Expert Opinion

Strategies for DNA vaccine delivery

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Strategies for gene delivery comprise a diverse range of live and synthetic approaches; DNA delivery for the purposes of immunisation in turn comprises a large part of this research. This review mainly discusses synthetic systems for application in the delivery of plasmid DNA vaccines, outlining polylactide-co-glycolide, liposome, chitosan and complex combination delivery systems. Areas of promise for DNA vaccine candidates include immune modulation of allergic responses and veterinarian application. The potential for realistic consideration of DNA vaccines as an alternative to existing approaches is dependent on the development of efficient DNA vaccine vectors and improved systems for DNA vaccine delivery. DNA vaccine technology may yet prove to be an important asset in an environment where there is a critical need for therapeutic and prophylactic strategies to combat a wide range of disease states.

Keywords: delivery system, DNA vaccine, liposome, polymer, virosome

Expert Opin. Drug Deliv. (2005) 2(5):829-842

1. Guide to vaccination strategies: limitations and opportunities

The twentieth century was the theatre of continuing progress in the field of vaccination. By 1926, vaccines against typhoid fever, shigellosis, tuberculosis, plague, diphtheria, tetanus and pertussis had been developed [1], and the success of vaccination against smallpox requires no reiteration [2,3]. Vaccines available at that time fall within three main categories: live attenuated vaccines, which can be exemplified by attenuated influenza viruses produced by propagation in embryonated hen's eggs [4]; killed vaccines, notably the killed whole cell pertussis vaccine, dramatically reducing the incidence of whooping cough since its introduction in 1948 [5,6]; and subunit vaccines, exemplified by the tetanus and diphtheria toxoid vaccines [3]. The first recombinant vaccine intended for human use, the hepatitis B vaccine [3,7,8], was the result of major microbiological advances in the 1950s. These advances allowed the relatively low cost and rapid production of subunit vaccines in cultured microbes such as yeast and *Escherichia coli*. In addition, it became possible to develop vaccines against pathogens that could not be grown *in vitro*, such as the leprosy-causing *Mycobacterium leprae*, and the Plasmodia that are responsible for malaria [9,10].

Attenuated live vaccine vectors are necessarily complex and may retain (or reacquire) many of their immunomodulatory and pathogenic traits [11]. The potential for reversion to a pathogenic form and the viral persistence of the attenuated polio vaccine may provide cause for concern regarding the cessation of vaccination, as an unvaccinated population would be particularly susceptible to such an event [12]. Other currently available vaccines against viral diseases in humans include live attenuated measles, mumps, rubella, varicella and yellow fever vaccines. Together, these normally pathogenic entities comprise a diverse selection of life cycles.

The traditional approach to attenuation is the repeated passage of the virus in semipermissive cells or altered conditions, such as lower temperature. Mutations are selected for and the ability of the virus to cause disease in its original host is compromised but they retain immunogenicity and suitable mutants are able to

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protect against the wild-type virus. Potential problems, however, may relate to the viral life cycle; for example, in mammalian cells, replication of DNA is highly conserved due to 'proof reading' by DNA polymerase enzymes. The replication of an RNA genome (e.g., poliovirus) is inherently inaccurate due to the lack of this mechanism and as a result it is unlikely that any copy of a viral RNA genome is exactly the same as the template from which it was copied. The high mutation rate can be circumvented by the cloning of the whole genome of seed strains into virus-producing cells as complementary DNA (genetic stabilisation), which has been achieved for poliovirus production; however, the problem of reversion to virulence after administration still remains for poliovirus and other RNA viruses.

Similarly to attenuated vaccines, inactivated vaccines are not free of limitations and raise both safety and efficacy concerns. The risks of incomplete inactivation, contamination during preparation and variable potency are also present in this class of vaccines. The inactivated polio vaccine that was introduced by Salk in 1955 suffered from variable potency and contamination with the SV40 virus [13,14]. The inactivated pertussis vaccine causes considerable adverse reactions, which include local inflammation, as well as systemic reactions, seizures and possible encephalopathy [5].

Subunit vaccines have gained credence in their potential to ameliorate safety concerns; vaccines based on subunits lack the extraneous materials often present in heat or chemically inactivated preparations and, hence, are often far less reactogenic and thus better tolerated by vaccinees. The downside of using the subunit approach is their relatively low immunogenicity as compared with attenuated and killed organisms, meaning that effective adjuvants are required [15]. Safety has been particularly improved with the advent of recombinant technology, which allowed the production of safer and less expensive subunit vaccines.

However, with many pathogens it is not possible to identify an antigen or antigens that are capable of inducing protective immunity [3].

At present, there is an urgent need for vaccines to protect from > 20 diseases, including AIDS, tuberculosis and hepatitis C [1]. Efforts to develop new vaccines have recently benefited from an improved understanding of immune function on the molecular level [16]. This has permitted researchers to depart from an empirical approach to a rational strategy for the design of novel, efficient vaccines [17]. Aided by an increased availability of molecular techniques, current vaccine research is focused not only on the identification of new antigens, but also on how to present them to the immune system in order to achieve protective immunity.

DNA vaccines represent one approach in which much research is aimed at improving delivery by the application of more effective delivery systems. The elucidation of effective delivery systems for DNA vaccines will likely have implications for the delivery of other nucleic acid moieties such as antisense oligonucleotides and small interfering RNA; certainly, the barriers to effective delivery offer similar hurdles [18]. Although this review presents a generally supportive view of DNA vaccine research, the achievements of vaccines so far using more traditional approaches cannot be underestimated for their phenomenal contribution to the reduction of mortality and morbidity worldwide.

