Formulating Poly(Lactide-co-Glycolide) Particles for Plasmid DNA Delivery

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ABSTRACT: Biodegradable poly(lactide-co-glycolide) (PLGA) particles have shown significant potential for sustained and targeted delivery of several pharmaceutical agents, including plasmid DNA (pDNA). Here, we survey current approaches to PLGA particle preparation for pDNA delivery and discuss recent progress on optimizing formulation development. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:2448–2461, 2008

Keywords: DNA delivery; formulation; gene delivery; microparticles; plasmid DNA; poly(lactide-co-glycolide) (PLGA); polymeric drug delivery systems

INTRODUCTION

In the field of gene therapy, viral vectors have significantly higher transfection efficiencies than nonviral vectors.1,2 Viral vectors, however, have several drawbacks. These include the requirement of cell mitosis for most retroviruses, immunogenicity of adenoviruses, safety concerns with HIV-like viruses and packaging constraints of adeno-associated viruses (AAV). Promising results with some viral vectors such as AAV have progressed into human clinical trials, but the long-term safety concerns of viral vectors remain and therapeutic levels of expression remain transient in the clinic.3–6 This is highlighted by the recent (July 2007) death of a participant in a gene therapy trial a few days after the intra-articular injection of an AAV vector. In contrast, nonviral vectors display substantially reduced immunogenicity. Advantages to nonviral vectors include ease of scale-up, storage stability, and improved quality control.7–9 The most promising nonviral vectors have been liposomes and several classes of cationic polymers that have been subject to extensive review elsewhere.10–12 However, limitations of liposomes and cationic polymers such as polyethyleneimine (PEI) include poor storage stability and high toxicity, respectively. In addition, the efficacy of nonviral gene delivery methods is still far below that of viral methods. Rapid fragmentation and elimination of free pDNA is observed when injected intravenously in mice. When pDNA is delivered using a nonviral vector, the expression of genes is transient thus necessitating readministration of the pDNA.13–15 These limitations in nonviral vectors have prompted the development of novel delivery systems capable of protecting pDNA, localizing its delivery and offering sustained delivery.13

A promising polymer for providing sustained pDNA delivery is poly(lactic-co-glycolic acid) (PLGA, Fig. 1), which has a demonstrated FDA
approved track record as a vehicle for drug and protein delivery.\textsuperscript{16–18} In addition, therapeutic agents, either entrapped, adsorbed or chemically coupled onto a PLGA matrix, have shown rapid endo-lysosomal escape and sustained intracytoplasmic delivery.\textsuperscript{17} Biodegradable PLGA particles are biocompatible and have the capacity to protect pDNA from nuclease degradation and increase pDNA stability.\textsuperscript{19–21}

PLGA particles typically less than 10 µm in size are efficiently phagocytosed by professional antigen presenting cells (APCs). PLGA particles, therefore, have significant potential for immunization applications.\textsuperscript{22,23} For example, intramuscular immunization of p55 Gag plasmid adsorbed on PLGA/cetyltrimethylammoniumbromide (CTAB) particles induces potent antibody and cytotoxic T lymphocyte responses. The particles showed a 250-fold increase in antibody response at higher DNA doses and more rapid and complete seroconversion, at the lower doses, compared to other adjuvants, including cationic liposomes.\textsuperscript{24,25}

In a study on the persistence and distribution of pDNA after parenteral delivery, pDNA encapsulated PLGA particles (average 5 µm) were injected into mice by a number of different routes of administration. Primary deposition occurred at the site of injection following intramuscular (IM) and subcutaneous (SQ) injection.\textsuperscript{26} In contrast, intravascular injection (IV) leads to a higher number of transfected APCs and greater accumulation in the lymphoid organs and the reticuloendothelial system. IM administration results in faster clearance of the encapsulated pDNA, with detection up to 60 days postinjection, while pDNA is detected up to 100 days post SQ injections.\textsuperscript{26}

The mechanism of cellular uptake of PLGA particles is size dependent. Nanoscale particles are internalized by endocytosis.\textsuperscript{27} For example, endocytosis of PLGA particles by human arterial smooth muscle cells takes place via nonspecific internalization through clathrin vesicles or by fluid-phase pinocytosis.\textsuperscript{28} After their uptake into early endosomes, the PLGA particles are transferred to secondary endosomes/lysosomes, where destabilization of the membrane takes place at the point of contact with PLGA.\textsuperscript{28,29} Particle release from the endosome is also achieved because polymeric delivery systems cause swelling of the endo-lysosomal vesicles and their rupture, allowing for escape into the cytoplasm.\textsuperscript{30,31}

