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ZEIN NANOSPHERES FOR GENE DELIVERY

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Particulates incorporating DNA provide for protection and sustained release of DNA, and thus are promising candidates for DNA delivery systems. Among the routes of administration for gene delivery, the oral route is perhaps the most appealing as it is associated with patient comfort and compliance and allows for targeting to intestinal targets for therapeutic and vaccination applications. With the goal of realizing the potential of an oral DNA delivery system, zein, a hydrophobic protein from corn that is biocompatible and degraded enzymatically, was investigated. This thesis describes the formulation of zein nanospheres encapsulating DNA by a coacervation technique and their characterization. Zein/DNA nanospheres ranged from 57.8 ± 3.9 nm to 396.8 ± 16.1 nm and from -21.8 ± 4.2 mV to -46.6 ± 1.6 mV for hydrodynamic diameter and zeta potential measured in water, respectively. Spheres formed at all ratios aggregated to some degree in PBS, with 20:1 and 40:1 zein:DNA spheres flocculating; aggregation was found to be dependent on salt concentration. DNA encapsulation efficiency was as high as 65.3 ± 1.9% with a maximum loading of 6.1 ± 0.2 mg DNA/g zein. DNA that was encapsulated and released retained its integrity. Release studies indicated that zein was degraded primary enzymatically with slow release of DNA in PBS, and faster release in pepsin containing media, and nearly instantaneous release in simulated intestinal fluid. Spheres demonstrated similar biocompatibility at high and low concentrations, and

showed cellular association and evidence of internalization. Possible improvements to this delivery system include increasing loading, improving stability against aggregation, increasing resistance to enzymatic degradation, and improving internalization, nuclear localization, and transfection. The spheres formed and characterized as described in this thesis show great potential for oral gene delivery, and with judicious modification should be rendered even more likely to be applied clinically in oral gene delivery as well as tissue engineering and intramuscular injection applications.

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List of Abbreviations

ANOVA	Analysis of variance
APCs	Antigen-presenting cells
BALB/c	Bagg Albino mice with genotype c/c at the color locus
Caco-2	Human colon carcinoma cells
CpG	Cytosine—phosphate—guanine
CTLs	Cytotoxic T lymphocytes
dDAVP	Desmopressin
ddH_2O	Double-distilled water
Der P	Dermatophgoides pteronyssinus
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DNases	Deoxyribonucleases
EtOH	Ethanol
FBS	Fetal bovine serum
FT-IR	Fourier transform infrared spectroscopy
GALT	Gut-associated lymphoid tissue
GI tract	Gastro-intestinal tract
HBsAg	Hepatitis-B-Virus surface antigen
HEK-293T	Human embryonic kidney cells
hGH	Human growth homone
HIV	Human immunodeficiency virus

HL-7702	Hepatic immortal cell line
HUVECs	Human umbilical vein endothelial cells
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin 10
M-cells	Microfold cells
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N/P ratio	Ratio of nitrogen to phosphorous atoms in a gene delivery complex
NLS	Nuclear localization signal
PBS	Phosphate buffered saline
PCL	Poly (ε-caprolactone)
pCMV/β-gal	Plasmid expressing beta-galactosidase with a cytomegalovirus promoter
PdI	Polydispersity index
pDNA	Plasmid DNA
pDsRed2-N1	Plasmid encoding for the red fluorescent protein, DsRed2
PEGylation	Covalent attachment of polyethylene glycol

PEI	Polyethyleneimine
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PS-K	Polysaccharide-K
PTH	Parathyroid hormone
PVP	Polyvinylpyrrolidone
SEM	Scanning electron microscopy
SEM	Standard error of the mean
SIF	Simulated intestinal fluid
SPI	Soy protein isolate
TE	Tris-EDTA buffer
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
USP	United States pharmacopeia

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Chapter 1

1.1 Introduction

While the application of nonviral gene delivery to gene therapy, tissue engineering, and DNA vaccination comes with numerous advantages, there are many hurdles that still must be overcome to achieve such therapies with clinical relevance. Gene therapy involves the correction of deleterious aberrant genes or supplementing these defects with genes expressing the correct product. Also, DNA vaccination modulates an immune response that can result in subsequent immunity when challenged by infection by transfecting cells with DNA that encodes an antigen, which is then produced by the cell. Because the oral route is the least invasive route and is associated with high patient compliance, it is appealing for both nonviral gene therapy and DNA vaccination. Nonviral gene delivery via the oral route for gene therapy and DNA vaccination has the potential to revolutionize the treatment and prevention of various ailments, by eliminating the need for administration by healthcare professionals and enhancing convenience to the patient. The feasibility of oral delivery has been demonstrated in numerous studies in animals. Barriers to efficient oral gene delivery, specifically the low pH in the stomach, degradation by enzymes in the stomach and intestine, and inefficient uptake in the intestine, have prevented the application of nonviral gene delivery. Therefore, there is a need to realize the potential of a novel material to overcome these obstacles. Zein, a protein from corn, is readily available and has physical properties which can be tailored to specific applications by modifications such as crosslinking, plasticizing, and copolymerization. It also has biological properties including enzymatic degradation and

biocompatibility of the protein and degradation products that make it well suited for the development of nonviral gene delivery systems specifically for the oral delivery of DNA.