2. DNA vaccines

DNA vaccines were born from the discovery that the injection of plasmid DNA into mouse muscle resulted in the expression of the reporter transgene in transfected muscle cells [19]. It was subsequently demonstrated that the injection of plasmid DNA encoding an antigenic protein resulted in an induction of an immune response [20]. The process is now commonly known as genetic immunisation, and the DNA molecule that encodes the antigen is termed a DNA vaccine. Since their first appearance, DNA vaccines have instigated an exponential increase in related research, and vaccine research in general has also seen a marked increase in the diversity of potential applications, together heralding an era of new and exciting opportunities in this research field.

The appeal of DNA vaccines is due to a number of potential advantages over conventional vaccines [21]. These are outlined below and some negative aspects are also included for a more balanced view:

- Low cost and ease of production. Different protein antigens exhibit a wide variation in physicochemical properties, and as such require different and complex methods of production and purification. In contrast, plasmid DNA possesses essentially the same physicochemical properties regardless of the antigen they encode, and can be purified by a single method. However, it should be noted that production of DNA to good manufacturing practice is essential for regulatory authorities, requiring detailed documentation of processes and purity. This may be particularly important if high doses continue to be required for clinical trials.
- Increased stability. Plasmid DNA vaccines are relatively more stable on storage than conventional protein vaccines, and their cost of storage and transport could potentially be lower than most current vaccines. This could broaden their availability to developing countries, where limited funding often hampers vaccination programmes. One of the major reasons for the exploitation of delivery system technology is the inherent instability of DNA in the biological milieu. Effective delivery systems will contribute immensely to facilitating enhanced stability *in vivo*.
- Ease of manipulation. The development of molecular techniques such as cloning allows the rapid construction and alteration of DNA vaccine plasmid vectors. It is possible for a single plasmid to be designed to express more than one antigen, and to restrict the expression of the transgene to certain cell types [22]. In addition, other genes encoding adjuvants such as cytokines may be included [23].

- Only express antigen of interest. DNA vaccines share the advantage of subunit vaccines over their live and killed counterparts in that they permit the administration of a single antigen, and as such can be used to focus the immune response to an antigen or epitopes known to confer protective immunity. In addition, as with subunit vaccines [24], plasmid DNA vaccines have the potential for the delivery of chimeric antigen [25] or fusion proteins based on multiple antigenic determinants.
- Increased safety. Again, similar to recombinant subunit vaccines, they can be manufactured to high standards of purity and, as a result, safety. In addition, they do not suffer from the risks involved with the use of live vaccines in terms of reversion to virulence, or side effects associated with these and killed whole cell vaccines.
- Increased or altered immunogenicity. DNA vaccines have an important advantage over subunit vaccines, in their superior ability to induce both an antibody and cell-mediated immune response [26].

Subunit vaccines tend to induce a mostly antibody-mediated immune response, which is generally more effective in combating extracellular microbes and toxin neutralisation. This is because of the presence of the antigen in the extracellular environment, which suggests to the immune system the presence of an extracellular pathogen. Such a humoral response is often not sufficient to protect from intracellular pathogens that tend to require a predominantly cell-mediated immune response. DNA vaccines mimic intracellular pathogens in that the antigen is endogenously expressed, and as a result they are more efficient at eliciting the cell-mediated immunity that is required for their removal.

However, with reference to regulatory concerns, the possibility of germ-line alteration, expression of the antigen in inappropriate tissue sites, inappropriate immune responses and immunopathology, longevity of antigen expression, and the induction of tolerance or autoimmunity as well as the generation of acute or chronic inflammatory responses, autoimmune sequelae and destruction of normal tissues potentially associated with the aberrant expression of some proteins have all been raised as issues of concern [11]. In addition, the coexpression of cytokine genes may require the assessment of unintended adverse consequences, such as generalised immunosuppression, chronic inflammation, autoimmunity or other immunopathology. The FDA have also advised that in order to limit the possibility for chromosomal integration, homology of plasmid DNA sequences to known sequences in the human genome should be examined, described and strong homology avoided if possible. They recommend sensitive studies such as polymerase chain reaction (PCR) using primers derived from the vaccine in order to examine tissue distribution and distinguish between integrated and nonintegrated plasmids in the analysis of genomic DNA.

Potentially, DNA vaccines are capable of providing the immunogenicity of live attenuated vaccines, with the safety of a recombinant subunit vaccine. Despite their possible advantages, however, DNA vaccines have yet to fulfill their promise [27]. They have yet to be successful in humans and large animals, in which their potency remains low [28]. To date, their success has been generally limited to small animal models and the relatively high and multiple doses required for DNA vaccines to be effective in comparison to protein vaccines renders scaling up for administration to humans and large animals impractical [28].

Electroporation has been well reviewed elsewhere [11,29]; this review is mainly focused on formulation strategies for plasmid DNA delivery for the purposes of immunisation. The key goal for DNA vaccines remains to somehow increase the immunogenicity of these agents in large animals.

3. Strategies for optimisation

The challenges faced by researchers have given rise to a wealth of strategies for the optimisation of DNA vaccines. These can be loosely divided into two main categories.