Once the particles have escaped the endo-lysosomal compartment, the slow release of the encapsulated pDNA predominantly occurs in the cytosol.\textsuperscript{28,32} For example, in NIH 3T3 cells, pDNA loaded particles are mainly detected in the cytoplasm of transfected cells, while the released pDNA is detected around and inside the nucleus.\textsuperscript{27} Sustained pDNA delivery using PLGA particles has shown significant potential in transfecting human arterial smooth muscles\textsuperscript{28,33} and airway epithelia.\textsuperscript{34} When PLGA particles are compared to conventional transfection reagents such as FuGENE\textsuperscript{TM}, initially lower transfection efficiency is obtained from the PLGA particles. An exponential decline in transfection level is noticed for the FuGENE\textsuperscript{TM} transfection agent, whereas sustained levels are generated from the particles.\textsuperscript{33} In vitro transfection of 293 cell lines with human placental alkaline phosphatase-pDNA loaded particles shows significantly higher expression of alkaline phosphatase compared to naked pDNA.\textsuperscript{27} In vivo assessment of alkaline phosphatase expression, after IM injection into rat tibialis muscle, results in higher expression of naked pDNA compared to particles at 3 and 7 days.\textsuperscript{27} However, at 28 days, pDNA loaded particles produced higher levels of alkaline phosphatase activity.

The lack of toxicity of PLGA particles and the burst release associated with early availability of DNA followed by more sustained release represent important factors in prolonging the time span of transgene expression.\textsuperscript{25} However, a significant hurdle in the development of PLGA delivery systems has been the difficulty of entrapping hydrophilic DNA type molecules within the hydrophobic PLGA. Methods used to entrap DNA into PLGA particles include spray-drying and oil-in-water solvent evaporation methods.\textsuperscript{20,35,36} The DNA can become damaged during the encapsulation procedure. Additionally, during degradation, the encapsulated DNA can become inactivated as a result of the acidic environment created by
the accumulation of oligomers within the particles.\textsuperscript{20,35}

This review focuses on the approaches that have been investigated to improve the overall biological activity and delivery of encapsulated pDNA including optimizing formulation parameters such as the molecular composition of PLGA, pH, surfactant type and concentration, and use of cationic excipients to promote transgene expression and to protect DNA during the harsh conditions required for particle formation.

**CURRENT APPROACHES AND LIMITATIONS TO PLGA PDNA PARTICLE FORMULATION METHODS**

PLGA particles can be prepared by several methods, including spray-drying, emulsion/evaporation, double-emulsion/evaporation, salting out, emulsification-diffusion, solvent displacement/nanoprecipitation, and emulsion-diffusion-evaporation methods.\textsuperscript{37} The method selected will be dependent on the size and loading efficiency required. For genetic immunization applications, particles prepared in the 1–10 $\mu$m region show preferential uptake by target APCs such as macrophages or dendritic cells.\textsuperscript{37} In contrast, if the targets are nonphagocytic cells, then particles need to be prepared in size ranges below 300 nm to allow for efficient clathrin pit-mediated endocytosis.\textsuperscript{38} The choice of formulation methodology has a direct impact on the target size of the PLGA particles. Solvent displacement or nanoprecipitation are techniques that more readily allow for PLGA particles to be prepared at the sub-300 nm size range.\textsuperscript{17,39–41} The physico-chemical characteristics of the PLGA particles are dependent on the method employed for encapsulation. Table 1 summarizes some of the current approaches to formulating PLGA pDNA particles and the corresponding particle size and entrapment efficiencies obtained.

The double-emulsion solvent-evaporation technique is one of the most common methods used for encapsulating pDNA. The method involves the use of three phases: (1) an inner water phase containing the DNA to be incorporated, (2) an intermediate organic phase consisting of a polymer/organic solvent solution, and (3) an outer water phase containing an emulsifying agent.\textsuperscript{20,33,42,43} The organic solvent is removed and the particles are isolated and dried. Using the double-emulsion solvent evaporation method for preparing PLGA particles, we have shown that PLGA particle size can be carefully controlled by varying the stirring rate and the surfactant concentration. Lowering the poly(vinyl alcohol) (PVA) surfactant concentration from 9% to 0.1% (w/w) resulted in the average particle size increasing from 1.6 to 9.7 $\mu$m at 3400 rpm stirring speed. Increasing the stirring speed from 3400 to 10000 rpm decreased the average particle size from 9.7 to 1.3 $\mu$m at a fixed surfactant concentration of 0.1% (w/w). PLGA particles could be prepared at sizes below 300 nm when the stirring rate was at 10000 rpm and a 9% PVA surfactant concentration was used.\textsuperscript{44,45} The double emulsion method tends to produce solid particles with very small pDNA containing “pockets” well dispersed throughout the matrix. The method is, however, usually associated with low pDNA loading and encapsulation efficiencies.\textsuperscript{43}

