BIOMATERIALS AND GENE THERAPY

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I. Introduction

Gene therapy seeks to alter or control the course of cellular action through the introduction of nucleic acids into a specific cell population. All gene therapy efforts to date in humans have been limited to somatic cells, such that the treatment is confined to the individual and not passed along to offspring. Gene therapy was initially conceived to treat inherited genetic disorders of a single gene, such as cystic fibrosis, adenosine deaminase deficiency associated with severe combined immunodeficiency (SCID), and hemophilia. For some conditions, the delivery of a gene encoding a protein that is pathologically absent can restore normal function. Such is the case

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with hemophilia, in which one of a number of blood clotting factors may be deficient, but effectively replaced through delivery of a gene encoding the factor (Connelly and Kaleko, 1997, 1998; Corr *et al*., 1996). In other cases, suppression of a defective gene product may be the preferred course of action. A candidate condition for such selective gene silencing is osteogenesis imperfecta, in which the production of an abnormal collagen molecule interferes with the structural integrity of connective tissue (Marini and Gerber, 1997). In either case, the original concept of gene therapy involved the delivery of nucleic acids (DNA, RNA, or oligonucleotides) into an individual to correct a genetic insufficiency.

A. Gene Therapy and Protein Delivery

Gene therapy has since evolved beyond addressing genetic disorders to include the delivery of genetic material to obtain desired cellular responses. For example, gene therapy has been employed to elicit a specific immune response or to guide tissue regeneration. Indeed, the delivery of genes encoding therapeutic proteins provides potential for overcoming the limitations associated with the delivery of the proteins themselves.

1. Limitations of Protein Delivery

Although the delivery of recombinant proteins for therapeutic purposes holds great theoretical potential, several limitations are associated with their delivery. First, the production and purification of recombinant proteins is expensive, especially on the scale that is generally required for efficacy. Second, the inclusion of large amounts of protein poses the risk of toxicity through rapid release of the factor from the carrier. Additionally, the long-term effects of a supraphysiological dose of a recombinant protein are not known (Oakes and Lieberman, 2000). Diffusion of the factor out of the local site may have an undesired effect at secondary locations. Proteins require the maintenance of complex tertiary and quaternary structure for bioactivity. In many cases, the bioactivity of these factors is diminished through exposure to the harsh conditions generally encountered in the processing of the delivery vehicle. Additionally, the half-life of the factors is often quite limited in the extracellular environment to which they are exposed upon release. Loss of protein structure may result in a reduction of bioactivity and an increase in the risk of immunogenicity. Further, proteins are subject to high rates of renal and hepatic clearance. These limitations associated with the delivery of proteins themselves have led to

the exploration of gene therapy to deliver the genes encoding therapeutic proteins.

2. *Advantages of Gene Delivery*

The delivery of plasmid DNA containing genes encoding specific proteins presents several potential advantages over the delivery of the proteins directly. First, the chemistry of DNA grants it inherent stability and flexibility. As a result, DNA may require less complex storage and present an extended shelf-life relative to the corresponding proteins. Additionally, the stability of plasmid DNA allows for its use with traditional drug delivery routes (Goldstein and Bonadio, 1998). Plasmid DNA can be easily manufactured via bulk processes that are potentially more economically feasible than the production and purification of the related proteins (Eastman and Durland, 1998). Once cells are transfected with plasmid DNA, the encoded gene product is produced *in vivo* by the cells. The retention of the gene by the cells provides a means for prolonged expression of the desired factor. Further, the encoded proteins produced by the cells *in vivo* have the potential to retain biological activity to a greater degree than delivered factors. As the desired factor must be produced by cells *in vivo*, the risk of toxicity is lower than with outright delivery of the factor.

B. Methods of Gene Delivery

1. *Ex vivo Gene Therapy*

The initial approach to gene therapy involved manipulation of gene expression *ex vivo*. Toward this end, the desired target cells are identified and subsequently removed from the subject, transfected *in vitro*, then reintroduced into the patient. A number of protocols have been established for the *ex vivo* transfection of a wide variety of cell types. This method allows specific cell targeting and high transfection efficiency. However, the process is time consuming, complex, and costly. Additionally, the method is not applicable to all situations, such as those in which an immediate modification is required.

2. *In vivo Gene Therapy*

Alternatively, gene therapy can be performed through *in vivo* manipulation of gene expression. In this case, the desired genes must be directly delivered to the appropriate cell population in the subject *in vivo*. This strategy offers several advantages over the *ex vivo* approach. For instance,

the *in vivo* approach does not require the isolation or culture of cells, thereby reducing cost, complexity, time, and donor site morbidity. Further, *in vivo* gene therapy approaches hold the potential for generating products that can be used off of the shelf. *In vivo* gene therapy, however, faces the major obstacle of identifying a safe and efficient gene delivery vehicle that can target specific cell populations (Anderson, 1998).

3. *Limitations of Naked DNA Delivery*

The direct injection of uncomplexed, naked plasmid DNA (pDNA) represents the simplest technique for gene transfer. Although the effectiveness of naked DNA toward inducing gene expression has been demonstrated (Wolff *et al.*, 1990), several limitations are associated with the delivery of naked DNA. A single injection of a bolus dose of pDNA typically generates expression levels that reach a maximum within one month, followed by a drop to low stable levels that can be detected for up to two years (Jiao *et al.*, 1992; Jong *et al.*, 1997; Wolff *et al.*, 1990). Some efforts to increase the efficacy of direct administration of naked DNA involve increasing the distribution of the injected DNA (Mumper *et al.*, 1996) or enhancing the level of DNA uptake by cells at the time of injection (Danko *et al.*, 1994; Vitadello *et al.*, 1994).

It has been shown that naked DNA is highly vulnerable to degradation by endonucleases, which can degrade DNA within 30 min in the extracellular spaces (Kawabata *et al.*, 1995). In addition, uncomplexed DNA introduced into the plasma via intravenous injection is quickly cleared from the circulation by scavenger receptors in the liver, which compromises its extravasasion into target tissues and organs (Pouton and Seymour, 1998; Takakura *et al.*, 1998; Yoshida *et al.*, 1996). The lymphatic system rapidly clears naked DNA that is injected directly into tissues (Choate and Khavari, 1997; Levy *et al.*, 1996; Pouton and Seymour, 1998). DNA is a large molecule with a high degree of negative charge derived from the phosphate groups of its backbone (Ledley, 1996). Indeed, plasmid DNA constructs used for gene therapy typically have a molecular weight on the order of 10^6 Da and a hydrodynamic radius greater than 100 nm (Ledley, 1996). These properties significantly impede its ability to cross biological barriers, such as the endothelium, the plasma membrane, and the nuclear membrane of cells (Ledley, 1996). The cellular plasma membrane holds a net negative charge, which likely repels large, negatively charged DNA molecules, thereby hindering the cellular uptake of DNA. Thus, entry into the cell is a major barrier faced by naked DNA, yet studies have demonstrated gene expression following introduction of naked DNA *in vivo* (Wolff *et al.*, 1990). It follows then that naked DNA can cross the plasma

membrane barrier, but this likely occurs with very low efficiency. Proposed mechanisms for the cellular entry of naked DNA involve either an unidentified cell membrane transporter (Budker *et al.*, 2000) or the process of potocytosis (Wolff *et al.*, 1992).

Once the DNA enters the cell, however, several additional barriers to expression must be overcome. The DNA enters the cytosol upon crossing the plasma membrane and then migrates toward the nucleus in a process likely governed by diffusion (Luo and Saltzman, 2000a). This movement toward the nucleus is quite slow, which prolongs the duration of exposure of the DNA to the cytosol (Luo and Saltzman, 2000a). Nucleases present in the cytosol can rapidly degrade the DNA during its intracellular migration. Although finding the nucleus is a major obstacle in itself, the DNA must enter the nucleus and be expressed for the therapy to be successful. These barriers to effective expression of naked DNA have inspired the search for a gene delivery vehicle that might overcome some or all of these obstacles. In general, an optimal gene delivery system should enhance DNA stability, offer cell or tissue specificity, improve the bioavailability of the DNA, and promote cellular uptake and intracellular trafficking (Han *et al.*, 2000; Mahato *et al.*, 1999; Truong-Le *et al.*, 1998).

4. *Viral Gene Therapy*

Viral gene therapy takes advantage of the highly evolved and efficient transfection mechanism of viruses to transfer genetic material into a host cell. The high transfection efficiency of viral carriers has resulted in their wide exploration in gene therapy studies. Viral vectors are generated by removing a section of the viral genome, so as to render the virus replication incompetent. A gene encoding the desired product is then inserted into the void created by the removal of the viral gene segment, which is usually of very limited size. Thus, there is a limit to the amount of genetic material that can be incorporated into a viral vector (Smith, 1995). Additional limitations include safety concerns and the cost and difficulty associated with production. Just as viruses have evolved to transfect mammalian cells, mammalian cells have evolved to resist viral transfection (Verma and Somia, 1997). Thus, viral vectors are highly immunogenic, which restricts their repeated use for gene delivery (Capan *et al.*, 1999a). Additionally, some viral vectors incorporate genetic material into the host genome, thereby generating the risk of oncogenesis and mutagenesis, although this possibility is often considered to be quite small. Further, a chance exists that viral vectors will recombine *in vivo* and activate the complement immune response. The risks and limitations of viral vectors have led to the study of nonviral methods for gene delivery.