3.1 Improving plasmid DNA vectors

The ongoing improvement in the understanding of the molecular mechanisms of immune function has led to attempts to guide the immune system to the desired response for a given DNA vaccine. Cytokines are known to play an important role in determining the outcome of an immune response and have enjoyed frequent use as adjuvants in the experimental arena. Genes encoding cytokines have been cloned on plasmid DNA vaccines and have successfully influenced the immune system towards a cellular or humoral bias [23].

It is thought that the ability of a DNA vaccine to induce an immune response is directly related to the level of antigen that is produced and made available to the cells of the immune system [28,30]. The amount of antigen that is produced *in vivo* can be directly increased by a method known as codon optimisation. In this process the sequence of the gene encoding the microbial antigen is altered to represent optimally expressed codon sequences; ameliorating interspecies-specific differences in codon usage and any regulation of gene expression that is designated by suboptimal codon sequences, thus allowing optimum expression in the eukaryotic host cell [31]. An interesting approach to optimising expression or function of a gene-encoded protein is the technique of molecular breeding or gene shuffling. In this process, it is possible to create reassembled chimeric genes from a selection of related or naturally existing homologous gene sequences. Reassembled chimeras are cloned into an expression vector for selection and potential inclusion in further rounds of molecular breeding. The technique takes advantage of crossovers, deletions, insertions, inversions and point mutations as occurs in natural evolution (hence the term molecular breeding). This

technology has already been exploited for the generation of enzymes with markedly enhanced activity [32] in addition to enhancement of the functions of a diverse range of proteins such as green fluorescent protein (GFP) [33] and IFN-γ [34]. The method has also been called directed evolution and Tobin *et al.* [35] used the phrase 'the rational basis for irrational design', which seems to capture the essence of the technology. This technique is thought to be superior to other methods that employ random mutagenesis, such as error-prone PCR, as molecular breeding may potentially combine the beneficial mutations from the naturally existing variants of a given gene. Molecular breeding has yet to make a significant impact in the area of genetic vaccines [11] despite the potential for the generation of antigenic epitopes with increased immunogenicity and/or protective efficacy, perhaps because the screening of candidate antigen genes is necessarily expensive in terms of resources and time if it is compared with the screening of an enzyme for example.

Alternatively, vectors encoding or comprising of self-replicating RNA may help to ameliorate potential safety risks such as chromosomal integration and alleviate the concerns associated with persistent expression of cytokines or costimulatory molecules [36] and represent markedly improved vectors for the delivery of genetic information, generating more effective immune responses with potentially improved pharmacokinetics and safety. Such systems offer significant diversity with packaging of RNA replicons into pseudoinfectious virus-like particles [37], the delivery of naked replicon RNA [38] or the delivery of DNA encoding the virally derived RNA replicon [11,39-41]. The utilisation of these vectors is based on immune recognition of processes not present in the host (RNA replication and the formation of double-stranded RNA) and are normally confined to virally infected cells.

3.2 Improving plasmid DNA delivery systems

An alternative and complementary approach is to improve the efficiency with which DNA reaches the host cells for transgene expression and the induction of immunologically relevant events. Delivery systems for DNA vaccines have been the subject of much research over the last decade. In addition, modulation of the immune response with adjuvants (distinct from the genetically encoded adjuvants outlined above) using immunostimulatory substances such as the cytokine-inducing imiquimod and monophosphoryl lipid A has also been used and yielded promising results [42,43], depending on the mode and route of administration. This strategy also offers a potential for combination with delivery system technology.

A DNA vaccine needs to be able to transfect cells efficiently and achieve expression of the encoded antigen. In addition, the vaccine needs to be able to alert the immune system to its presence, and thus initiate an immune response. The consensus at present is that the delivery of DNA to antigen-presenting cells (APCs) is particularly important [44], and, accordingly, systems that are able to deliver DNA to APCs offer good potential for vaccine delivery systems. Barriers to effective delivery include nuclease enzymes in the extracellular medium, the hostile environment of the endosome and that the DNA must reach the nucleus in order to be transcribed.

For the administration of naked DNA, it has been reported that there is a rapid migration from the injection site following intramuscular injection coupled with plasmid degradation such that only occasional detection of plasmid DNA can be observed after 8 h in mice [45]. The primary strategies implicit in DNA vaccine delivery systems are thus to aid the passage of DNA through these biological barriers, target cells of the immune system and safely deliver plasmids to the nuclei of relevant cells for expression.

4. Particulate DNA vaccine delivery systems

It was discovered early on that antigens presented in solution were less immunogenic than proteins presented in particulate form [3]. From the particulate delivery systems that have gained interest for the delivery of both subunit protein antigens and DNA [46,47], two types of particulate vaccine delivery system have dominated the field: those based on biodegradable polyester particles and liposomes.

4.1 Biodegradable polyester particles

Biodegradable polyester microspheres and nanoparticles have been used for the encapsulation and delivery of protein antigens for more than two decades [48]. They possess a number of attractive properties, including biocompatibility, biodegradability and technical versatility, which allow particles to be designed to meet a variety of specifications [49]. Their ability to release the encapsulated antigen in a controlled manner over extended periods of time has suggested that they have the potential to eliminate the need for multiple vaccination doses [48,50,51].