PLGA particle preparation using methods such as double-emulsion solvent-evaporation can cause nicking of the pDNA during the formulation process. For instance, shear forces generated by homogenization, ultrasonic radiation, and high

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**Table 1. Summary of Recent Approaches to Formulating PLGA pDNA Particles and Corresponding Particle Size and Entrapment Efficiencies Obtained**

<table>
<thead>
<tr>
<th>Method</th>
<th>Particle Size</th>
<th>Encapsulation Efficiency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray drying</td>
<td>&lt;10 $\mu$m</td>
<td>57 ±13</td>
<td>23</td>
</tr>
<tr>
<td>Spontaneous emulsification solvent diffusion (SESD)</td>
<td>200–300 nm</td>
<td>NA</td>
<td>41</td>
</tr>
<tr>
<td>Double-emulsion solvent-evaporation</td>
<td>0.1–10 $\mu$m</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>Double-emulsion solvent-evaporation</td>
<td>72 nm</td>
<td>91.25</td>
<td>100</td>
</tr>
<tr>
<td>Modified solvent evaporation</td>
<td>1.0 $\mu$m</td>
<td>92.0</td>
<td>24</td>
</tr>
<tr>
<td>DNA-organic phase self-emulsification (DOPSM)</td>
<td>1.0–2.0 $\mu$m</td>
<td>76</td>
<td>55</td>
</tr>
<tr>
<td>Emulsion-diffusion-evaporation</td>
<td>181 nm</td>
<td>NA</td>
<td>80</td>
</tr>
<tr>
<td>Cryopreparation</td>
<td>4.8 $\mu$m</td>
<td>89</td>
<td>47</td>
</tr>
<tr>
<td>Modified phase inversion/solvent diffusion method</td>
<td>&lt;100 nm</td>
<td>40</td>
<td>66</td>
</tr>
</tbody>
</table>
interfacial tension at the o/w interface can produce deleterious effects on supercoiled pDNA, resulting in conversion into open circular or linear forms. Supercoiled pDNA yields markedly higher levels of expression than linear or open circular pDNA. It is worth noting that some reports of bioactivities of extracted encapsulated plasmid with a decreased supercoiled fraction show they were comparable to those of unencapsulated DNA. This is consistent with studies in which transfectivity of the linear, open circular or nicked molecule has been shown to be nearly equal to or slightly lower than that of the supercoiled DNA.

Ultrasonic dispersion of pDNA and double stranded DNA in water causes time dependent damage resulting in breakage into single strands or oligonucleotide formation. Ultrasonication is believed to cause this disruption via the intense mechanical pressures placed on the sonicated medium. As the gaseous cavitation bubbles collapse, it causes the liquid to become intensely agitated, resulting in breaks in the double-stranded DNA. At the same time, chemically reactive species such as \( \text{H}_2\text{O}_2 \) and free radicals are produced, which can also harm the sonicated material. Sonication using a probe can result in the most harmful effects.

Although sonication is reported to have deleterious effects on DNA, the presence of PLGA during fabrication provides a degree of protection to the DNA at the w/o interface in comparison to free DNA. As a result, moderate use of sonication to increase the DNA loading of PLGA particles is possible. An alternative method for preventing degradation due to ultrasonication can be achieved by adding physiological concentration of ions, such as phosphate buffered saline (PBS) or 0.1 M NaHCO\(_3\), the use of which will be discussed in greater detail in “The Use of Excipients” Section. Reducing the time of sonication is the most effective method of preserving the integrity of double-stranded DNA but this also results in lower encapsulation efficiencies and a higher burst effect. This is a result of reduced dispersion during the o/w emulsion formation.

As mentioned earlier, the mechanical or shear forces generated during many of the fabrication steps are believed to denature or inactivate pDNA. The spontaneous emulsification solvent diffusion method (SESD) is a method for preparation of PLGA nanoparticles using minimal mechanical force. In this method, PLGA is dissolved in an organic phase consisting of two organic solvents, a poorly water-miscible solvent and a freely water-miscible solvent, such as acetone. This solution is slowly poured into the emulsifier containing the stirred aqueous phase. Acetone quickly diffuses into the aqueous phase, and the resulting particles formed are solidified by solvent evaporation (e.g., methylene chloride). In this method, high shear forces are not required because the acetone rapidly diffuses into the aqueous phase causing a decrease in the interfacial tension between the organic and aqueous phase thus forming small droplets. The SESD method is restricted to applications using relatively small particles, ranging from 200 to 300 nm. A process of microencapsulation called DNA-organic phase self-emulsification (DOPSM), is a modification of the SESD method. The method is based on the concept that hydrophobic drugs have higher PLGA encapsulation efficiencies. Hydrophobic complexes between cationic lipids, such as cetyl-dimethylammonium bromide (CDAB), and plasmid DNA (pDNA) are prepared in the organic phase. The DNA-containing organic phase is mixed with PLGA in acetone, and the resulting solution dropped into a 1% aqueous PVA solution. This results in spherical particles that are 1.0–2.0 \( \mu \text{m} \) in size.