1.2 Nonviral gene delivery

Gene delivery, the introduction of exogenous DNA to a cell, has applications in several areas. This thesis focuses on applications in gene therapy and DNA vaccination. To this end there are two main modes of gene delivery: viral and nonviral. While viral methods are effective, eliciting a high rate of transduction and long-term expression, there are associated safety concerns. The primary problems with viral vectors are their immunogenicity, lack of specificity and potential for insertional mutagenesis (Thomas, Ehrhardt, & Kay, 2003). The use of viral vectors for gene therapy in clinical trials has been tied to a death and the development of a leukaemia-like disorder in several subjects (Thomas, Ehrhardt, & Kay, 2003). There are also technical problems with viral vectors including limits to the size of the transgene that they can carry and issues related to their production (Gao, Kim, & Liu, 2007). Because of these technical and safety issues, the introduction of plasmid DNA (pDNA), which is nonintegrative and nonreplicating, has lower immunogenicity, has more flexibility in transgene capacity, and has the potential for industrial production, is an appealing alternative to viral gene delivery. The use of nonviral vectors for gene delivery is hindered, however, by relatively low transfection efficiency as several intra- and extracellular barriers exist that prevent high levels of gene delivery and expression. Nonetheless, the biocompatibility and potential of scaled-up production of these delivery vehicles has spurred on research aimed at overcoming these barriers and improving transfection efficiency (Mintzer & Simanek, 2009).

There are two main classifications of nonviral gene delivery techniques, namely physical and chemical methods. In physical methods of nonviral gene delivery, DNA may be delivered unprotected and uncondensed, termed naked DNA delivery, bypassing the first hurdle of complexation or encapsulation. However, naked DNA delivery using electroporation, gene gun or direct injection techniques is of little clinical relevance for gene therapy due to low levels of transfection (Mintzer & Simanek, 2009). These techniques may be appropriate for applications were limited transfection is acceptable, namely, vaccination (Cotten & Wagner, 1993). Under physiological conditions DNA is negatively charged as are the plasma membranes of the cell and the resultant repulsive forces as well as the large size of plasmid DNA prevent widespread internalization of naked DNA (Segura & Shea, 2001). In chemical methods of nonviral gene delivery, the first step is typically complexation, which involves the condensation and charge neutralization of DNA by positively charged polymers, lipids, dendrimers, or polypeptides (Mintzer & Simanek, 2009). DNA can also be encapsulated in nano- or microparticles (Mintzer & Simanek, 2009) which can protect DNA from degradation, facilitate *in vivo* distribution, and release of DNA in a sustained manner (Panyam & Labhasetwar, 2003). Although various chemical and particulate-based methods exist for nonviral gene delivery, the barriers that must be overcome are, for the most part, universal.

The barriers to efficient gene delivery by particles and vectors include internalization, endosomal escape, nuclear localization, and expression. Packaged DNA must first be

internalized by endocytosis, fluid phase pinocytosis, or phagocytosis (Agarwal & Mallapragada, 2008; Pichon, Billiet, & Midoux, 2010). Endocytosis may occur by clathrin-dependent or –independent (i.e. caveolae mediated) pathways for complexes and nanospheres (Mintzer & Simanek, 2009), while microspheres are taken up by phagocytosis (Agarwal & Mallapragada, 2008). DNA delivery vehicles may also be targeted to specific receptors that mediate internalization (Pichon, Billiet, & Midoux, 2010). Once internalized, the DNA must escape the endosome without being degraded by low pH or enzymes. This escape is believed to occur by disruption of the endosomal membrane, or through a proton sponge effect that causes the endosome to rupture (Mintzer & Simanek, 2009). The DNA must then navigate through the cytosol to the nucleus. This process is to some degree impeded by the cytoskeleton which may act as a sieve (Mintzer & Simanek, 2009) with nonspecific binding to cytoskeletal proteins impairing DNA mobility (Pichon, Billiet, & Midoux, 2010). There is also the possibility of degradation by enzymes in the cytosol (Mintzer & Simanek, 2009). In non-dividing cells nuclear internalization through pores in the nuclear membrane is necessary for the transgene to gain access to the transcription machinery, whereas, in dividing cells vectors can enter the nucleus when the nuclear membrane breaks down and reforms (De Laporte, Rea, & Shea, 2006). Complexes and free DNA are too large to pass through nuclear pores and are therefore likely transported into the nucleus by ATP-dependent pathways similar to those responsible for the transport of proteins into the nucleus or enter the nucleus during mitosis (Mintzer & Simanek, 2009). Nuclear localization signals can help mediate this process (Pichon, Billiet, & Midoux, 2010). At some point during the endosomal escape/nuclear localization process, the DNA must disassociate from the complex or be