The advantages of biodegradable polyester particles may also extend to DNA vaccination [52]. Similar to protein antigens, DNA vaccines also appear to benefit from the controlled release afforded by encapsulation within the particles. In addition, the encapsulated DNA is shielded from the hostile external environment, thus improving the efficiency of its delivery [52]. Finally, the particulate nature of the delivery system targets it to professional APCs of the immune system, whose primary function is to process the expressed antigen and orchestrate an immune response to its presence [53].

Biodegradable polymers have had extensive application in medicine. The use of non-synthetic, biodegradable materials as implants to promote post-traumatic tissue healing dates back to Roman times. Galen developed a reputation circa 150 AD by using catgut as a suture to treat the wounds of gladiators. It took several centuries for the clinical introduction of fully synthetic sutures, in the form of the biodegradable polymer polyglycolide (PGA), which was marketed in 1970 by the name Dexon.

In addition to PGA, the field is currently dominated by polylactide (PLA), and their copolymer polylactide-co-glycolide (PLGA). These polymers are synthesised from cyclic diesters of

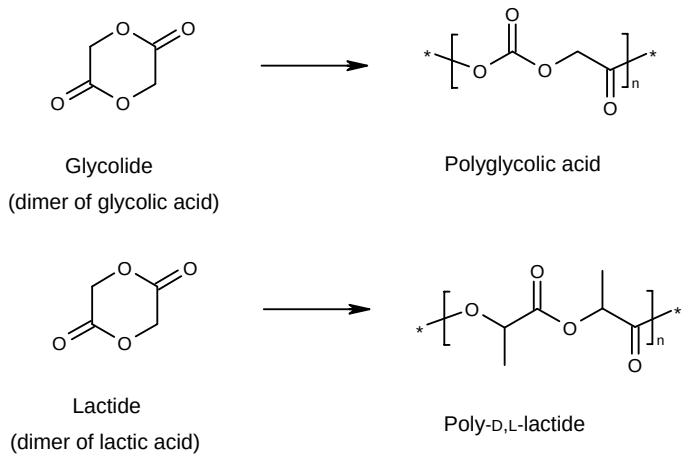

Figure 1. Synthesis of polyglycolide and polylactide by ring opening polymerisation of their respective diesters.

lactic acid and glycolic acid by a process known as ring opening polymerisation (Figure 1).

These polymers are generally thought to be degraded by the uptake of water, followed by hydrolysis of the ester bonds. The hydrolysis seems to be passive rather than enzymatic in nature, and is thought to be the primary mechanism responsible for polymer degradation both *in vitro* and *in vivo*, and passive hydrolysis is largely governed by the ability of the polymer to absorb water [54]. Generally, the degradation rate is higher for PGA, taking several months, by virtue of its larger capacity to absorb water, which renders it is more susceptible to hydrolysis. In contrast, it is slower for the more hydrophobic PLA, which may require > 1 year to degrade.

The rate of polymer hydrolysis is also affected by a number of other factors. The most important of these is pH: PLA and PLGA exhibit higher degradation rates at low and high pH values. The degradation rate may also be affected by the presence of catalysts, the degree of crystallinity and hydrophobicity of the polymer, as well as the polymer molecular weight. In the case of PLA, the rate of hydrolysis is also dependent on the enantiomeric composition of the polymer [55]. It is thus possible to synthesise PLGA copolymers that conform to a certain degradation profile, by altering polymer characteristics such as molecular weight and PLA/PGA content, as well as by the inclusion of catalysts as excipients.

As a result of their versatility, these polymers have been used in an extensive range of medical applications in the last three decades. PGA, PLA and their copolymers have been employed as implants in craniofacial reconstruction [56], spinal surgery [57] and general tissue engineering [58]. Similarly, PLA and PLGA have enjoyed much interest as the main components of particulate systems in drug delivery. Numerous methods have been developed for the production of PLA and PLGA particles, including emulsion-evaporation, nano-precipitation, cross-flow filtration [59], salting-out techniques [60], emulsion–diffusion methods [61], or jet milling [62] and spray drying.

In the case of gene vaccine delivery, the particles can be loaded with plasmid DNA by means of a double-emulsion method, using the internal aqueous phase for entrapment of the hydrophilic plasmid DNA. As PLGA and PLA are insoluble in water, the technique requires the use of organic solvents and stabilising agents, most commonly polyvinyl alcohol (PVA) [63].

The delivery of DNA using PLGA microparticles has shown promise following oral administration [64], and, more recently, immunoprophylactic protective effects against anaphylaxis were shown using DNA-loaded PLGA particles delivered subcutaneously [65]. Other work includes modification of the microparticle surface charge to enable complexation of DNA to the particle surface.

4.2 Liposomes

Liposomes were first identified by Bangham *et al.* in the 1960s [66], and were initially used in order to provide a model for the study of biological membranes [67]. In the following years, liposomes successfully demonstrated their potential as adjuvants and delivery vehicles for a wide spectrum of therapeutic substances [68,69].