An alternative approach to minimizing shear stress during homogenization and preserving the supercoiled structure is to utilize a cryopreparation modification of the double emulsion method. This technique is able to increase overall DNA encapsulation to approximately 89%. In this process, the temperature of the DNA-containing primary emulsion is lowered below the freezing point of the aqueous inner phase resulting in a solid particle suspension prior to homogenization. A similar cryo-based approach has been developed in which the two phase mixtures of polymer in methylene chloride solution and DNA-lecithin suspensions are frozen in liquid nitrogen immediately after vortexing, followed by lyophilization and then resuspension in methylene chloride. The particle suspension is sonicated and then introduced into a petroleum oil bath. Finally the particles are filtered and lyophilized.

The choice of freeze drying or spray drying after emulsification can have a significant impact on the biological activity. Particles produced by emulsification/spray-drying exhibit more diversity in shape and size than those produced by emulsification/freeze-drying. These particles also
show higher pDNA encapsulation efficiencies than particles produced by emulsification/freeze-drying. Cell transfection assays have confirmed the biological activity of encapsulated DNA is significantly higher for spray dried particles.58 While these methodologies have generated significant improvements in pDNA entrapment in PLGA particles, optimization of several other formulation parameters can also significantly affect pDNA entrapment and functional release.

**OPTIMIZING PLGA PDNA PARTICLE FORMULATION PARAMETERS**

**Molecular Weight and Lactide: Glycolide Ratio of PLGA**

The first step in preparing PLGA pDNA particles is selecting the appropriate molecular weight (MW) and grade of PLGA. Polyglycolic acid (PGA), polylactic acid (PLA), and their copolymers poly(lactic-co-glycolic acid) (PLGA) are the most commonly used family of biodegradable polymers. Random PLGA copolymers with different ratios of lactide (LA) and glycolide (GA) exhibit different degradation rates, and can thus be tailor-made for specific applications.59–63 Amorphous polymers are more hygroscopic than crystalline polymers. Copolymers are generally more amorphous than their homo-polymers and become increasingly susceptible to hydrolysis as the two monomer contents reach equivalence.64,65 PLGA with different compositions, represented by the ratio of lactide to glycolide, have been investigated for pDNA delivery. PLA particles containing pDNA encoding for luciferase show poor release and lower transfection compared to 50:50 (LA:GA) and 75:25 (LA:GA) PLGA copolymers.33 This can be explained by the high hydrophobicity of PLA which reduces DNA loading efficiency and delays hydrolysis and DNA release.33 Most DNA entrapment studies in the literature use lactide:glycolide ratios of either 50:50 or 75:25 PLGA.20,33,51,66 Due to the hydrophilic nature of DNA, the more hydrophilic PLGA polymer grades, such as those with a higher fraction of glycolide monomer and those having uncapped end groups, show higher DNA loading.23

The MW of PLGA plays a major role in the efficiency of DNA encapsulation and its subsequent release in vitro. Higher encapsulation efficiencies are observed using higher MW copolymers. For example, increasing the MW of PLGA (50:50) from 12 to 143 kDa results in particles with smaller sizes, less negative zeta potentials, greater percentages of DNA release, as well as enhanced transfection efficiencies.67 In addition, increasing the MW of PLGA from 12.5 to 50 kDa results in a twofold increase in DNA loading using the double emulsion method.20,33,43 This is attributed to the higher viscosity of the higher MW polymer solutions and the shorter time needed for the particles to solidify.46 Increasing the MW of the polymer solution also improves its emulsifying properties.33 As a result, higher MW PLGAs have higher supercoiled DNA contents and improved protection against DNase I degradation. In addition, supercoiled DNA is released more slowly from the particles.20,46,68

In a comparison of the release of pLuc pDNA from particles prepared using 50:50 PLGA with MW either 9 or 25 kDa,43 nearly 50% of the pDNA is released immediately from the low MW particles, and complete release occurs within 5 days. The high MW particles, in comparison, show no initial release and most of the pDNA release occurs between 4 and 6 weeks. Plasmid DNA release is the result of hydrolysis, bulk erosion, and formation of a pore network in microparticles. Therefore, the more rapid degradation of the 9 kDa PLGA results in complete pDNA release over a shorter time-frame. Similar rapid release patterns are observed when the polymer concentration parameter during fabrication is decreased resulting in less dense, more hydrolytically unstable particles.