released from the nano- or microsphere to allow expression. This disassociation is thought to take place at different points in transport for different types of vectors (Pichon, Billiet, & Midoux, 2010). *In vivo* transfection is further complicated by instability of complexes under physiological conditions and adsorption of serum proteins prior to cellular internalization (Mintzer & Simanek, 2009). The design of particulates and complexes able to effect high levels transfection must take into account these barriers and include tactics to overcome them. Particulates designed in this way have the potential to result in high levels of transfection over extended periods of time as the DNA is protected and released. As transfection is transient, particulates ability to extend the duration of expression makes them appealing for the delivery of DNA.

1.2.1 Particulate DNA delivery

Particulates for DNA delivery, including nanoparticles or microparticles, can consist of systems where the active compound, DNA, is entrapped within the particles or adsorbed or conjugated to the surface of the particles (Panyam & Labhasetwar, 2003). Particulates for gene delivery have been utilized as tools to administer DNA in a controlled, sustained, and localized/targeted manner (Agarwal & Mallapragada, 2008; Panyam & Labhasetwar, 2003). Encapsulation of DNA into particles has the potential to improve *in vivo* response and transfection by shielding the CpG methylation patterns of plasmid DNA from the immune system, shielding the plasmid from degradation by pH and enzymes, and increasing residence time (Agarwal & Mallapragada, 2008). Polymeric particulates can encapsulate naked DNA or polyplexes, which are composed of DNA complexed with a cationic polymer (Agarwal & Mallapragada, 2008). Nanoparticles and

microparticles that are small enough to be internalized by endocytosis or phagocytosis could potentially serve as intracellular gene depots. Unless the particles themselves are located in the nucleus, transfection by this released DNA requires nuclear localization. Microspheres which cannot be internalized by endocytosis could be designed to remain in the extracellular area of tissues and release their encapsulated DNA; this would also serve as a controlled and local delivery mechanism (Pannier & Shea, 2004). Release of entrapped DNA occurs due to a combination of diffusion through and degradation of the polymer matrix (Agarwal & Mallapragada, 2008). Particulates encapsulating DNA have the potential to be used in gene therapy and DNA vaccination.

1.2.2 Gene therapy

The definition of gene therapy includes the modification of, removal of, or addition of genetic material specifically aimed at affecting a phenotypic change in patients with genetic defects (Drugan, Miller, & Evans, 1987). The state of the art of gene therapy entails either the introduction of the corrected gene or the direction of cells to produce a protein or regulatory RNA which would block the harmful effects of the defective gene (Berns, 2010). These techniques can be used to treat genetic diseases like cystic fibrosis, hemophilia, and cancer (Bowman, Sarkar, Raut, & Leong, 2008; Montier et al., 2004; Schatzlein, 2001). While most studies in gene therapy involve the use of viral vectors, nonviral vectors remain promising tools for gene therapy. Current efforts in the realm of nonviral gene delivery are aimed at developing selective, safe, and efficient methods of delivering genes to an individual's cells for the correct production of gene products associated with genetic disease (S. Li & Huang, 2000). Gene therapy is a possible

application of the DNA-encapsulating particulates produced by the methods outlined in this thesis.