Dehydration rehydration vesicle (DRV) technology [70] has also been used with some promise for the delivery of plasmid DNA for the purposes of immunisation [71]. DRV formulation is able to generate submicron-sized liposomes incorporating most of the DNA in a way that prevents DNA displacement through anion competition, indicating that much of the DNA is entrapped within the aqueous compartments in between bilayers [72]. Moreover, this technique allows the use of liposomes that do not complex with plasmid DNA easily and a wider diversity of formulation components. Effective uptake by APCs and the expression of transgene encoded protein (GFP) has been shown; DRV liposomes

facilitated an enhanced expression of the plasmid both in the injected muscle and the draining lymph nodes [73]. This is in support of the notion that liposomes promote immune responses to DNA vaccines by facilitating their uptake by APCs in the lymphoid tissues. Recent studies have used this liposomal entrapment technique for the comparison of pCI and pcDNA3 plasmid constructs encoding infectious bursal disease virus antigens [74]. Entrapment in liposomes enhanced the protective capability of the DNA vaccine, and the bursal disease polyprotein expressed by the pCI vector induced a better immune response than that engendered by the pcDNA3. The pCI vector contains an intron from β-globulin that can enhance the expression of foreign genes in mammalian cells, which may have aided the enhancement of observed immune responses *in vivo*.

Liposomes incorporating cationic lipids can interact strongly with DNA leading to the formation of liposome–DNA complexes commonly referred to as lipoplexes [75,76]. The combination of cationic and fusogenic lipids is able to greatly enhance DNA transfection in cell culture, and has provided a promising approach to gene delivery [77,78], also giving comparatively robust and protective immune responses in the mouse model [79-81]. Complexation of DNA with cationic liposomes is thought to enhance the efficiency of DNA uptake and expression by cells *in vivo*, resulting in improved immune responses [82]. In addition, the vesicles are able to function as adjuvants, as they activate macrophages and induce cellular immune responses [83,84]. In contrast to double emulsion polymer formulations, the loading of cationic liposomes with plasmid DNA occurs spontaneously as a result of their opposite charges, and is achieved by the simple mixing of the vesicles with the plasmid. Loading efficiencies approaching 100% are thus attainable in an aqueous environment that is entirely safe for the plasmid.

4.3 Virosomes

Virosomes can be described as semisynthetic complexes derived from nucleic acid free viral particles. The term virosomes has been used to describe agents ranging from essentially reconstituted viral coats to more synthetic vesicles with limited viral components. Initially described 30 years ago using influenza virus haemagglutinin and neuraminidase relocated on the surface of unilamellar liposomes [85], virosomes retain their fusogenic activity and thus deliver the incorporated compound (antigens, drugs, genes) inside the target cell. In the context of DNA delivery, modified immunopotentiating reconstituted influenza virosomes (IRIVs) composed of spherical, unilamellar vesicles, and prepared using a mixture of natural and synthetic phospholipids and 10% envelope phospholipids originating from influenza A/Singapore/6/86 and influenza surface glycoproteins, have been used for the delivery of DNA (encoding mumps virus haemagglutinin) to dendritic cells [86] showing uptake of fluorescently labelled DNA by cells with dendritic phenotype *in vivo*. Subsequent work from the same group showed an enhancement of immune responses against carcinoembryonic antigen using virosomes delivering a gene encoding the costimulatory molecule CD40L [87]. This represents an interesting strategy for the enhancement of immune responses to any virosome-delivered antigen. Sendai virus envelope glycoproteins have also been used to create fusogenic liposomes [88], and their potential for the delivery of DNA or indeed other nucleic acid molecules has been noted (e.g., antisense DNA oligonucleotides or small interfering RNA).

4.4 Comparison of delivery systems and alternative strategies and opportunities

The above cited advantages of cationic liposomes as gene delivery vectors are offset by their instability in the biological medium, and both PLA and PLGA particles offer advantages in this respect. Biodegradable polyester particles are generally rigid and capable of providing effective protection to their DNA load from the external environment [89]. However, these polymers form hydrophobic particles that bear a slight negative charge [90]. As a result, they do not efficiently encapsulate a hydrophilic macromolecule such as plasmid DNA [91]. In addition, the methods required for encapsulation are generally harsh, requiring homogenisation steps and the use of organic solvents, which may be damaging to the DNA [49,53]. In contrast, cationic liposomes, as outlined above, are capable of spontaneously interacting with the anionic nucleic acid and forming lipoplexes [75]. Therefore, unlike double emulsion solvent techniques, cationic liposomes can associate with DNA by a relatively simple mixing process, which is highly efficient. However, the vesicles are inferior to the rigid particles in terms of stability: cationic liposomes suffer from instability *in vivo*, mainly due to their charge and fluid, lipid membrane nature [92-94]. In order to ameliorate the low entrapment for microparticles, cationic components have been used in the emulsion itself, either as additions to the organic phase [95] or as addition to emulsification agents in the aqueous phase [96]. The modification of polymer particles with cationic polymers such as chitosan results in cationic particles that can be used for complexing DNA in the same manner as cationic liposomes [96]. This strategy has been studied extensively, showing positive results in the enhancement of DNA delivery and immune responses to transgene encoded antigen [16]. Cetyltrimethylammonium bromide (CTAB) has been used in the external aqueous phase in the formulation of PLGA microparticles and dimethyl dioctadecyl ammonium (DDA) bromide and dioleoyl trimethylammoniopropane (DOTAP) in the organic phase [97]. Markedly increased transgene-specific antibody titres (HIV-1 p55 gag) were shown for the complexed DNA formulations in comparison with naked DNA or plasmid in conjunction with microparticles alone, and CTL responses were increased using these cationic particle delivery systems. In addition, it was shown that PLGA–CTAB particles of 300 nm were more effective than larger particles (1 and 30 μm) in the elicitation of transgene

specific serum IgG antibody responses [97]. DDA has also been used in the formulation of PLGA particles < 200 nm for the adsorption of DNA [98] with increased *in vitro* transfection in comparison to naked DNA and, interestingly, increased transport rates for these cationic nanoparticles in reconstituted pig gastric mucus in comparison to that of similar sized polystyrene nanoparticles.