**Effect of Surfactant**

Both PVA and polyvinyl pyrrolidone (PVP) have been commonly used as emulsion stabilizers in the process of preparation of PLGA particles. PVA is a very safe polymer with low acute oral toxicity (LD50 values are in the range of 15–20 g/kg) and poor absorption from the gastrointestinal tract. In addition, PVA is not mutagenic or clastogenic.69 In a range of 1–7% (w/v) PVA solution used as stabilizer, 4% (w/v) PVA solution yielded the maximum pDNA loading.46 Increasing the surfactant concentration results in higher viscosity solutions that produce smaller particle sizes, due to the enhanced emulsification properties. This occurs because the precipitation of surfactant molecules on the surface of the o/w droplets prevents coalescence during solvent removal and...
polymer solidification. It also reduces the surface energy during the formation of the secondary emulsion. In contrast to this observation, Hsu et al. have shown that smaller particles made with higher PVA concentrations contained more DNA than larger particles made with the lower PVA concentrations, but with a similar ratio of supercoiled to nicked DNA. These results were consistent with the observations of Prabha and Labhasetwar, in which the loading efficiency of particles formulated using 0.5% (w/v) PVA solution was lower compared to 2% (w/v) and 5% (w/v) PVA solutions. At a 4% (w/v) surfactant concentration both PVA and PVP provided a degree of protection to DNA from nucleases. However, the loading efficiency obtained using PVP was almost half of that compared to using PVA. Some residual PVA remains associated with PLGA on the surface of the particles thus changing the surface characteristics, and reducing cellular uptake. The PVA used as a surfactant in PLGA particle preparations is normally a copolymer of poly(vinyl acetate) and poly(vinyl alcohol). The degree of hydrolyzation of PVA has a significant impact on the DNA loading and the level of surface-associated PVA. In addition, reducing the PVA MW leads to smaller size particles and higher DNA loading.

Because the surfactant used in the PLGA particle can have a significant impact on the resulting surface characteristics, the surfactant can also have a pronounced effect on the cellular transfection of the particles by affecting the endolysosomal escape. In studies examining the effect of PVA concentration on transfection efficiency, it was shown that both 2% and 5% (w/v) PVA solutions resulted in the same DNA loading but that the 2% PVA solution demonstrated 20-fold higher transfection and elevated cellular uptake. This was linked to changes in the surface charge. The influence of the PVA concentration was further highlighted when a 0.5% PVA solution resulted in a lower DNA loading than a 5% PVA solution but generated higher transfection efficiencies. We have also identified that 0.5% PVA is the optimum concentration of surfactant for generating the strongest transfection efficiencies in PLGA PEI pDNA particles. In addition, a higher degree of hydrolyzation (89% compared to 80%) and a higher MW PVA (83–146 kDa compared to 13–23 kDa) were both shown to enhance transfection efficiencies.

**Effect of DNA Type and Loading Rate**

The type of DNA loaded determines its stability upon encapsulation. For example, when the spray-drying method was used for encapsulation, Calf thymus DNA was more stable than pDNA which was in turn more stable than Salmon DNA. In addition to stability issues, the type of DNA can also affect the release profiles. For example, pDNA displays the highest burst effect, possibly due its supercoiled nature, which may be more difficult to integrate between polymer molecules. The burst release of pDNA is also related to the encapsulation efficiency. Particles with a theoretical loading of 1% and 2% yielded loading efficiencies of 53.0% and 65.9%, respectively, which were shown to have a burst effect in the first 10 min of about 40.4% and 76.6%, respectively. The rest of the pDNA was released slowly over 12 weeks.

Increasing the nominal loading of DNA in the spray-drying method led to increased stability but reduced encapsulation efficiency, whereas the aqueous phase volume had no effect on these characteristics. Increasing the loading of the microparticles from 0.3% to 0.9% resulted in both increased supercoiled DNA content and increased protection against DNase. However, a high concentration of pDNA in the organic phase solution can encourage agglomeration of microparticles due to DNA–DNA interactions on the surface of microparticles.

**Effect of pH**

Changes in the structure of the pDNA can be induced by the acidic micro-environment of degrading PLGA particles. The nature of the structural change in pDNA is related to the time-point at which it is released. The highest proportion of supercoiled pDNA is released in the initial burst. Greater proportions of nicked DNA and lower proportions of supercoiled pDNA are released at later time-points. This is presumably due to pDNA exposure to the acidic microenvironments of the PLGA particle that build up over time.

