DNA complexes embedded within the matrix to achieve long-term expression in seeded cells. In these systems, plasmid DNA is pre-complexed with polyethylenimine, then embedded within a degradable scaffold. Both porous poly(lactic-*co*-glycolic) polyesters[82,83] and collagen hydrogels[84,85] have served as the macroscale matrix.

Gene delivery from 2D and 3D surfaces is the most nascent area of research activity in the

field, and its future success will be evaluated on a case-by-case basis. From a functional genomics perspective, the transfection efficiency of a system may be adequate, whereas for a tissue-engineering application the transfection efficiency of the same system may be inadequate.

Looking to the future, scaffold characteristics may be optimized by judicial design of the matrix using biomolecular constructs. For example, protein-based materials, or genetically engineered polymers, enable precise control of the matrix composition[86]. Through exquisite control of the chemical composition and mechanical properties of the scaffold material, constructs with optimally engineered properties may be created to retain tight control over its chemical and physical characteristics, thereby gaining greater control over protein expression and cell growth.

THE FATE OF DELIVERED DNA

Once within the cell, plasmid DNA can exist in a number of locales in either a functional or non-functional state. A large fraction of endocytosed DNA is trafficked to lysosomal compartments, enzymatically degraded and rendered non-functional. If the DNA escapes from the endosomal/lysosomal pathway, it can either remain cytosolic (and non-functional) or trafficked to the nucleus. Nuclear uptake of plasmid DNA can occur in two ways, through nuclear pores (when the cell is not dividing) or by sequestration on nuclear reformation following cell division. Once within the nucleus, standard plasmid DNA can remain episomal (not inserted into the cell's chromosomal DNA), or can insert into the host cell's chromosome. The functional activity of episomal DNA typically diminishes over a period of days if the host cells are dividing. However, if the host cells are postmitotic or slowly dividing, the functional activity of the episomal DNA can last for months[87]. There is also evidence suggesting that optimizing the promoter sequence for a target tissue can significantly increase the functional time-course of episomal plasmid DNA[88].

Some investigators have explored the engineering of plasmid DNA systems to integrate into the host chromosome, with the goal of retaining greater control over expression and to reduce the potential of proto-oncogene activation that can occur with random insertion. For example, the *Sleeping Beauty* transposon system is a series of two inverted terminal repeats that directs the transfer of plasmid DNA segments into precise areas of the host-cell chromosome[89]. *Sleeping Beauty* has been investigated for a range of potential clinical applications, including diseases of the liver and lung, cancer, and for the stable transfection of hematopoietic stem cells[90]. Another approach for increasing the expression persistence of plasmid DNA is to use integrase enzymes that catalyze chromosomal integration. The ϕC31 integrase enzyme, which comes from the *Streptomyces* phage ϕC31, catalyses the unidirectional insertion of an engineered plasmid into specific sites in the chromosome[91]. The potential clinical utility of the ϕC31 integrase system has been studied for diseases of the liver and retina, as well as treating recessive dystrophic epidermolysis bullosa. It is important to note, however, that although *Sleeping Beauty* and the ϕC31 integrase system increase the probability of site-specific integration into the chromosome, the risk of non-specific integration remains, and this risk must be weighed against potential clinical benefit of the treatment.

POTENTIAL CLINICAL APPLICATIONS

Currently, the clinical trial landscape is dominated by viral vectors[92]. Approximately 65% of clinical trials use a viral vector for DNA delivery. It is clear that the use of polymeric materials to deliver DNA is in its infancy, and its introduction into the clinical trial mainstream will depend on improving their delivery efficiency.

The exception is in the field of DNA vaccination where at least two microscale systems are currently in clinical trials. MGI Biologics (Lexington, Massachusetts) has completed a Phase II clinical trial (using a PLGA-based microsphere system to deliver a therapeutic DNA vaccine against human papilloma virus, and has completed a Phase I/IIa trial using the delivery system for a therapeutic DNA vaccine against cancer[93]. Chiron Corporation (Emeryville, California) has entered a Phase I clinical trial, in collaboration with the United States' National Institutes of Health, for the delivery of a DNA vaccine against HIV infection. For this vaccine, the DNA is absorbed to the surface of cationic PLGA microspheres. The human data is not yet available, but the vaccine is promising based on the data obtained in rhesus macaque monkeys[94]. As the efficiency profile of polymer-based gene-delivery reagents improves, it is anticipated that these systems will begin to be used to treat a range of genetic and acquired diseases.

CHALLENGES AND THE FUTURE

Functional biomaterials continue to grow in sophistication, leading to enhanced specificity and applicability for specific tasks. The drawback of this progress is that the ability to broadly apply these new materials to different fields, either in gene delivery or other areas, diminishes with each level of enhanced specificity. Nucleic acids are not only restricted to DNA. For example, small duplex RNA sequences have gained prominence as a new form of nucleic acid therapeutic. These small duplex RNA, or short interfering RNA (siRNA), can inhibit protein synthesis within a cell through the RNA-interference pathway[95–97]. It is not clear whether the design criteria traditionally used to generate new DNA-delivery reagents will translate to the design of materials for different nucleic acid classifications, and clever new generations of materials and constructs have begun to be developed for such purposes[98–100].

From a technology standpoint, the combinatorial approach described for the development of nanoscale materials span only a small fraction of the potential parameter space that could be created from the large number of available building blocks. It is apparent that the high-throughput, combinatorial synthesis work reported to date falls short of fully understanding the structure–function relationships that govern DNA

delivery. The future of the field will bring to bear new synthetic and robotic methods to increase the total number of structures that can be investigated. In addition, the incorporation of statistical and predictive computational methods at the library design phase could help lessen the synthetic burden by helping investigators choose the library components in a more rational and thorough fashion. Kinetic Monte Carlo methods have been used to assist library design and to predict the structure–function relationships of combinatorial small-molecule libraries, and it is likely that a similar approach could be equally beneficial to the design of DNA-delivery-vector libraries[101,102].

The materials needed to advance both the micro- and macroscales may also benefit from recent advances in combinatorial methods. The use of combinatorial approaches to alter and optimize the functional characteristics of biomaterials has been reported, and it is likely that a similar approach will be used to optimize larger length-scale systems as well[103,104]. Combinatorial approaches have recently been applied to the discovery of new material composites through the generation of biomaterial microarrays, and it is foreseeable that such efforts could be applied to gene delivery from scaffolds[105].

It is an exciting time for the field of functional polymeric materials. The delivery of nucleic-acid-based therapeutics to cells is only a small fraction of an ever-growing field, which is positioned at the interface of a number of disciplines, including (but not limited to) materials science, chemistry, engineering, physics, biology and medicine. Perhaps the greatest obstacle to advancing the field to the next level is the challenge associated with coordinating an integrated effort among these disciplines, as well as the realization that each field's contribution is commensurate with the other.

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