5. Nonviral Gene Therapy

The past decade has seen numerous attempts toward the development of a nonviral vector that can match the level of specificity and gene expression offered by viral vectors, while improving the safety, gene insert capacity, and immunogenicity. Although naked DNA can be introduced and has been shown to be effective *in vivo* (Wolff *et al.*, 1990, 1992), many limitations are associated with naked gene delivery. Physical methods of nonviral gene therapy have been explored including microinjection, electroporation, and biolistic delivery with the gene gun. Microinjection involves the direct injection of genes into the nuclei of individual cells one at a time. As a result, high efficiency and specificity are attained with microinjection, but the process requires a great deal of time and effort. Thus, the method is not practical for *in vivo* DNA delivery or for the transfection of large numbers of cells *in vitro*. Electroporation induces temporary pore formation in the membranes of living cells through brief exposure to high-voltage electrical pulses. Genes may enter the cells through the transient pores, which typically close on the order of minutes. Electroporation is among the most efficient methods of gene transfer, but the high mortality of cells following exposure to the high-voltage pulses limits its utility (Luo and Saltzman, 2000a). The gene gun, however, utilizes DNA-coated gold particles that are driven through the cell membrane at high velocity (Yang and Sun, 1995; Yang *et al.*, 1990). This method allows for the simultaneous introduction of DNA into many cells and has been used effectively in DNA vaccination applications (Fynan *et al.*, 1993; Qiu *et al.*, 1996). The particle bombardment approach to DNA delivery, however, requires invasive proceedures to expose tissues and organs that are not readily accessible. Delivery of DNA with polymeric materials provides an alternative method for nonviral *in vivo* gene therapy, with the potential to circumvent the limitations of physical methods for DNA delivery.

II. Biomaterials for Nonviral Gene Therapy

A. Cationic Lipids

The first use of lipid molecules with an associated positively charged head group for gene transfer was described in 1987 (Felgner *et al.*, 1987). The use of cationic lipids has since grown to become the most widely investigated method for condensing plasmid DNA for nonviral delivery (Segura and Shea, 2001). Cationic lipids are generally composed of a hydrophilic lipid anchor linked through an intermediate group to a cationic

head group. The hydrophilic lipid group strongly influences the physical properties of the lipid bilayer, including the flexibility and the kinetics of lipid exchange within the bilayer (Felgner *et al*., 1994). A cholesterol or fatty acid group generally comprises the lipid anchor. The intermediate linker group plays a large role in determining the chemical stability and biodegradability. Additionally, the linker can affect the transfection efficiency of the cationic lipid, as it provides a site for the potential attachment of side chains to augment cellular targeting, uptake, and intracellular trafficking of the complex (Byk *et al*., 1998a). In general, longer linker lengths correspond to increased gene transfer (Byk *et al*., 1998a). The head groups vary widely in structure and charge, with multivalent groups generally having higher transfection efficiencies than the monovalent equivalents (Felgner *et al*., 1994; Lee *et al*., 1996). The generation of novel cationic lipids from libraries of components has improved understanding of the structure–activity relationships of these materials as gene carriers (Byk *et al*., 1998a,b; Lee *et al*., 1996). However, a more detailed understanding of the correlation between lipid structure, DNA complexation, and cellular interaction is required for optimization of cationic lipid gene delivery vectors.

The interaction of plasmid DNA with cationic lipids results in a tightly condensed complex (termed a lipoplex), in which the cationic lipids completely or partially cover the DNA. This interaction is driven by the entropy increase derived from the release of water molecules and counter ions associated with the surfaces of the DNA and lipid molecules (Radler *et al*., 1997). The formulations of cationic lipids used for gene delivery typically include a zwitterionic or neutral lipid in addition to the cationic lipid group to enhance transfection (Ferrari *et al*., 2002). Properties such as the size and stability of the lipoplexes are influenced by the charge ratio of the amines on the cationic lipid to the phosphates groups on the DNA more so than the composition of the lipids (Xu *et al*., 1999). Large aggregates (greater than one micrometer) are formed from the complexation of DNA and cationic lipids at a neutral, or one-to-one, charge ratio (Xu *et al*., 1999), hence positive or negative charge ratios are typically used for gene delivery. As cell membranes carry a net negative charge, lipoplexes with a positive charge ratio are generally used to delivery DNA for *in vitro* studies, as they promote electrostatic interaction with the cell membrane. It has been proposed, however, that *in vivo* studies may require different charge ratios for efficacy due to possible interactions with components of the physiological environment (Mahato *et al*., 1999). Indeed, DNA can be released from complexes due to the binding to the lipid of polyanionic species with a sufficient charge density, such as heparin (Xu and Szoka, 1996). Thus, DNA may be prematurely released from cationic lipids and subject to nuclease

degradation before reaching cells *in vivo*. Conjugation of poly(ethylene glycol) (PEG) to the lipid has been shown to reduce the extent of protein binding and complement activation of the lipoplexes, however gene expression of the modified complexes is significantly reduced (Filion and Phillips, 1998).

Initial theories proposed that lipoplexes enter the cytoplasm after fusing directly with the plasma membrane (Felgner *et al*., 1987; Smith *et al*., 1993). Current thought, however, suggests that complex entry follows endocytosis, which can be enhanced with the use of endosomolytic agents such as chloroquine (Zuhorn *et al*., 2002; Zabner *et al*., 1995). Although the mechanism of cellular entry is not well understood, high efficiency of delivery has been demonstrated with several cell types *in vitro* (Bebok *et al*., 1996; Zabner *et al*., 1995). Following cellular entry of the lipoplex, the next important barrier is the release of the plasmid DNA from the lipid (Xu and Szoka, 1996). The final obstacle is nuclear entry of the DNA for expression. Indeed, it has been proposed that the dissociation of DNA from the lipid and subsequent entry into the nucleus represent the most significant hurdles for gene expression with cationic lipid carriers (Zabner *et al*., 1995).

Despite their current wide use in nonviral gene therapy, cationic lipids present several limitations. First, the structure and transfection mechanism of cationic lipoplexes are not well characterized. Second, although the lipoplexes are able to enter cells efficiently, they exhibit poor cell targeting and gene expression. Additionally, cationic lipoplexes are highly toxic upon repeated use (Han *et al*., 2000), and induce a strong anti-inflammatory response *in vivo* (Filion and Phillips, 1997; Tan and Huang, 2002). As a result of these limitations, interest in polymeric gene carriers has grown in recent years.

B. Cationic Polymers

Another nonviral approach to gene delivery is the use of polycationic polymers to form electrostatic complexes with plasmid DNA. The polycationic polymers have a high density of primary amines, which endow the molecules with a large quantity of positive charge in the physiologic pH range. As a result, DNA is able to electrostatically complex with the polymers and condense into small particles capable of entering cells. The primary amines on the polymer chain also provide a site for chemical modification with peptides or ligands through which enhanced cell targeting and transfection may be achieved via receptor-mediated endocytosis (Erbacher *et al*., 1999; Gottschalk *et al*., 1994; Wagner *et al*., 1990). Many different cationic polymers have been investigated for use in gene

delivery, and they range in structure from linear to highly branched. The most widely investigated cationic polymers for gene delivery are poly(L-lysine) (PLL), poly(ethylenimine) (PEI), and poly(amidoamine) dendrimers.

Poly(L-lysine) has been used to complex with DNA in a variety of salt conditions and has demonstrated gene transfer both *in vitro* and *in vivo* (Duguid *et al*., 1998; Gonsho *et al*., 1994). PLL is typically synthesized on a solid support through a series of protecting/deprotecting steps to generate fairly monodisperse polypeptides. Charge ratios of amine groups on PLL to phosphate groups on DNA between 3:1 and 6:1 are typically used to generate complexes. High molecular weight PLL forms smaller complexes with DNA than lower molecular weight PLL. These smaller, tighter complexes offer greater protection of DNA from the effects of sonication and salt concentration (Adami *et al*., 1998), yet have a higher associated cytotoxicity relative to the larger complexes (Duguid *et al*., 1998). Complexes of PLL and DNA are subject to aggregation under physiological conditions. Chemical modification of PLL with such molecules as dextran (Maruyama *et al*., 1998), poly(ethylene glycol) (Choi *et al*., 1998; Lee *et al*., 2002), and poly[*N*-(2-hydroxypropyl)methacrylamide] (Oupicky *et al*., 2000) has been shown to reduce aggregation of the complexes without compromising the ability of the complex to form. The modification of PLL with PEG has also been shown to decrease the high degree of cytotoxicity associated with PLL alone (Choi *et al*., 1998; Lee *et al*., 2002).