PLGA, regardless of the grade or molecular composition of the polymer used, will decrease the pH of the aqueous solution during fabrication. Low pH during the fabrication process induces degradation of DNA by shifting the equilibrium between double stranded and single stranded DNA toward the single stranded. This causes
rapid loss of transfectivity when pH \leq 3.35,71
In addition, depurination reactions take place at low pH, causing irreversible damage to the DNA.23,35,72 Using excipients such as NaHCO₃ helps to buffer against these changes in pH. Other excipients such as cationic agents can condense pDNA providing significant protection to the pDNA during entrapment.

The Use of Excipients

Excipients used for formulating DNA into PLGA particles can be broadly categorized as: (a) cationic agents that condense the DNA to protect it from the harsh formulation processes and facilitate uptake and expression of the DNA and (b) buffering agents that protect the pDNA against the microacidic environment of degrading PLGA particles. There are excipients that do not fit into these two categories such as the use of chloroquine to avoid enzymatic digestion by lysosomal enzymes. Chloroquine, is an endosomalytic agent, that also aids in transporting the released plasmid to the nucleus after being released in the cytosol. Another example is the incorporation of acetylated bovine serum albumin (BSA) in the DNA solution during the double emulsion technique which is thought to facilitate the release of DNA.33,57

Table 2 shows the chemical structure of cationic excipients that are commonly used for pDNA formulation in PLGA particles. These include CTAB, CDAB, dimethyl dioctadecyl ammonium bromide (DDA), 1,2-dioleoyl-1,3-trimethylammonio-propane (DOTAP), cationic DDAB, poly(L-lysine) (PLL), chitosan and PEI. Polymers such as chitosan and PEI are often used as standalone nonviral vectors for pDNA delivery. For example, the cationic polymer PEI comes in two forms: linear and branched. The repeat unit of the polymer is two carbon atoms followed by a nitrogen atom. In the branched form, PEI has 1°, 2°, and 3° amines that can be protonated, allowing PEI to serve as a buffer through a wide pH range. The positive charge of the PEI results in effective binding to the negatively charged pDNA, and this condensation protects the pDNA from digestion in serum and as the complex enters cells. Once in the endosomal compartment, PEI can act as a buffer or “proton sponge” to induce osmotic swelling and cause release from the endosome. This is necessary to avoid degradation of the pDNA when the endosome fuses with the lysosome. Previous studies have found that PEI/DNA complexes with higher buffering capacities give better transfection efficiencies.73

To investigate the effect of CTAB on adsorption efficiency and release of DNA, PLGA particles with increasing amounts of CTAB (0.2–3%, w/w) were prepared by controlling the washing cycles.25 Increasing the CTAB concentration, increased DNA loading efficiency. However, above 0.6% CTAB, the in vitro release decreases. For example, 100% loading is obtained using 1% CTAB, but minimal in vitro release is observed at 24 h.25 The size of the particles is unaffected, while the zeta potential increases.

PLGA particles containing PLL complexed pDNA have been prepared by the double-emulsion method.21,34,46 Particles with a size range of 3.1–3.5 \mu m are obtained, with a decrease in the negative surface charge.21 This results in PLL complexed release of DNA and delayed second phase release.74 A higher percentage of supercoiled pDNA and protection against endonuclease activity of DNAse 1 is observed for the PLL-complexed pDNA compared to uncomplexed pDNA.21 The release of pDNA-PLL complex from PLGA particles is also dependent on the stabilizer type and concentration and the highest release is found using 7% PVA.46 A significant burst effect was observed with these particles, likely due to the increased surface area associated with smaller particle sizes.

Blends of PLGA with PEI have been evaluated as gene delivery systems where PEI can be considered a cationic excipient.75 For example, particles containing both PEI and PLGA have been investigated as pulmonary gene delivery vehicles.76,77 The particles are prepared by adding 10% (w/v) PLGA in dichloromethane to 1% (w/v) PEI in acetone, followed by the addition of Tween-80 and acetone.76 The organic phase is poured into an aqueous phase of Poloxamer-188 and stirred. Loading of pDNA is performed by vortexing the pre-formed particles in the presence of an aqueous pDNA solution. Particles ranged in size between 207 and 231 nm, had a positive zeta potential and loading efficiencies as high as 99%. The cell viability could be improved by reducing the PEI-DNA ratio.76 When PLGA particles containing PEI are prepared by a diafiltration method that uses no surfactant,75 the optimum PLGA: PEI ratio for maximum pDNA adsorption was found to be 5:1. Nanometer scale particles are produced due to the reduced interfacial tension at the w/o interface in the presence of PEI,
particle aggregation tendency after lyophilization is also reduced. This has been attributed to the positive charge imparted on the particles which cause electrostatic repulsion between particles.\textsuperscript{75}