Other attempts to combine the strengths of the two (liposomal- and polymer-based) systems have led researchers to the development of hybrid lipid/polymer vectors that aim to improve *in vivo* efficacy. This can be achieved by the initial complexation of DNA with a cationic polymer such as poly-L-lysine prior to microencapsulation [99]. Alternatively, the modification of liposome DNA complexes by the precipitation of PLA has also been explored [100]. Liposomes modified with mannan have been found to enhance HIV-1-specific cell-mediated immune responses induced by DNA vaccination [101]. However, non-water soluble polymers were not previously used for this purpose. This is perhaps due in part to formulation constraints when using organic solvents, overcome in the cited work [100] by the apparent increase in solubility and protection of the plasmid DNA mediated by the incorporation of plasmid into the liposomal formulation and influenced by the choice of lipid, polymer and solvent. Howard *et al.* [102] encapsulated pegylated polyethylenimine–DNA complexes in PLGA microparticles using a double-emulsion solvent evaporation technique. The use of organic solvents and the multicomponent nature of such composite delivery systems leads to difficulties with formulation techniques and product analysis; however, the cited paper offers a good template for initial analysis of these agents. Coating of liposome–DNA complexes with a hydrophilic polymer has been achieved by covalently linking the multivalent reactive copolymer of poly-*N*-(2-hydroxypropyl) methacrylamide (pHPMA), and this has been shown to confer resistance to *in vitro* protein binding [103]. This strategy builds on previous work in which polymers were adsorbed without covalent attachment; it will be interesting to note whether promising *in vitro* results extrapolate well to *in vivo* studies.

There is an increasing volume of work highlighting chitosan and its derivatives as a formulation agent for gene and DNA vaccine delivery. Initially outlined a decade ago [104], and used for immune protection in a mouse model of peanut allergy [105], these systems are reviewed well elsewhere [16,106] and the most recent work outlined in Section 5 (Table 1).

An extensive investigation into DNA delivery using naked liposome- or chitosan-formulated DNA by different routes of administration (intramuscular, intraperitoneal and intravenous injection, immersion and anal intubation) in fish showed markedly different biodistribution of transgene expression (luciferase) and detection (using PCR) according to the formulation and route employed [107]. The highest luciferase levels in the head, kidney and liver samples were detected after intraperitoneal injection of DOTAP–dipalmitoly phosphatidylcholine (DOPE) lipoplexes. In the spleen, the highest average value was detected after intraperitoneal injection of naked DNA (although not significantly better than DOTAP–DOPE lipoplexes) and naked DNA appeared to be more effective than the formulated DNA following intravenous administration. Intraperitoneal administration is the usual route of vaccination in fish and so may be of particular interest. It was also concluded that the administration dose and volume, as well as the size of the fish, may have an effect on the distribution and expression of plasmids, and the study as a whole provides an interesting insight to differences of transgene expression facilitated by delivery system and route.

Very early studies into the routes of vaccination with DNA identified the gene gun as a particularly promising method of administration [108]. Delivery via gene gun has also been shown to be superior to the intramuscular route in rabbits; facilitating notable protection in comparison with intramuscular administration [109]. Comparative investigation has highlighted the qualitative differences of gene gun and intramuscular administration [110], and the importance of the priming event for DNA vaccines in defining subsequent qualitative immune responses following boosting. Gene gun administration, in addition to being an efficient way in which to deliver DNA for the purposes of vaccination, has an implication for the type of immune response observed.

5. Recent trends

Some of the current research trends of interest with application (or potential application) for delivery of DNA vaccines from recently published work are shown in Table 1.

The recent trends in the delivery of DNA vaccines illustrate the interest that surrounds chitosan and its derivatives as the basis of delivery system design. By virtue of the versatility offered by differently modified chitosans, this agent can be included in or as a diversity of carriers. The recent work also shows that more traditional approaches of intramuscular and gene gun administration still feature strongly as a first line of assessment of the potential of new DNA–vaccine constructs. Delivery of plasmid DNA using bacterial ghosts represents an additional strategy that has superficial similarity to virosome delivery. The cited work combines the preparation of the delivery vehicle with amplification of plasmid DNA and the loading of this nonliving bacterial vector with DNA in a single process [116].

6. Conclusion

There is no doubt that DNA vaccines are able to elicit immune responses that may possess qualitative advantages over protein and subunit vaccines, and there is good potential for their implementation in diseases where target (such as in allergy [126]) or protective antigens are well defined. Many delivery systems, however, have outlined increased *in vitro* transfection and have not been taken beyond preliminary

Table 1. Some current research trends with application for delivery of DNA vaccines.