Another cationic polymer that has been investigated for gene delivery applications is poly(ethylenimine) (PEI). It presents a high density of positive charge, as nitrogen represents every third atom along its backbone. Both linear and branched forms of PEI exist, but only two-thirds of the backbone nitrogens can carry a charge in the branched form, whereas all of the nitrogens can be charged in the linear form (Garnett, 1999). In either form, the effectiveness of PEI in complexing and transferring DNA into a variety of cell types has been demonstrated both *in vitro* and *in vivo* (Boussif *et al*., 1995). The PEI/DNA complexes have been shown to attach to the cell surface and aggregate into clumps, which are subsequently endocytosed (Godbey *et al*., 1999b). The endocytosed PEI enters the nucleus in an ordered structure, whether or not it is complexed with DNA (Godbey *et al*., 1999b). It has been proposed that PEI may protect DNA from nuclease degradation in and enhance release of the complex from the lysosome through buffering action and swelling, which leads to lysosomal rupture (Boussif *et al*., 1995). In this process, termed the proton sponge effect, the previously uncharged amines of the PEI are protonated by an influx of positively charged protons. The resulting charge gradient leads to the entry of negative ions, such as Cl^-, into the endosome. The accumulation of ions within the endosome leads to osmotic swelling and

rupture of the endosome, allowing release of the PEI/DNA complexes into the cytosol. A study by Godbey *et al.* (2000), however, challenged the proton sponge hypothesis for PEI transfection. This study demonstrated that the pH in the lysosome remained constant over the time frame needed for nuclear entry of the DNA, and endosome–lysosome fusion was not observed. Thus, it was suggested that PEI/DNA complexes escape from the endosomes before the pH drops to acidic coniditons (Godbey *et al.*, 2000). Further, the molecular weight of PEI affects gene transfer, with transfection efficiency increasing with the molecular weight of PEI (Godbey *et al.*, 1999a). However, complexes of PEI and DNA are cytotoxic and prone to aggregation.

Dendrimers of poly(amidoamine) (PAMAM) have also received attention as polycationic nonviral gene delivery vehicles. The diameter and surface charge of the starburst PAMAM dendrimers is a function of the number of synthetic cycles, also known as generations, used in the synthesis (Haensler and Szoka, 1993). Higher transfection efficiencies have been observed for DNA complexed with fifth- and sixth-generation PAMAM dendrimers when compared to lower generations (Haensler and Szoka, 1993). PAMAM dendrimers are generally synthesized from core molecules of ammonia or ethylenediamine to which methyl acrylate and ethylenediamine are successively added (Tomalia *et al.*, 2002). The core molecules from which the synthesis originates determine the shape, density, and surface charge of the resulting dendrimer, which can be either ''fractured'' or ''intact,'' depending on the number of branch arms extending from every branch point. Fractured dendrimers may have as few as zero and as many as two arms extending from each branch point; while intact dendrimers extend two arms from every branch point (Tang *et al.*, 1996). Electrostatic interaction between the phosphate groups of plasmid DNA and the terminal primary amines of the PAMAM dendrimers generates condense complexes that are capable of mediating gene delivery (Bielinska *et al.*, 1997; Haensler and Szoka, 1993; Qin *et al.*, 1998). This gene transfer, however, has generally lacked cell specificity.

Although cationic lipids and cationic polymers present several advantages over viral techniques and the administration of naked DNA for gene therapy, a number of limitations are generally associated with these materials. In contrast to viral vectors, virtually any size of plasmid DNA can be delivered with cationic complexes. The complexed DNA is partially protected from degradation by endonucleases and sonication, yet dissociation of the DNA from the carrier can be difficult. Additionally, these complexes are highly efficient at entering cells in a nonspecific manner, yet the delivered genes are not efficiently expressed. A complete understanding of the cellular entry, trafficking, and expression of these vectors has not

been developed. Further, cationic DNA carriers generally exhibit significant cytotoxicity. These limitations have led to the investigation of controlled release from polymer constructs.

C. Polymers for Controlled Delivery

The nonviral methods for gene therapy discussed above present a short period of time during which the DNA is available for cellular entry, which is adequate for some applications. However, other applications require a long-term or sustained presence of the transfection agent for efficacy. As a result, technology for the controlled release of proteins from biocompatible polymers has recently been applied to the delivery of DNA. In this method, plasmid DNA is entrapped within a biocompatible polymeric material. The polymer construct may then either hold the DNA *in situ* while cells migrate into the scaffold to encounter the DNA, or the DNA may be released from the scaffold in a controlled manner. The polymer entrapment method for controlled DNA delivery offers several potential advantages over the other nonviral gene transfer techniques. To begin, physical entrapment may protect DNA from degradation until its release or cellular uptake. Second, long-term localized release may be achieved through the polymeric constructs without need for repeated administration, or the construct may be tailored to deliver the DNA in a rapid, bolus fashion depending upon the particular application. Additionally, cellular or tissue specific targeting may be achieved through introduction of the polymer at the desired site. The localization of the DNA within the scaffold provides a safety mechanism for ending the therapy as the implant could be easily retrieved. Although the potential applications of controlled release of plasmid DNA from polymer constructs are expansive, the majority of work to date has focused on the application of the technology toward genetic vaccination and tissue engineering. Indeed, polymeric microparticle delivery systems have been widely studied for DNA vaccines, while large constructs have been developed for tissue engineering or guided tissue regeneration applications. The current discussion will focus on the development of polymeric materials for the entrapment and controlled delivery of plasmid DNA toward these applications.

III. Polymer Based Particles for Controlled DNA Release

Polymeric-based particles have been utilized for the encapsulation and release of proteins and peptides for numerous applications. Recently,

however, attention has been granted to the use of polymeric particles for the encapsulation and release of genetic material. The search for a nonviral delivery method for genetic immunization has prompted much of the investigation of microparticles for gene delivery. Microparticles and nanoparticles have been fabricated from both natural and synthetic materials, including, chitosan, gelatin, and poly(D,L-lactic-*co*-glycolic) acid (PLGA) for the encapsulation and release of plasmid DNA.

A. Genetic Immunization

Direct introduction of naked plasmid DNA has been explored as a novel and effective method of vaccination (Donnelly *et al.*, 1997; Hassett and Whitton, 1996; Shiver *et al.*, 1996). DNA vaccinations, in opposition to conventional protein immunization, provide a means for extended expression of antigens and the elicitation of both humoral and cellular immune responses (Donnelly *et al.*, 1997). This strategy emerged following reports that direct injection of naked plasmid DNA into muscle cells could induce gene expression (Wolff *et al.*, 1990). The approach for genetic immunization involves the direct introduction of plasmid DNA into host cells, such that the encoded antigenic protein is expressed and induces a desired immune response (Wang *et al.*, 1999). Indeed, an initial report of genetic immunization demonstrated the generation of cytotoxic T lymphocytes and protective immunity in mice following intramuscular injection of plasmid DNA encoding the influenza A nucleoprotein (Ulmer *et al.*, 1993). Since that report in 1993, protective immunity following DNA immunization has been demonstrated in numerous independent studies (Donnelly *et al.*, 1997). Indeed, the effectiveness of DNA vaccines has been demonstrated in both small and large animals, including porcine, bovine and equine models (Donnelly *et al.*, 1995; Porgador *et al.*, 1998; Singh *et al.*, 2000). Recent studies in humans and nonhuman primates, however, have required large doses (milligrams) of plasmid DNA to induce antibody and cytotoxic T lymphocyte responses (Calarota *et al.*, 1998; Letvin *et al.*, 1997; MacGregor *et al.*, 1998; Wang *et al.*, 1998). Although the demonstrated effectiveness of genetic immunization is promising, the use of milligram quantities of DNA is not appealing from an economic perspective, especially if multiple administrations are required.

The activation of a T-cell response and subsequent efficacy of DNA vaccines is ultimately determined by professional antigen presenting cells, such as macrophages, dendritic cells, and Langerhans cells (Corr *et al.*, 1996; Doe *et al.*, 1996; Iwasaki *et al.*, 1997; Ulmer *et al.*, 1996). It follows that successful DNA vaccination strategies should seek to target DNA

delivery to these cells or to tissue rich in these cells, such as the lymph nodes, spleen, skin, and sub-mucosal tissues (Lunsford *et al.*, 2000). It has been shown that naked DNA introduced through intramuscular and intradermal injection remains localized at the site of injection (Lunsford *et al.*, 2000; Nichols *et al.*, 1995; Parker *et al.*, 1999; Winegar *et al.*, 1996). Delivery of DNA encapsulated in polymer particles may facilitate the selective transfection of phagocytotic cells, such as macrophages, by size exclusion, as microspheres between 1 and 10 μm in diameter are too large to enter cells by endocytosis, but small enough to be phagocytosed (Eldridge *et al.*, 1989; Tabata and Ikada, 1990).

B. Advantages of Encapsulation Over Injection

The use of biodegradable polymeric microparticles presents several potential advantages for the delivery of plasmid DNA (pDNA). First, encapsulation of pDNA within or adsorption to the surface of microparticles offers increased resistance to degradation via endonucleases (Capan *et al.*, 1999a). Additionally, biodegradable microparticles provide a means for sustained, localized delivery of pDNA in a controlled manner, which may increase the level of pDNA retention in the tissue (Davis *et al.*, 1993a). High concentrations of pDNA retained within the tissue may enhance the transfection efficiency of the local cells by increasing the physical concentration of pDNA at the cell surface (Luo and Saltzman, 2000b). Another advantage of microparticle delivery is the potential for minimally invasive administration of the carrier by means such as direct injection or oral delivery.