Transfection of HEK 293 cells by the PLGA/PEI particles, determined by β-galactosidase activity, had about 50% of the activity of PEI alone. However, enhanced cell viability was achieved.\textsuperscript{75}

\begin{table}
\centering
\caption{Chemical Structures of the Most Common Cationic Excipients Used in PLGA pDNA Particle Formulation}
\begin{tabular}{ll}
\hline
Excipient & Chemical Structure \\
\hline
CTAB & \includegraphics[width=0.5\textwidth]{ctab.png} \\
CDAB & \includegraphics[width=0.5\textwidth]{cdab.png} \\
DDA & \includegraphics[width=0.5\textwidth]{dda.png} \\
Chitosan & \includegraphics[width=0.5\textwidth]{chitosan.png} \\
PEI & \includegraphics[width=0.5\textwidth]{pei.png} \\
PLL & \includegraphics[width=0.5\textwidth]{pll.png} \\
\hline
\end{tabular}
\end{table}
PLGA particles can also be covalently surface functionalized with branched PEI for efficient delivery of surface-loaded pDNA to APCs.\textsuperscript{78} Conjugation of PEI is carried out using modified EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride)/NHS (N-hydroxysuccinimide) chemistry.\textsuperscript{78} Compared to the physical blending of PEI with PLGA, covalent conjugation of PEI reduces batch-to-batch variation in zeta potential, leading to a more reproducible loading of pDNA, imparts cationic surface charge with minimal PEI content and minimizes the free PEI-related cytotoxicity.\textsuperscript{78} We have recently compared formulation strategies for preparing PLGA PEI pDNA particles and evaluated them for buffering capacity, cell uptake, transfection efficiency, and toxicity. PLGA PEI pDNA particles were prepared by blending PLGA and PEI and then entrapping pDNA in the particles using the double emulsion water in oil in water solvent evaporation technique (PA). In a second approach, we prepared PEI-pDNA polyplexes and then entrapped them in PLGA particles using a double emulsion solvent evaporation method (PB). We then compared these two formulation approaches against PLGA particles with PEI conjugated to the surface using carbodiimide chemistry (PC). N:P ratios of 5 and 10 were selected for preparation of each group. Gel electrophoresis demonstrated that all PLGA particle formulations had strong pDNA binding capacity. An MTT assay showed that in vitro cytotoxicity of PLGA PEI particles was significantly lower than PEI alone. PLGA PEI particles mediated higher cellular uptake efficiency and consequently higher transgene expression than unmodified PLGA particles in COS7 and HEK293 cells. The formulation strategy (PB) of preparing PEI-pDNA polyplexes prior to entrapment in PLGA particles resulted in the highest pDNA loading. This was 2.5-fold higher than unmodified PLGA particles. The highest transfection efficiencies with this formulation approach were achieved using an N:P ratio of 5. This formulation strategy also produced 9-fold higher transfection efficiencies than unmodified PLGA pDNA particles in HEK293 cells and 1800-fold higher in COS-7 cells.\textsuperscript{70} In addition, we have also evaluated the potential of conjugating polyamidoamine (PAMAM) dendrimers of varying generations to the surface of PLGA particles. The buffering capacity and zeta-potential of the PLGA PAMAM particles increased with increasing generation level of the PAMAM dendrimer conjugated. Conjugation of the PAMAM dendrimer to the surface of the PLGA particle removed generation dependent cytotoxicity in HEK293 and COS7 cell lines. PLGA PAMAM pDNA particles displayed similar cytotoxicity profiles to unmodified PLGA pDNA particles in COS7 cells. A generation 3 PAMAM dendrimer conjugated to PLGA particles significantly increased transfection efficiencies in comparison to unmodified PLGA particles.\textsuperscript{79} Finally in terms of cationic excipients, complexation of pDNA with PLGA particles prepared using chitosan-PVA, and amphiphilic polymers, such as poloxamer and poloxamine as surfactants in the aqueous phase can also protect pDNA during entrapment.\textsuperscript{80,81}

Buffering agents reduce the effects of pH on DNA denaturation. Adding 0.1 M NaHCO\textsubscript{3} as a buffering agent was shown to prevent a shift in pH, which improves the encapsulation and stability of DNA.\textsuperscript{23} In this approach, the more hydrophilic PLGA polymers show increased protection against DNA degradation, which is probably due to more efficient exchange of fluids, which more readily removes the acidification by-products of PLGA.\textsuperscript{23} The approach is not however without its complications since changes in pH can induce variation in the encapsulation efficiency when cationic excipients are used to protect the pDNA.\textsuperscript{55} For example, when the pH of a 1% PVA solution in the DOPSM technique is 5, an encapsulation efficiency of 76% is obtained. This value is reduced to 63% when the pH is increased to 7. At high or low pH, the leakage of DNA to the PVA solution is lower. This is related to the dissociation constant of the pDNA/cationic lipid complex, which is an important factor in stabilization of the complex during the encapsulation process.\textsuperscript{55} In addition, DNA released after the initial burst loses its bioactivity and double-stranded structure. This is presumably the result of the acidic microclimate in PLGA particles following the rapid depletion of the water soluble low MW NaHCO\textsubscript{3}.\textsuperscript{35,82} Another approach to counter the acidic environment of degrading PLGA particles is to incorporate MgCO\textsubscript{3} to neutralize the acidic oligomers created during degradation. This approach would therefore have potential for protecting entrapped pDNA.\textsuperscript{83}