Delivery system	Observations and comments
Trimethylated chitosan DNA complexes [111]	Consolidates earlier observations showing the potential of chitosan complexes *in vitro* and low toxicity. The degree of quaternisation may be important. Comparative *in vivo* analysis would be interesting.
Alginate chitosan nanoparticles [112]	Nano- and microparticle (323 nm – 1.6 μm) formation was affected by the ratio of alginate to chitosan, the molecular weight of the biopolymers and the solution pH.
Oral delivery of chitosan DNA in fish flakes [113]	β-Galactosidase expression could be observed in the stomachs, spleens and gills of fishes fed with flakes. This work builds on excellent results observed for DNA vaccines in fish that has excellent potential for the application of this technology. See protection against *Mycobacterium marinum* [114] following intramuscular inoculation of a (naked) DNA vaccine.
Nanoparticles formed with hydrophobically modified glycol chitosan [115]	Glycol chitosan was hydrophobically modified with 5β-cholanic acid. *In vitro* as well as *in vivo* data showed promising results for transgene expression. Hydrophobic modification (although not for the first time shown here) gives greater diversity for formulation work using chitosan. Potential for use in the context of DNA vaccine delivery.
Bacterial 'ghosts' as plasmid carriers [116]	A hybrid protein integrates in the bacterial membrane and binds to sites on the pSIP (plasmid) vector. Lysis of live bacteria (and formation of 'ghosts') can be induced by increasing culture temperature. High efficiency in the transfection of macrophages and primary dendritic cells makes this technology promising.
Attenuated *Salmonella typhimurium* [117]	Delivery of DNA vaccines using live bacterial vectors is a strategy that has showed much promise. This approach has been well reviewed elsewhere [11,118,119]. The initial impact of this strategy seems to have lost impetus since some excellent publications following initial success with this elegant mode of delivery for DNA vaccines [120-123].
Intramuscular delivery of naked DNA [124]	Many studies, especially when assessing a new plasmid construct, still use delivery of naked DNA intramuscularly. The cited work here is of particular interest because they show good protection data in their animal model (guinea-pigs) against a significant pathogen (foot and mouth disease virus) following intramuscular administration of naked DNA. This mode of administration has shown positive results in many models.
Gene gun delivery of β-amyloid peptide encoding plasmid DNA [125]	Gene-gun-administered genetic immunisation with the *β-amyloid$_{42}$* gene in wild type, BALB/c and Alzheimer's transgenic mice elicited immune responses without a significant T-cell-mediated (damaging) immune response to the β-amyloid peptide. However, further analysis is required to evaluate whether DNA vaccination will be able to ameliorate β-amyloid deposition.

in vivo evaluation. From the work reviewed here, it would seem that liposomal- and polymer-based delivery systems have failed to meet initial expectations and produce good results in larger animal models. A diverse array of alternative strategies is apparent that borders on non-DNA strategies, by, for example, the use of attenuated bacteria. Promising recent trends have outlined continuing work using synthetic or nonviral delivery systems with chitosan derivatives showing much potential, in particular due to their low toxicity, and combination systems would appear to offer benefits. However, promising systems need to be taken further and proven in relevant models before they can be selected for further development. Strategies for DNA vaccine delivery are summarised in Figure 2.

7. Expert opinion

The delivery of DNA presents numerous challenges, as it requires the successful passage through complex biological barriers and hostile environments. Although currently outlined DNA vaccine delivery systems ameliorate some of these problems, unless there are significant improvements in plasmid DNA efficacy by improved design of plasmid vectors, neither polymer particles nor cationic liposomes can be seen as satisfactory carriers for DNA vaccination with the present state of the art technology. A PLGA–CTAB formulation has recently been tested in macaques, which demonstrated that seroconversion could be achieved but that milligram quantities of plasmid DNA were required [127]. Nevertheless, the authors cite the poor efficacy of DNA vaccines for the induction of antibody responses in primates, even following large doses on multiple occasions [128] against which their results are undoubtedly encouraging. This work also highlighted that the optimal regimen for the administration of DNA in primates has not been agreed [127]. There is a need for the development of new and safer vaccines. In their identification, however, the development of adjuvants required for subunit vaccines has been problematic and clinical efficacy elusive. In addition, the public remains suspicious of vaccines in general and there is certainly apprehension about anything involving genetic-related technology. The risk/benefit analysis that is