C. PLGA for Release of DNA in Vaccination Applications

The majority of work toward the development of microparticles for DNA encapsulation and release has involved the polymer poly(D,L-lactic-*co*-glycolic) acid (PLGA). Since the FDA approved its use as a suture material, PLGA has been widely investigated for medical applications ranging from implant fabrication to drug release systems. Indeed, the biocompatibility of PLGA is generally accepted as the material degrades in the body via hydrolysis to yield lactic acid and glycolic acid, both of which are metabolized by natural pathways. The degradation of PLGA can be controlled through varying the ratio of lactic to glycolic acid in the polymer and varying the molecular weight of the polymer chains. An increase in the ratio of glycolic acid in the copolymer results in a decrease in the hydrophobicity of the polymer and an increase in the degradation rate

(Heller *et al.*, 1987), with a 50:50 ratio of lactic to glycolic acid degrading most rapidly (Gupta *et al.*, 1998). Similarly, a decrease in the molecular weight of the polymer chains results in an increase in the rate of degradation (Gupta *et al.*, 1998). Exposure of the polymer to water results in hydrolytic cleavage of the ester bonds via a bulk process, such that the polymer chains become successively smaller until they are soluble in the aqueous environment (Hausberger and DeLuca, 1995). This controllable, steady degradation mechanism has led to the application of PLGA for drug encapsulation and delivery (Mehta *et al.*, 1994). In recent years, investigators have explored the use of PLGA based nano- and microparticles for the encapsulation and release of plasmid DNA.

The initial investigation of PLGA microparticles for delivery of encapsulated plasmid DNA was conducted by Jones *et al.* (1997). This report demonstrated that encapsulated DNA was effectively protected from degradation following parenteral and oral administration and that cellular uptake was facilitated so that systemic and mucosal antibody responses were invoked. It was shown that the antigen was effectively presented so as to elicit IgM, IgG, and IgA antibody responses. Although this study demonstrated proof of principle, the encapsulation efficiency was limited to about 25%, and released DNA retained only about 25% of its bioactivity when compared to unencapsulated controls following *in vitro* gene expression assays. Further, the DNA, which was initially in a super-coiled isoform, was converted predominately to an open circular form. This pilot study promoted further investigation into the mechanism behind and control of DNA release from PLGA nano- and microspheres.

Luo *et al.* (1999) investigated the effects of adjusting the polymer molecular weight and the composition of poly(L-lactic acid) (PLA)/PLGA in the fabrication of microspheres upon the release of encapsulated DNA. A bi-phasic release profile was observed, with an initial burst of DNA followed by a slow release for all microsphere formulations. Three polymer formulations were examined for DNA release, namely PLA of low molecular weight (2 kDa), PLA of high molecular weight (300 kDa), and PLGA (50:50 ratio of lactide to glycolide). Microspheres fabricated from the lower molecular weight PLA were observed to release a greater amount of the loaded DNA than the lower molecular weight PLA. The microspheres made from PLGA, however, demonstrated the most rapid release, as 95% of the loaded DNA was released within two days. It was suggested that the inclusion of the glycolic acid into the material increased the hydrophilicity and, subsequently, degradation rate of the PLGA relative to PLA, which would accelerate the release of DNA. It was concluded that the release of DNA from PLGA microspheres depends on both polymer degradation and DNA diffusion. Indeed, a number of factors can influence the release of

DNA from polymeric microspheres, including the chemical characteristics and molecular weight of the material, the size and morphology of the microspheres, as well as the amount of DNA loaded into the microspheres (Luo *et al.*, 1999).

Several processing techniques have been successfully employed in the encapsulation of plasmid DNA within PLGA nano- and microspheres, including double-emulsion (water-oil-water) solvent-evaporation (Ando *et al.*, 1999; Barman *et al.*, 2000; Cohen *et al.*, 2000; Hao *et al.*, 2000; Lunsford *et al.*, 2000; Tinsley-Bown *et al.*, 2000; Wang *et al.*, 1999) and spray drying (Walter *et al.*, 1999, 2001). In the double-emulsion solvent-evaporation technique, the DNA is suspended in an aqueous solution, while the PLGA is dissolved in a partially water-miscible organic solvent, usually methylene chloride or ethyl acetate. An emulsion is then generated between the two phases through either sonication or homogenization. Polymers such as poly(vinyl alcohol) (PVA) and poly(vinylpyrrolidone) (PVP) may be used as emulsion stabilizers in the double-emulsion process and are deposited on the surface of the microspheres to prevent coalescence. The first emulsion is subsequently added to a much larger volume of aqueous solution and mixed in a similar manner to create the double-emulsion. The organic solvent is then evaporated from the double-emulsion, allowing the solidification of the polymer rich droplets into nano- or microspheres. The remaining aqueous solution may be either centrifuged or filtered to isolate the polymer spheres. Finally, the microspheres are lyophilized to remove the water from the interior aqueous phase, leaving the DNA within the PLGA matrix.

A study by Wang *et al.* (1999) investigated the effect of PLGA molecular weight upon the entrapment efficiency and release kinetics of plasmid DNA in microspheres created with the double-emulsion solvent-evaporation technique. The DNA entrapment efficiency increased as the PLGA molecular weight increased, ranging from 22.5% for 6000 Da PLGA to 53.3% for 50,000 Da PLGA. The microspheres fabricated from the low molecular weight PLGA, however, displayed the fastest and highest total amount of DNA release, with 20% of the encapsulated DNA being released over the course of 28 days *in vitro*. The microspheres made from PLGA of 30,000 Da or higher released less than 5% of the encapsulated DNA over the course of 28 days. The release profile in all cases was bi-phasic, with an initial burst followed by little subsequent release. The polymer particles ranged in size from 400 nm to 2 μm, with no observed effect from the molecular weight of the PLGA. The DNA extracted from microspheres was found to have a higher fraction of open circular and lower fraction of super-coiled DNA than unencapsulated controls. This indicates that degradation of the DNA was induced in the encapsulation process.

The low entrapment efficiencies for microparticles that released appreciable amounts of DNA and the observed denaturation of DNA following encapsulation prompted Tinsley-Bown *et al.* (2000) to optimize some of the processing parameters in the double-emulsion solvent-evaporation technique. The double-emulsion process was conducted with the use of ethyl acetate as the organic solvent, rather than methylene chloride, which had typically been used. Additionally, the temperatures and volume ratios of the reagents were adjusted from typical values. Further, emulsification was induced with a blender on low speed rather than through sonication or homogenization. It was concluded that the optimized processing parameters resulted in higher entrapment efficiencies than previous reports from their group (Jones and Farrar, 1995; Jones *et al.*, 1997) and others (Wang *et al.*, 1999) using the double-emulsion technique. The observed DNA incorporation in this study, however, ranged from only 19 to 54%, depending upon the PLGA formulation used and the total polymer concentration in solution. A trade-off effect for the blending speed was observed between particle size and the structural integrity of the encapsulated DNA. The optimized blender process resulted in approximately 30–40% of the DNA retaining its super-coiled structure in comparison to about 10% when a homogenizer was used in a previous study by the same group (Jones and Farrar, 1995; Jones *et al.*, 1997). The structural integrity of encapsulated DNA was shown to decrease significantly with time upon release *in vitro* relative to unencapsulated controls. The control DNA was only partially degraded to the open circular form after six weeks, whereas little of the encapsulated DNA was detected at the fifth week. It was suggested that the loss of DNA integrity may be a result of degradation induced by the low pH in the microenviroment within the PLGA matrix during degradation (Ando *et al.*, 1999; Walter *et al.*, 1999; 2001). Thus, this study proposed that DNA degradation may occur both in the encapsulation process and during the degradation of the polymer matrix.

A study by Ando *et al.* sought to determine the mechanism of degradation during the double-emulsion solvent-evaporation technique and to develop methods to counteract it (Ando *et al.*, 1999). They hypothesized that DNA was damaged by (1) exposure to sheer stresses in the homogenization or sonication steps of processing and (2) the formation of crystals of the buffer salts during lyophilization. A cryopreparation process was introduced to address the first hypothesis. Cryopreparation involves lowering the temperature of the primary emulsion below the freezing point of the aqueous phase, such that a solid particulate suspension is produced. They proposed that the shear stresses encountered by the DNA in the frozen phase should be minimal during the homogenization step to form the secondary emulsion. As a result, the DNA should retain its

super-coiled isoform. Additionally, it was suggested that freezing the inner phase should prevent diffusion of DNA from the microspheres during homogenization, thereby increasing the entrapment efficiency. The second hypothesis was addressed by including saccharides in the primary emulsion to hinder the formation of salt crystals during lyophilization. Increasing rates of homogenization were found to increase the degree of degradation of DNA. However, the cryopreparation technique preserved the super-coiled structure of DNA at all homogenization rates relative to samples made without the cryopreparation step. Additionally, it was found that the inclusion of ethylenediaminetetraacetic acid (EDTA) and lactose in the DNA solution was an important factor in maintaining the super-coiled structure of DNA during cryopreparation. When microspheres were prepared using optimized conditions, the average particle diameter was 4.8 μm, 88% of the DNA retained its super-coiled isoform, and the encapsulation efficiency was 89%, versus values of 4.5 μm, 39% and 29%, respectively for microspheres fabricated with the standard double-emulsion method. The inclusion of saccharides into the primary emulsion was adopted by other studies for the encapsulation of DNA in PLGA microparticles (Barman *et al*., 2000; Hao *et al*., 2000). Although this study addressed the problems associated with DNA degradation during microparticle fabrication, the issue of protection against DNA degradation during polymer degradation remains to be addressed.