**Covalent Modification of PLGA**

Improving formulation properties of PLGA by covalent modification of the polymer backbone involves two primary approaches: (1) pegylation of...
the polymer to increase its hydrophilic properties in order to increase the loading efficiency of hydrophilic DNA and (2) cationic modification of the PLGA to provide the polymer backbone with greater capacity to condense the DNA molecules.

Monomethoxy(polyethylene glycol)-PLGA-monomethoxy(polyethylene glycol) (mPEG) is a modified PLGA with increased hydrophilicity. Encapsulation of pDNA with this polymer has been carried out using the double-emulsion solvent-evaporation technique and resulted in spherical particles with sizes of approximately 300 nm. A decrease in the supercoiled content is observed, although no fragmentation occurs. Increasing the monomethoxy(polyethylene glycol) (mPEG) content of the copolymer results in smaller particle sizes that are attributed to the enhanced emulsification properties of copolymers containing PEG and the hydrophilic nature of the mPEG component which leads to easier dispersion and improved affinity of the polymer to pDNA. Similar benefits have been observed with other PEO-type modifications of PLGA.

Amine-modified branched polyesters circumvent many of the disadvantages of encapsulation using unmodified PLGA, such as the need for high shear forces and the acidic environment created by polymer hydrolysis. Complexation between amine-modified polymer and DNA happens via electrostatic interactions and shear forces necessary for particle fabrication can be avoided by using a gentle solvent displacement method.

Diethylaminopropylamine (DEAPA) substituents on polyesters produces an acetone soluble polymer, which is advantageous for the encapsulation process because it allows solubilization of the pDNA in the acetone/water mixture by ionic interaction with the tertiary amine modification. The amphiphilic properties of this type of polymer produces nano sized particles with positive zeta potentials. Transfection efficiency is increased in direct proportion to the amine substitution and inversely with the length of PLGA chain, which may be due to the proton sponge effect of the polymer or the hydrogel effect that leads to swelling of the polymer.

Other recently developed amine modified polymers that condense pDNA using similar principles include amine-modified PVA backbones grafted with PLGA. Innovative approaches of combining the advantages of pegylation and cationic polymers include physically combining poly(ethylene glycol)-grafted chitosan with PLGA using a modified conventional emulsion solvent evaporation method.

CONCLUDING REMARKS

PLGA is a biocompatible, biodegradable, and FDA approved polymer that has significant potential for sustained delivery of functional pDNA. Limitations to PLGA particle delivery system development include exposing pDNA to the harsh processes and solvents used in fabricating micro- and nanoparticles. In this review, we have surveyed current formulation strategies that are used to improve the protection of the pDNA entrapped and delivered in PLGA particles. Significant progress has been made with advances in the knowledge of excipients, surfactants, and many other parameters. Other formulation parameters that were only briefly mentioned but are equally important include the ratio and nature of the organic phase and the polymer concentration used in the solvent phase. In addition, an area of increasingly active research is the combination of cell targeting strategies or nuclear localization signals with PLGA particles. PLGA pDNA particles have significant potential for the treatment of a variety of ailments from foot and mouth disease to allergies and cancer. Optimizing the formulation parameters described in this review or utilizing cationic excipients has potential to substantially improve the efficacy of PLGA particles further. For example, PLGA particles containing PEI have shown significant potential in genetic vaccination applications for treatment or prevention of a variety of diseases that include Listeria Monocytogenes, glaucoma, lymphoma, and prostate cancer. A relatively unexplored area of potential for PLGA particles is the capacity to coentrap pDNA and other drugs within the particle matrix for codelivery of multiple therapeutic agents. We have shown that codelivery of antigens and CpG DNA in PLGA microparticles is critical for enhancing antigen-specific immune responses. Other studies have shown that 5-FU and antisense EGFR (epidermal growth factor receptor) plasmids can be readily coloaded in biodegradable PLGA/O-carboxymethyl-chitosan (O-CMC) nanoparticles for potent anti-cancer activity. Future approaches to PLGA particle formulation will require a combinatorial approach that incorporates and optimizes all of the formulation parameters detailed in this review.

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