1.2.3 DNA vaccination

DNA vaccination requires the transfection of cells that results in the production of an antigen. DNA vaccines preserve the native protein structure of the antigen, induce cytotoxitc T lymphocytes (CTLs), and lack the potential virulence and toxicity associated with attenuated organisms or recombinant viral or bacterial vectors (O'Hagan, Singh, & Ulmer, 2004). Because DNA vaccination is typically performed using intramuscular injection or a gene gun, the cells usually transfected and responsible for antigen production are myocytes in the muscle and keratinocytes or Langerhans cells in the skin. The antigen can then be presented to cells of the immune system eliciting an antibody response. Additionally, CTLs are induced in two ways. The antigen can be processed and produced by antigen-presenting cells (APCs) or the antigen can be produced by non-APCs and transferred to APCs. Plasmids used in DNA vaccination also have adjuvant properties due to their CpG methylation motifs. Despite its advantages and successes in animal models, DNA vaccination in clinical trials has primarily been limited to naked DNA delivery methods and has not been found to be efficient in most cases of human application. However, there have been a couple of promising results offering proof of concept for this vaccination technique (O'Hagan, Singh, & Ulmer, 2004). The development of a zein nano- or microsphere capable of transfection would result in a possible delivery system for DNA vaccination. Important to the utility of particulates in both gene therapy and DNA vaccination is an appropriate route of administration.

1.2.4 Oral route of administration

The oral route of administration is associated with high patient compliance and convenience. Because nonviral gene delivery is transient and would require repeated administration for gene therapy applications, ease of administration and patient comfort are particularly important. The cellular structure of the intestine epithelium and gut-associated lymphoid tissue (GALT) is crucial to the oral absorption profile of a compound or delivery system. A single layer of epithelial cells makes up the intestinal epithelial barrier. It is composed of primarily enterocytes with goblet cells, which secrete mucus, interspersed. GALT consisting of lymphoid follicles are found throughout the GI tract (gastro-intestinal tract). Organized clusters of these follicles are called Peyer's patches which are overlayed with a layer of epithelium that is set apart from other intestinal epithelium by the presence of M-cells (microfold cells, (Clark, Jepson, & Hirst, 2001).

For the oral route of administration, biodegradable particulate carrier systems have the potential to improve bioavailability, target certain organs, improve gastric tolerance of agents that are stomach irritants and serve as a carrier for antigens in oral immunization compared to unencapsulated drugs (Desai, Labhasetwar, Amidon, & Levy, 1996). The oral route has the additional advantages of presenting a large surface area of intestinal epithelium for transfection and allowing treatment of regional disorders by providing access to the luminal side of the intestine (Bhavsar & Amiji, 2007). Particulates are considered a viable tool for the protection of DNA from the harsh environment of the stomach and intestine. However, the limited oral absorption of these particulates has

hampered their efficiency (H. Chen & Langer, 1998). Mucoadhesion, or association with the mucous layer of the GI-tract, has been proposed as a means of increasing the residence time of particulates encapsulating DNA as well as their proximity to the underlying intestinal epithelium. Chen and Langer point out that the turnover time of the mucous layer is only slightly longer than the normal intestinal transit time (H. Chen & Langer, 1998). However, it does appear that bioadhesion to the underlying epithelium is an effective way of increasing residence time (Mathiowitz et al., 1997). Other means of improving transfection in the GI-tract include targeting glycoproteins and receptors of the intestine, using chemical enhancers to promote absorption, and using mucolytic agents to reduce the mucous layer (Page & Cudmore, 2001). An ideal particulate gene delivery system would incorporate properties allowing it to protect the payload of DNA in the stomach and intestine and facilitate a high level of uptake by cells lining the intestine. To this end, zein was investigated in the studies described in this thesis as a possible oral delivery system. As described below, zein is a promising candidate material for oral delivery.

1.2.4.1 Oral gene therapy

Oral gene therapy has the potential to treat patients suffering from diseases directly associated with the GI-tract (e.g. familial adenomatous polyposis, cystic fibrosis, various colon cancers, inflammatory bowel disease, and Crohn's disease) (Page & Cudmore, 2001) as well as systemic diseases (Rothman, Tseng, & Goldfine, 2005). The use of oral administration to treat diseases affecting the GI-tract is quite appealing as this route allows direct access to the affected tissue and thus no further transport of the gene or the

encoded protein is required. The diseased state of these tissues may also allow for additional targeting due to different receptor expression compared to healthy tissue (Page & Cudmore, 2001). Local nonviral gene therapy within the GI-tract was demonstrated by Bhavsar and Amiji with their nanoparticles-in-microsphere oral system. Using a murine model of acute colitis it was demonstrated that delivery of murine IL-10 expressing plasmid resulted in a reduction in the levels of proinflammatory cytokines and certain chemokines. This therapy also resulted in an increase in body weight, good clinical activity scores, restoration of colon length and weight, and reduction in inflammatory response (Bhavsar & Amiji, 2008). This study indicates a possible use of zein/DNA nanospheres for gene therapy for diseases affecting the GI-tract.