Other studies have sought to encapsulate plasmid DNA that has been complexed with another polymer, such as PLL, within PLGA nano- and microspheres by the double-emulsion solvent-evaporation technique (Capan *et al*., 1999a,b). The first of the studies demonstrated that PLL-complexed DNA encapsulated within PLGA microspheres retained a significantly higher percentage of its super-coiled form relative to encapsulated, uncomplexed control DNA (76.7–85.6% vs 16.6%, respectively) (Capan *et al*., 1999a). The encapsulation efficiency and total percentage of the loaded DNA released was lower for encapsulated, PLL-complexed DNA than for encapsulated, uncomplexed controls. It was also observed that the encapsulated, PLL-complexed DNA was protected from enzymatic degradation *in vitro*. The second study investigated the influence of various formulation parameters on the size of the microparticles, the degradation of DNA, the loading efficiency, and the *in vitro* release kinetics (Capan *et al*., 1999b). A complex ratio of DNA to PLL of 1:0.33 (w/w) was encapsulated in all formulations. The hydrophobic PLGA resulted in a higher entrapment efficiency (46.2%) and maintenance of super-coiled structure (64.9%) of DNA than microspheres fabricated from either a lower molecular weight PLGA (26.1 and 45.1%, respectively) or a more hydrophilic formulation of PLGA (15.9 and 58.7%, respectively). The hydrophobic PLGA, however,

demonstrated lower cumulative release of the encapsulated DNA over 38 days *in vitro* (54.2%), relative to the low molecular weight and hydrophilic PLGA formulations (95.9 and 84.9%, respectively). The release profiles for all formulations were bi-phasic, with an initial burst release. A decrease in particle size (6.6 to 2.2 μm) was observed as the concentration of PVA was increased from 1 to 7%, however, the fraction of super-coiled DNA was significantly reduced. Additionally, microspheres stabilized with PVA demonstrated a higher DNA–PLL entrapment efficiency than those stabilized with PVP at the same concentration (46.2 vs 24.1%). It was shown that the encapsulated, PLL-complexed DNA was protected from degradation by DNase I *in vitro*, yet a loss of super-coiled isoform was observed. This study suggests that the type and concentration of the surfactant, as well as the properties of the PLGA, can affect the encapsulation and release of PLL-complexed DNA. Although these studies demonstrated that the entrapment of PLL-complexed DNA protected the DNA from enzymatic degradation and reduced the conversion of super-coiled DNA to open circular and linear isoforms, the entrapment efficiencies were low, relative to those obtained with cryopreparation (Ando *et al*., 1999) and optimized double-emulsion solvent-evaporation processing parameters (Tinsley-Bown *et al*., 2000).

Following the general concept introduced by Capan *et al*. (1999a,b), another group investigated the encapsulation of DNA in PLGA nanospheres fabricated through the double-emulsion solvent-evaporation technique, but with the inclusion of calcium in the surfactant phase (Cohen *et al*., 2000). This approach resulted in a high encapsulation efficiency of approximately 70%. It was suggested that the inclusion of calcium in the surfactant phase electrostatically hindered the diffusional escape of DNA from the primary aqueous phase during the processing, thereby increasing the encapsulation efficiency. The typical bi-phasic release pattern was observed with the calcium modified nanospheres in this study. The nanospheres released approximately 80% of the encapsulated DNA over the course of 28 days *in vitro*, and a loss of super-coiled isoform was observed with time. Despite the loss of super-coiled structure, however, the *in vitro* bioactivity of the DNA was not significantly affected. It was proposed that some of the DNA was exposed on the surface during the emulsification process, and that the immediate release of this exposed, soluble DNA accounted for the observed initial burst release, with the remainder of the release being controlled by polymer degradation. The use of calcium may provide protection of the encapsulated DNA from degradation, in a similar fashion to PLL, yet circumvent the toxicity commonly associated with PLL. This was the first study to demonstrate the direct transfection of cells *in vitro* with DNA released from nanoparticle carriers.

Higher expression levels were associated with the encapsulated DNA than with the administration of naked DNA *in vitro*, but standard liposomal transfection yielded the highest level of gene expression. *In vivo*, however, the encapsulated DNA exhibited higher levels of sustained expression of a marker gene at 28 days, than that associated with naked DNA or DNA delivered with liposomes. Thus, this study indicated the potential for DNA encapsulated in degradable nanospheres to elicit sustained gene expression *in vivo*.

An alternative approach to PLGA microsphere fabrication for DNA encapsulation was explored by Walter *et al*. (1999, 2001). They proposed the use of a spray-drying technique. In this method, a primary emulsion is generated much as in the double-emulsion technique, with an aqueous solution of DNA dispersed in an organic phase of PLGA and solvent. The dispersion is then spray-dried, and the resulting particles are washed, filtered, and vacuum dried. The initial study (Walter *et al*., 1999) examined the integrity and functionality of three types of DNA (plasmid DNA, high molecular weight linear DNA, and low molecular weight linear DNA) during fabrication, after encapsulation, and after *in vitro* release. As ultrasonication is employed to generate the primary emulsion in the spray-drying technique, the effect of ultrasonication on each of the DNA types was investigated. It was found that both plasmid and high molecular weight linear DNA were degraded by ultrasonication. However, the inclusion of the buffer $NaHCO_3$ or of phosphate buffered saline solution prevented the conversion of plasmid DNA to the open circular form and significantly improved transfection activity relative to DNA in water alone. Additionally, exposure of plasmid DNA to an acidic environment (pH 3.5) resulted in a conversion from the super-coiled to the open circular isoform. Further, a significant reduction in biological activity was observed following exposure of the DNA to pH values of 3 or lower. A reduction in the duration of ultrasonication reduced the degree of DNA degradation, but decreased the encapsulation efficiency and increased the initial burst release *in vitro*. Increased nominal loading of DNA resulted in a reduction in the encapsulation efficiency, but an increase in the DNA stability. Comparison of DNA extracted from microspheres following fabrication with the DNA released in the initial burst phase indicated that the structural integrity of the DNA is independent of its location within the microparticle. A bi-phasic release pattern was typically observed in this study, with an initial burst phase followed by a sustained release governed by polymer degradation. Only negligible amounts of double-stranded DNA (dsDNA) were detected in the second release phase, according to analysis with the PicoGreen® dye, which specifically binds to dsDNA. A complete loss of bioactivity was associated with plasmid DNA released from day 16

onward *in vitro*, indicating a high degree of DNA degradation in the second release phase. It was proposed that the acidic microenvironment created in the degradation of the PLGA results in a significant degradation of the entrapped plasmid DNA. This study demonstrated the ability to protect DNA from degradation during microsphere fabrication, yet demonstrated the need for a method to stabilize DNA against acidic degradation as the PLGA is hydrolyzed.

The second study using the spray-drying fabrication technique (Walter *et al.*, 2001) investigated the effect of PLGA properties, such as molecular weight and hydrophilicity, upon the encapsulation efficiency and release kinetics of DNA *in vitro*. Additionally, the delivery of the microspheres to phagocytotic cells, namely human-derived macrophages and dendritic cells was examined *in vitro*. The molecular weight and the hydrophobicity of the PLGA had a marked effect on the encapsulation efficiency and *in vitro* release of plasmid DNA. Higher encapsulation efficiency and faster release of intact DNA were associated with the hydrophilic formulations in relation to the more hydrophobic formulations. Although all formulations displayed similar release in the burst phase, the hydrophilic formulations released DNA more rapidly in the second phase than the hydrophobic formulations. This faster release was shown to result in higher amounts of dsDNA in the second release phase. In addition, the *in vitro* release kinetics were examined under various pH values, ranging from 5.4 to 7.4. The initial burst phase of release was similar at all pH values, as 10–12% of the encapsulated DNA was released in the first 2.5 h. The release of DNA in the second phase was enhanced by acidic conditions. The DNA was found to lose its super-coiled structure and biological activity with time at all pH values. Additionally, this study demonstrated that the hydrophilic PLGA microspheres were phagocytosed with almost the same efficiency as the hydrophobic formulations in dendritic cells. The macrophages showed a slightly higher uptake of the hydrophobic microparticles, but still phagocytosed the hydrophilic microspheres. It was concluded, then, that all of the formulations held a degree of hydrophobicity that fell within the optimal range for cellular uptake. Once inside the cells, the hydrophilic microspheres were completely degraded within 9 days with a dispersion of the fluorescently marked DNA throughout the cell. Thus, the hydrophilic formulations were able to release the DNA within 2 weeks, a time frame compatible with the life span of the cells. The hydrophobic microspheres, however, were still intact after 13 days, having released little DNA at that time point. The degradation of the microspheres and subsequent release of plasmid DNA within the cell occurred more rapidly than in PBS. Indeed, the acidic environment encountered by the particles in the lysosomes may accelerate PLGA hydrolysis in a manner consistent with that observed in

the acidic *in vitro* release component of this study. The phagocytosed microparticles did not induce significant necrosis or apoptosis in either cell type. Although this study demonstrated that PLGA microparticles can be phagocytosed by cells to release their encoded plasmid DNA, the activity of the reporter gene could not be monitored once the cells were transfected. Thus, further investigation is warranted to elucidate the barriers to gene expression encountered by the released DNA.