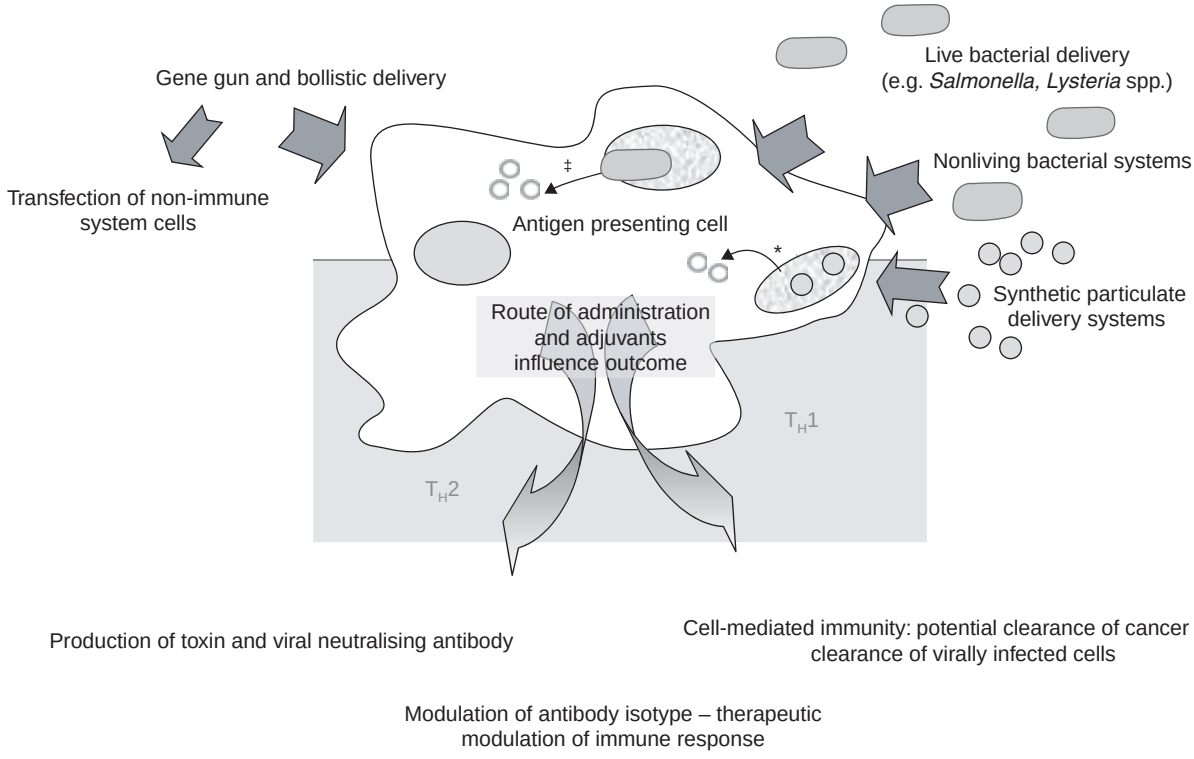

Figure 2. Schematic representation of several strategies for DNA vaccine delivery. The outcome is dependent on the delivery system strategy (including 'non-genetic' adjuvants), route of administration and facets of the plasmid vector (including genetic adjuvants).
*Fusogenic lipids facilitate. ‡Bacteria cause lysis and release.
$T_H1/2$: T helper cell type 1/2.

implicit in the implementation of vaccines in humans underpins a good rationale for the use of novel technologies in order to identify protective vaccines when traditional approaches such as treatment or vaccination have proven to be inadequate. Such opportunities include the most difficult and chronic conditions and diseases such as tuberculosis, HIV, cancers and allergy. Interestingly, DNA vaccines have shown good promise for the modulation of allergic responses and, in a similar fashion, were shown to modulate immune responses directed against the β-amyloid peptide that has demonstrated a central role in the neurodegeneration of Alzheimer's disease. Therapeutic immunisation in patients with Alzheimer's was shown to be effective, but studies were discontinued owing to the development of an autoimmune, cell-mediated meningoencephalitis. The altered immune response facilitated by DNA vaccination [125] provides an alternative, if experimental, immunisation method for therapy and prevention of Altzheimer's disease. Application in the therapeutic modulation of ongoing immune responses is one of the most promising areas for the implementation of DNA vaccine technology, due to the ability of DNA-encoded antigen to exert such a profound effect in the qualitative alteration of immune responses. In addition, the application of DNA vaccines in the treatment of cancer has attracted much attention [129] and may justify the use of more adventurous delivery strategies, such as electroporation. It is true that more efficient delivery of DNA using one system will allow more efficient delivery of alternative DNA molecules, but it should be noted that immune responses directed against encoded antigen will differ according to mode and route of administration (e.g., the quality of immune response engendered by intramuscular administration of naked DNA will be different to that engendered by gene gun administration) and accordingly, the delivery strategy will impact on the ultimate vaccine efficacy.

Not much has been made of the potential reaction from the public if an effective vaccine comes close to clinical acceptance. There can be a significant distrust of medical science where technology may be difficult for clear understanding by the general public. The potential for resentment against technologies that involve genetic modification of living organisms, particularly humans, is great, and convincing people that gene technologies are safe and reliable may be a tough challenge [130].

In terms of efficacy, generally but particularly where the need for new vaccines is less, DNA vaccines will need to be proven against the harsh reality of a multitude of existing and new technologies for vaccine development, many of which offer significant potential. Continuing developments with

alternative novel technologies, such as bacteriophage vectors offering low host immunogenicity against the vaccine [131], live viral and bacterial vectors expressing heterologous antigens, or highly attenuated and safer live vaccines, are all strategies that offer excellent opportunities for development. DNA vaccines may benefit from their ability to function well in prime-boost systems as DNA vaccines can induce a T-cell response that can be strongly boosted by recombinant viral vectors [132]. This potential application takes advantage of the ability of synthetic delivery systems or naked DNA to avoid immune responses directed against the carrier that impact heavily on multiple administrations of viral or other immunogenic carriers. Presumably, virosomes and other viral or bacterially derived plasmid DNA carriers could have similar limitations. In support of live vectors, recent work has shown that substitution of the DNA prime with an alternative recombinant virus was able to facilitate good results against experimental malaria challenge in humans [133]. Expression of virally delivered heterologous DNA by the host or delivery of heterologous antigens as proteins expressed by viruses or bacteria are very interesting strategies that significantly predate the advent of DNA vaccines [134,135].

Veterinarian vaccines look likely to yield the first DNA vaccine product, given the excellent protective immune responses that have been observed in fish. In any case, DNA vaccination will continue to be a consideration for experimental evaluation of antigens of interest for many disease-causing organisms and immunologically related disease states even though DNA vaccine vectors offer much scope for improvement.

As with other vaccines (and highlighted by the recent mire of patent disputes surrounding new GlaxoSmithKline and Merck human papillomavirus vaccines [136]), intellectual property issues need to be resolved early on in development.

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