Hirosue *et al.* (2001) employed a third processing technique to encapsulate plasmid DNA in PLGA nanospheres. They explored the use of a modified phase inversion/solvent diffusion method, in which the cationic lipid dimethyl dioctadecyl ammonium bromide (DDAB) was used as an excipient. This process allowed for particles to be formed without exposure to the shear stresses commonly employed in the double-emulsion and spray-drying techniques. The phase inversion/solvent diffusion involved the addition of a solution of the condensing agent (DDAB) in a solvent (trifluoroethanol, TFE) to a solution of the PLGA in TFE. This resulting solution was mixed by inversion. Subsequently, a solution of DNA in water was slowly introduced to the mixture with a pipette. The nanospheres were formed upon rapid addition of ethanol (50% v/v) to the resulting mixture, thereby allowing phase inversion to occur. The suspension was then diluted with water, and the solvents were removed under reduced pressure. This process resulted in nanospheres that were less than 150 nm in diameter, regardless of the molecular weight of the PLGA used. Free DNA and DNA/DDAB complexes were separated from the nanospheres through use of a density separation method. In contrast to the bi-phasic release characteristic of previous studies, the release of DNA from the phase inversion nanospheres was approximately zero-order, with no burst effect observed. The nanospheres released up to approximately 20% of the encapsulated DNA after 30 days *in vitro*. Although the released DNA retained some bioactivity, it was significantly less than unencapsulated plasmid control DNA. It was suggested that the manner of entrapment likely governs the release kinetics. In contrast to the double-emulsion method, the phase inversion process likely results in the formation of a uniform matrix of polymer, lipid, and DNA, from which release would be expected to be near constant with no initial burst effect (Hirosue *et al.*, 2001). Although this study presented an extended, zero-order release, the structural integrity and bioactivity of the DNA may significantly decrease with time. Additionally, the extended release is likely not practical for DNA vaccination applications, in which a need for rapid release of DNA has been suggested (Tinsley-Bown *et al.*, 2000; Walter *et al.* 1999, 2001).

A subsequent study by Perez *et al.* (2001) investigated the use of poly (ethylene glycol)-(D,L-lactide) (PEG–PLA) nanospheres for encapsulation

and release of plasmid DNA. They proposed that the use of nanospheres over microspheres might preserve the stability of encapsulated DNA, as acidic oligomers created in the degradation of the polymer could potentially diffuse out of the smaller construct with greater ease. They encapsulated plasmid DNA alone or with either PVA or PVP through one of two methods: double-emulsion solvent-evaporation or water-in-oil (w/o) solvent diffusion. The w/o solvent diffusion method involved the creation of an emulsion of aqueous DNA solution in an organic polymer solution by sonication. Subsequently, the emulsion was poured into ethanol under stirring to precipitate the polymer particles. The solution was diluted with water, and the solvents were removed through reduced pressure. Finally, the nanoparticles were collected by centrifugation. The nanospheres had negative ζ potential and were in the range of 150–300 nm in diameter, and the processing method influenced both of these parameters. High encapsulation efficiencies were obtained (60–90%), independent of the presence of PVA or PVP. The w/o solvent diffusion technique resulted in slightly higher encapsulation efficiencies than the double-emulsion (80–90% vs 60–80%). The encapsulation efficiencies of the nanospheres fabricated through double-emulsion were higher than generally observed. Indeed, it was proposed that high encapsulation efficiencies were the result of an interaction between plasmid DNA and the PEG chains of the polymer. The preparation technique was found to significantly affect the release kinetics: w/o solvent-diffusion nanospheres released the DNA rapidly (within an hour), whereas double-emulsion nanospheres followed the typical bi-phasic release over the course of 28 days *in vitro*. Thus, this study demonstrated high encapsulation efficiency through the interaction of DNA with PEG chains of the polymer. Further, in accord with previous studies, it was concluded that the preparation technique strongly influences the DNA release kinetics (Hirosue *et al*., 2001).

A debate exists in the literature regarding whether DNA should be adsorbed to the surfaces of synthetic polymeric particles or be encapsulated within them. The studies discussed to this point have involved the encapsulation of the plasmid DNA within the particle, which has been proposed to promote DNA stability and allow for possible surface modification of the carriers (Hirosue *et al*., 2001). Other studies, however, have investigated the adsorption of plasmid DNA to the surface of the particle (Maruyama *et al*., 1997; Singh *et al*., 2000). It has been suggested that surface adsorption could reduce the exposure of the DNA to the harsh conditions typically involved in the encapsulation process and increase the amount of DNA available for early release (Singh *et al*., 2000). Indeed, Singh *et al*. fabricated PLGA microparticles with a cationic surface, through the inclusion of either dimethyl dioctadecyl ammonium bromide (DDAB) or

1,2-dioleoyl-1,3-trimethylammonio-propane (DOTAP) in the polymer phase or the addition of cetyltrimethylammonium bromide (CTAB) to the primary emulsion of the double-emulsion process. It was found that the inclusion of any of these cationic surfactants resulted in a positive ζ potential, with CTAB generating particles with the highest positive surface charge. Additionally, a high loading efficiency was observed for particles containing CTAB over those with DDAB or DOTAP (92, 68 and 62%, respectively). The DNA was released from PLGA–CTAB microspheres with an initial burst effect (35% released at day 1), followed by a slower release rate (75% of adsorbed DNA was released by day 14). The released DNA was shown to lose super-coiled structure with time. It was shown that DNA released from PLGA–CTAB nanospheres following intramuscular injection into a rat model resulted in an enhanced seroconversion relative to naked DNA controls. Additionally, a size effect of the particles was observed, with DNA released from 300 nm spheres generating a significantly higher Ig titer than from 1 μm to 30 μm spheres (10,000, 4000, and 0 titers, respectively). Although this study demonstrated that DNA adsorbed to particle surfaces can elicit an immune respone in DNA vaccination applications, the system requires further characterization.

1. Natural Polymers for Release of DNA in Vaccination Applications

Some of the initial studies toward the investigation of polymeric nano- and microparticles involved the complex coacervation of DNA with natural polymers such as gelatin and chitosan (Leong *et al.*, 1998; Mao *et al.*, 2001; Roy *et al.*, 1999; Truong-Le *et al.*, 1998, 1999). Complex coacervation is a process in which oppositely charged macromolecular species are mixed in aqueous solution. The electrostatic interaction between the macromolecules drives a spontaneous phase separation to yield a phase rich in polymer (termed the coacervate) and a supernatant. The complex coacervation of gelatin and chondroitin sulfate has been used to encapsulate a variety of proteins and pharmaceuticals. Investigators have also explored the use of DNA as a polyanionic species for producing microspheres by complex coacervation with cationic macromolecules such as gelatin and chitosan (Leong *et al.*, 1998).

Gelatin is a natural material derived from the denaturation of collagen, and it has been widely applied in the food and pharmaceutical industries. It has the ability to act as an acid in the presence of a strong acid or a base in the presence of a strong base. Gelatin carries a net positive charge at pH values below 5, thus it can form a coacervate with DNA through electrostatic complexation in these conditions (Leong *et al.*, 1998).

The gelatin can subsequently be crosslinked to stabilize the complex. The crosslinked gelatin matrices are enzymatically degraded to release the DNA (Fukunaka *et al*., 2002; Truong-Le *et al*., 1998). An initial study by Truong-Le *et al*. (1998) investigated the release of plasmid DNA from DNA–gelatin microspheres generated through complex coacervation. Sodium sulfate was used in the process to facilitate the formation of coacervates through inducing desolvation in the water environment surrounding the polyelectrolytes (Leong *et al*., 1998). They obtained nanospheres in the size range of 200–700 nm with 25–30% (w/w) DNA loading. The higher molecular weight gelatin resulted in higher entrapment efficiency (~98%), likely through chain entanglement of the polyelectrolytes (Truong-Le *et al*., 1998). Although there was no evidence of DNA crosslinking to itself or to gelatin, increased crosslinking densities resulted in lower amounts of DNA released over the course of one hour *in vitro* (PBS with trypsin). It was found that the nanoparticles enhanced protection of DNA for up to 4 h in serum, but it was completely degraded by 12 h. Naked DNA incubated under the same conditions was completely degraded within half an hour. The release medium was found to affect the release kinetics, with enhanced cumulative release observed in serum versus PBS or water over the course of one week *in vitro*. For *in vitro* transfection analysis, an endosomolytic agent, chloroquine, was coencapsulated with the DNA, and human transferrin was conjugated to the nanosphere surface. The expression of a marker gene was greater and more prolonged over three weeks following intramuscular injection of the nanospheres into a mouse model, relative to controls of either naked DNA or DNA complexed with the cationic lipid Lipofectamine. This study demonstrated the encapsulation of DNA within gelatin nanospheres and subsequent gene expression following administration *in vivo*. Further, it was shown that other molecules could be coencapsulated with the DNA and that molecules could be conjugated onto the surface of the nanospheres to enhance DNA stability and cellular uptake.

A subsequent study by the same group sought to further characterize the gelatin–DNA nanosphere system through examination of the reaction conditions for particle formation, the protection offered to the DNA through encapsulation, and the effect of adding calcium as an additional coencapsulant on transfection (Truong-Le *et al*., 1999). The particle size was influenced by the temperature of the reaction, the size of the plasmid DNA, the concentration of sodium sulfate, and the speed of mixing employed. The encapsulated DNA was partially protected from DNase I induced damage at low concentrations. The optimal formulation for transfection included the coencapsulation of choloroquine and calcium with the DNA, as well as conjugation of transferrin on the nanosphere

surface. Over 50% of cultured cells transfected with the nanospheres exhibited expression of a model gene for a transporter protein. Further, the cells demonstrated effective transport through the generated transporter protein.

The use of chitosan–DNA nanoparticles as DNA carriers has also been explored (Aral *et al.*, 2000; Leong *et al.*, 1998; Mao *et al.*, 2001; Roy *et al.*, 1999). Chitosan is a natural material derived from the shells of crustaceans. The biodegradability and nontoxicity of chitosan has led to the wide application of this polysaccharide in medical and pharmaceutical applications. Recent studies have demonstrated the ability of chitosan to effectively complex with and partially protect DNA (MacLaughlin *et al.*, 1998; Richardson *et al.*, 1999). An initial study by Leong *et al.* (1998) examined the encapsulation of DNA in chitosan nanospheres generated through complex coacervation. This study demonstrated that transferrin could be conjugated to the surface of the chitosan–DNA nanospheres and that chloroquine could be coencapsulated with the DNA, however, unmodified chitosan–DNA nanospheres were as effective at transfecting cells *in vitro* as nanospheres with transferrin and nanospheres with transferrin and chloroquine. The *in vitro* transfection efficiency of the nanopsheres was less efficient than observed with Lipofectamine. This study, however, did not examine the *in vitro* release kinetics of DNA or *in vivo* gene expression. A subsequent study by the same group evaluated the efficacy of the chitosan–DNA nanoparticles in delivering DNA to a mucosal surface (Roy *et al.*, 1999). A murine peanut allergy model was used, and plasmid DNA encoding an anaphylaxis-inducing antigen was delivered orally via chitosan–DNA nanospheres. The DNA was delivered effectively to the small intestine of the mice through oral administration and elicited a high IgG2a response, which protected the sensitized mice from challenge with the peanut allergen. A third study by this group sought to further examine the preparation parameters for the chitosan–DNA nanospheres and characterize the physico-chemical properties (Mao *et al.*, 2001). In this study, several processing parameters were examined and optimized for nanoparticle production. It was found that the processing of the nanospheres has a negligible effect on the conformation of the DNA. Further, this study suggested that encapsulation within the nanospheres offered protection of the DNA from degradation. The *in vitro* transfection efficiency of the nanospheres was shown to depend on the cell type, yet was several times lower than that obtained with Lipofectamine–DNA complexes. This result corresponded with results from a previous study (Leong *et al.*, 1998). As in the previous study (Leong *et al.*, 1998), the enhancement of transfection through co-encapsulating chloroquine or conjugating transferrin to the surface was limited. Conjugation of the viral

KNOB protein, however, was shown to improve gene expression markedly (130-fold in HeLa cells). Additionally, it was shown that PEG could be conjugated to the surface of the nanospheres to prevent aggregation during lyophilization without a loss of bioactivity following one month in storage. The PEGylated particles, however, were cleared from mice at a slower rate than unmodified controls and were found to accumulate in the kidney and liver at 15 min after intravenous administration. There was no difference after one hour, however.

Another study by an independent group examined the effect of various processing parameters on the *in vivo* transfection efficiency of chitosan–DNA microspheres (Aral *et al*., 2000). Additionally, this study investigated the effect of plasmid size on the *in vitro* release kinetics and transfection properties. An initial burst phase was observed with all chitosan–DNA microsphere formulations, which was likely due to release of DNA from the surface of the microspheres (Aral *et al*., 2000). The effect of chitosan concentration on the release of DNA seemed to be dependent on the size of the incorporated plasmid. Plasmid adsorbed to the surface of the microspheres was released more rapidly *in vitro* than DNA entrapped within the microspheres. Further, larger plasmids were released more rapidly *in vitro* than smaller plasmids (7.2 vs 2.69 kilobases). Although the released DNA showed some conversion from super-coiled to open circular and linear conformations upon release, the entrapment was shown to protect the DNA from nuclease degradation *in vitro*. The DNA delivered with chitosan–DNA microspheres demonstrated significantly higher expression *in vivo* than naked DNA, with the highest expression associated with the low-dose chitosan–DNA formulation. The effect of plasmid size on transfection was not clear. The studies with chitosan–DNA microspheres demonstrate its ability to enhance transfection *in vitro* and *in vivo*, to protect DNA from degradation, and to allow for conjugation of cell-targeting moieties on the surface of the microspheres.

The use of polymeric nano- and microparticles for DNA entrapment and release has demonstrated promise for use in DNA vaccination applications. Several materials have been investigated, each with their own set of advantages and limitations. In general, the release kinetics of the DNA can be controlled through manipulation of the formulation parameters in fabrication of the carrier particles. PLGA particles demonstrate a degree of control over the release kinetics, and processing techniques have been introduced to reduce the extent of DNA damage during fabrication. However, protection of the DNA from the acidic microenvironment during particle degradation remains an issue. Further, there appears to be a trade-off between entrapment efficiency and release kinetics with the PLGA carriers. The natural materials, such as gelatin and

chitosan, offer the potential for less damage to the DNA during fabrication and allow for conjugation of cell-targeting moieties to the surface of the spheres. The use of natural materials, however, has an associated risk of potential immunogenecity of the material, as it is derived from a natural source. Additionally, the characterization and control of release from the natural materials requires further investigation.

The entrapment and release of plasmid DNA from polymeric nano- and microparticles has not been limited to DNA vaccination applications. Indeed, the delivery of DNA from nano- and microparticles for application in guided tissue regeneration applications has been explored. Labhasetwar *et al*. (1998) demonstrated effective expression of a marker gene in a rat osteotomy model following delivery of DNA-loaded PLGA nanospheres generated with a double-emulsion solvent-evaporation technique. This study demonstrated the potential for DNA delivery from a polymeric material introduced into a wound site. Another study demonstrated that tissue engineering scaffolds can be fabricated from DNA-loaded PLGA microspheres through either compression molding or a gas-foaming process (Nof and Shea, 2002). Additionally, it was shown that porous scaffolds could be fabricated with these techniques in conjunction with a salt-leaching method (Nof and Shea, 2002). This study evaluated the *in vitro* release of the incorporated plasmid DNA. The release from the scaffolds showed minimal burst effect in the initial phase of release when compared to the microspheres from which they were fabricated. This study demonstrated the potential for fabricating tissue engineering scaffolds from DNA loaded microspheres and the ability to control the release of DNA from the scaffold through manipulation of processing parameters. Indeed, these studies have led to the investigation of release of plasmid DNA from polymeric scaffolds to augment guided tissue regeneration in tissue engineering applications.

IV. Polymeric Scaffolds for Controlled DNA Delivery

A. Gene Activated Matrices

A method was recently developed to deliver plasmid DNA locally to cells involved in wound repair (Bonadio *et al*., 1999; Fang *et al*., 1996; Shea *et al*., 1999). The technique involves the introduction of a porous, biodegradable polymer matrix into the wound site (Bonadio *et al*., 1998). The scaffold (a gene activated matrix, or GAM), in its simplest form comprises plasmid DNA and the polymer matrix (Bonadio, 2000). Naked

plasmid DNA is physically entrapped into the polymer network during scaffold fabrication. It has been proposed that the matrix holds the plasmid DNA *in situ* as wound healing cells, mainly fibroblasts, infiltrate the construct from the periphery (Fang *et al*., 1996). The cells incorporate the DNA as they encounter it and begin to produce the encoded factor (Fang *et al*., 1996; Martin, 1997). The transfected fibroblasts then serve as local bioreactors, producing the encoded gene product at physiological levels in the wound site. This factor can, in turn, influence the course of events at the wound site (Fang *et al*., 1996). The ability of GAMs to transfect wound healing fibroblasts has opened the technology to application in a wide range of tissues, including bone (Bonadio *et al*., 1999; Fang *et al*., 1996; Patil *et al*., 2000), skin (Chandler *et al*., 2000; Shea *et al*., 1999), arteries (Klugherz *et al*., 2000), cardiac and skeletal muscle (Labhasetwar *et al*., 1998), tendon (Zhu *et al*., 1994), and cartilage (Samuel *et al*., 2002). The application of GAM technology in bone regeneration, however, will serve as the focus for the current discussion.

B. Wound Healing and Bone Regeneration

The wound healing response involves a complex cascade of events, yet is a conserved process between tissues and among mammals (Martin, 1997). A wide variety of cells participate in wound healing, including platelets, lymphocytes, macrophages, fibroblasts, endothelial cells, and various progenitor cells (Bonadio, 2000). The migration, proliferation, and differentiation of these cells are coordinated through the local action of cytokines and growth factors. The complex signal cascade and subsequent cellular response depends upon the nature of the tissue injury. The initial response to acute injury typically involves hemostasis and clearing of cellular debris from the site as part of an acute inflammatory response (Bonadio, 2000; Park and Lakes, 1992). Subsequently, granulation tissue appears from which either a scar is formed or tissue regeneration occurs. Tissue engineering strategies seek to guide the wound healing process toward the path of tissue regeneration.

The regenerative capacity of bone is robust and effective at addressing wounds under normal conditions. A proportion of fractures, however, present conditions that are not conducive to regeneration and place the fracture at high risk for nonunion or delayed union. For example, fractures located at sites of marginal vascularity and those associated with a large area of bone loss repair with difficulty if at all. As a result, a great deal of effort has been invested in the development of treatment methods for fractures and defects at risk of nonunion, as they would not likely heal

unaided. The treatment options range from fixation of the fracture coupled with pain management to attempts to augment bone regeneration.

The surgical transfer of autologous bone tissue to the defect site is the "gold standard" for augmentation of bone regeneration. The procedure is generally successful, however, the amount of autologous bone tissue available for transfer is limited, and often not of the desired shape. Additionally, the recruitment of distal bone from the patient requires the introduction of a second defect and creates a risk of donor site morbidity. The employment of allogenic bone tissue provides an alternative method to autografts. Although allograft material is more plentiful than autologous bone for grafting, processing of the allogenic tissue limits its osteoinductive properties and does not eliminate the risk of pathogen transmission.

C. Bone Tissue Engineering

The limitations associated with bone grafting techniques have led researchers to seek additional methods to augment bone repair. As a result, tissue engineering strategies have been developed to enhance bone formation in large defects. Such methods generally involve the employment of biocompatible materials with osteoinductive properties. Three general tissue engineering strategies for bone exist: (1) implantation of a scaffold that is conducive to bone tissue infiltration, (2) inclusion of bioactive molecules within a conductive scaffold, and (3) *in vitro* seeding of cells on a conductive scaffold prior to implantation (Murphy and Mooney, 1999). In some cases, combinations of the various strategies are employed to regenerate bone tissue.

1. Bioactive Factor Delivery

The approach of bioactive factor delivery for bone tissue engineering is of particular interest for the current discussion. The wound repair cascade in bone involves the generation of numerous factors, which influence and direct cellular migration, proliferation, and differentiation. Isolation of these factors and identification of their specific roles in the regeneration response has led to their implementation in bone tissue engineering scaffolds. Indeed, numerous studies have shown the *in vivo* osteoinductive potential of various recombinant growth factors including bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β), and insulin-like growth factor (IGF) [see Babensee *et al.* (2000) and Linkhart *et al.* (1996) for a review]. The limitations associated with the direct delivery of growth factors and cytokines has led to the exploration of

growth factor delivery via gene therapy. Although many methods of gene therapy exist, the delivery of uncomplexed, nonviral plasmid DNA is of particular interest in the current discussion.

2. GAMs and Bone: Small Animals

The first study to investigate the feasibility of GAMs for bone repair utilized a rat model (Fang *et al.*, 1996). In that study, collagen sponge GAMs were introduced into 5-mm segmental defects in rat femurs with external fixation. The first portion of the study involved the implantation of GAMs containing plasmids for the marker genes β-galactasidase and luciferase in order to assess the *in vivo* transfection of wound healing cells. The marker gene GAMs were implanted into 23 animals at various dosages. The study demonstrated the infiltration of fibroblasts into the GAM, uptake of the plasmid DNA by these cells, and functional expression of the encoded markers. The remainder of the study focused on the ability of GAMs containing plasmid genes encoding osteoinductive factors to augment bone regeneration. GAMs in this portion of the study contained either a BMP-4 plasmid or a plasmid encoding for the first 34 amino acids of parathyroid hormone alone, or both plasmids together. Each GAM resulted in new bone filling the gap. Interestingly, the GAMs containing both plasmids, which act synergistically *in vitro*, led to a faster regeneration response than either plasmid alone. It is important to note that control defects containing collagen matrices alone or collagen matrices with marker gene plasmids demonstrated no new bone formation. This work was the first to demonstrate that bone formation is augmented through the *in vivo* delivery of osteoinductive plasmid genes from GAMs. Additionally, this study demonstrated the feasibility of delivering two plasmid genes at once from a GAM to elicit a synergistic biological effect.

3. GAMs and Bone: Larger Animals

Another study was conducted to investigate the potential for GAM technology to be scaled-up to augment bone regeneration in larger animals (Bonadio *et al.*, 1999). Two canine models were used in this study. In the first model, GAMs were implanted into 8-mm diameter by 8-mm deep cylindrical defects drilled into the distal femurs and proximal tibias of large mongrel dogs. GAMs in the first portion of the study contained either a plasmid for β-galactasidase or no plasmid at all (control), in order to assess *in vivo* transfection and functional protein expression. The control defects were negative for β-galactasidase staining, whereas the defects receiving plasmid GAMs were positive. The authors estimated that the staining marked 30–50% of the available granulation tissue cells. Further, based

upon morphological criteria, the authors found most of the transfected cells to be fibroblasts.

A final portion of the study utilized the second model to examine the effects of plasmid dose and defect size on the regeneration of bone. A beagle tibia critical defect model (2-cm) with external fixation was used in this portion of the study. Collagen GAMs were formulated with and without (control) the inclusion of the plasmid for $hPTH_{1\text{-}34}$. Bone regeneration was not induced in defects receiving doses of 1.0–20.0 mg of plasmid. Defects receiving doses of 40.0 and 100.0 mg of plasmid, however, demonstrated significant bone formation relative to controls over the course of 12 weeks, yet no complete filling of the gap was observed. Smaller defects (1.6 and 1.0 cm) receiving GAMs with 100.0 mg of plasmid demonstrated new bone formation and complete filling of the gap over the course of six months. Control defects in all cases produced little or no new bone. This study demonstrated that bone regeneration can be substantially and reproducibly augmented in large defects through the use of GAMs. Further, the bone formation appeared to be dose dependent and to follow a predictable time course.

4. *GAMs and Bone: Summary*

These initial studies demonstrated the feasibility of nonviral DNA delivery via a porous, degradable matrix (GAM) for the regeneration of bone in small and large animals. Although these studies demonstrated functional results in various bone defect models, they have not examined in detail dose response or transfection efficiency. Additionally, the GAMs in these studies were fabricated using collagen sponges. It has been demonstrated, however, that other materials may be used as matrix carriers of plasmid DNA, including alginate (Ho and Neufeld, 2001; Quong *et al.*, 1996), poly(ethylene vinyl *co*-acetate) (Jong *et al.*, 1997), poly(lactide-*co*-glycolide) (PLGA) (Klugherz *et al.*, 2000; Shea *et al.*, 1999), and poly(vinyl alcohol) (Chandler *et al.*, 2000). Indeed, it has been proposed that GAMs may be optimized through the choice of the matrix material and the choice of the gene or genes required to propel the desired biological effect (Goldstein, 2000).

D. Release of DNA from Scaffolds

Scaffolds have also been investigated for the controlled release of plasmid DNA rather than holding the DNA *in situ* as with GAMs. Fukunaka *et al.* (2002) investigated the controlled release of plasmid DNA

from cationized gelatin hydrogels. They demonstrated that the release was controlled by the enzymatic degradation of the gelatin, which could be controlled by manipulating the degree of crosslinking or the water content of the gels. Another group demonstrated *in vivo* expression and controlled release of DNA from a collagen minipellet (Ochiya *et al.*, 1999). Park *et al.*, (2002) investigated the controlled release of plasmid DNA from mucoadhesive poloxamers (poly(carbophil) or poly(ethylene glycol)) in intranasal applications. The controlled release of plasmid DNA from nondegradable EVAc matrices was demonstrated by Jong *et al.*, (1997). An additional development involved the incorporation of plasmid containing collagen within the pores of a PVA matrix for tissue engineering to regulate angiogenesis (Kyriakides *et al.*, 2001). Regardless of the material of choice, polymeric scaffolds have been shown to be effective in the controlled delivery of plasmid DNA, so as to guide tissue formation in tissue engineering applications.

V. Conclusion

The concept of gene therapy has evolved in recent years to include the application of gene delivery to DNA vaccinations and guided tissue regeneration. Although several methods of gene therapy exist, the use of polymeric biomaterials for nonviral gene therapy has been a major area of focus in recent years. Methods have been developed for the controlled delivery of plasmid DNA from polymer nano- and microspheres as well as from tissue engineering scaffolds. Entrapment of DNA within polymeric carriers has been shown to enhance DNA stability, improve bioavailability, and promote cellular uptake. However, further investigation is warranted to improve the transfection efficiency and cell or tissue targeting.

VI. Abbreviations

DOTAP	1,2-dioleoyl-1,3-trimethylammonio-propane
BMP	bone morphogeneic protein
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
DDAB	dimethyl dioctadecyl ammonium bromide
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraacetic acid
$hPTH_{1-34}$	first 34 amino acids of human parathyroid hormone
FDA	Food and Drug Administration

GAM	gene activated matrix
IGF	insulin-like growth factor
PBS	phosphate buffered saline
pDNA	plasmid DNA
PAMAM	poly(amidoamine)
PLGA	poly(D,L-lactic-*co*-glycolic) acid
PEG	poly(ethylene glycol)
PEI	poly(ethylenimine)
PLA	poly(L-lactic acid)
PLL	poly(L-lysine)
PVP	poly(vinylpyrrolidone)
RNA	ribonucleic acid
SCID	severe combined immunodeficiency
TGF-β	transforming growth factor β
TFE	trifluoroethanol

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