Proof-of-principle for the oral delivery of DNA was provided by Rothman et al. Rothman and coworkers proposed oral gene therapy as a more convenient and easier to manufacture alternative to protein pharmaceutics for systemic therapy (Rothman, Tseng, & Goldfine, 2005). They further hypothesized that protein could be manufactured in the cells of the intestine, could be delivered into the bloodstream, and could act elsewhere in the body. For this application nonviral gene delivery requires frequent dosing like other oral medications, but this allows the dose to be altered on a daily basis and allows treatment to be stopped in the case of adverse side effects. To provide proof of this principle they administered naked DNA in a number of ways: injected into an isolated segment of the duodenum, through an indwelling catheter placed in the intestinal lumen, injected into a patent duodenum, and by oral gavage. Delivery of the luciferase gene to ligated, and unligated duodenums and via oral gavage showed that expression occurred in

the intestine and was significantly higher than control for all cases. It should be mentioned that transfection as a result of plasmid delivered by oral gavage was one-fourth to one-fifth of that of plasmid injected directly into the duodenum. Plasmid encoding human growth hormone (hGH) was used to assess the ability of orally administered plasmid to elicit the production of protein with subsequent delivery of the protein into the bloodstream. Twenty-four hours post-administration to the duodenum of rats, plasmid encoding hGH resulted in hGH levels at five times background levels in the blood. Five days of such treatment resulted in maintained elevated levels that fell to background upon the cessation of the treatment. The ability of the administration of plasmid DNA to affect a physiological change was evaluated using a plasmid encoding insulin in diabetic mice. Delivery of plasmid to isolated duodenal segments resulted in blood glucose levels that remained at or close to non-diabetic values for about two days before rising into the diabetes range. The effect was transient but reproducible with repeated dosing. These results led Rothman and coworkers to conclude that oral gene therapy had great potential but required improvement in DNA packaging and protection from degradation and improvements in the efficiency and reliability of transfection (Rothman, Tseng, & Goldfine, 2005).

Previously polyanhydride copolymers of fumaric and sebacic acid were used to form microspheres encapsulating pCMV/β-gal that were delivered orally. Histochemical staining of Peyer's patches showed that most transfected cells were located in the muscularis mucosae and adventitia below the Peyer's patches for encapsulated DNA, whereas there were was no false-positive staining in the Peyer's patches of rats that

received naked DNA or no DNA (Mathiowitz et al., 1997). Oral administration of the mEpo gene in chitosan nanoparticles resulted in a significant increase in hematocrit measured in the blood compared to control upon first and second feedings (J. Chen et al., 2004). Similarly Bowman et al. orally administered Factor VIII DNA in chitosan nanoparticles and achieved a physiological effect. Hemophilia A mice that were fed chitosan/DNA nanoparticles underwent a phenotypic correction in 13 out of 20 cases, compared to 1 out of 13 cases for naked plasmid (Bowman, Sarkar, Raut, & Leong, 2008). In another study, parathyroid hormone (PTH)(1-34) gene was orally admininstered to parathyroidectomized rats in polyethylene oxide-polypropylene oxide-polyethylene oxide polymeric micelles. This treatment resulted in 100% survival with normal activity and appetite and significantly higher serum calcium and serum PTH(1-34) levels as compared to controls (Chou, Huang, Chang, Liaw, & Hung, 2009). These studies demonstrate the feasibility and promise of oral gene therapy to treat a variety of systemic diseases. The possible treatment of systemic diseases makes the development of a particulate zein/DNA system capable of oral absorption and transfection extremely useful to individuals with a wide variety of diseases.

1.2.4.2 Oral DNA vaccination

The oral route of delivery is particularly appealing for vaccination because it is less invasive than current treatment methods and because it facilitates access to GALT. Immunization at GALT could provide an effective barrier to infections gaining entry through mucosal surfaces (Jones, Clegg, & Farrar, 1998). The high transcytotic capacity of M-cells also aids in the absorption of other particulates making them a common target

for delivery systems specifically for oral vaccination due to the proximity of M-cells to cells belonging to the immune system (Clark, Jepson, & Hirst, 2001). As microspheres are too large to be taken up by endocytosis, size exclusion can lead to their preferential uptake by phagocytic cells like M-cells. Hence, primarily microspheres have been studied as carriers for DNA vaccination (Agarwal & Mallapragada, 2008).

Oral DNA vaccination was first demonstrated in 1997 by Jones and coworkers with orally administered DNA encapsulated in poly(lactic-co-glycolic acid) (PLGA) microspheres eliciting both a systemic and a mucosal antibody response (Jones, Corris, McDonald, Clegg, & Farrar, 1997). These results have led to many studies using synthetic and natural polymers to encapsulate and deliver DNA via the oral route for DNA vaccination. Jones et al. compared oral DNA vaccination to intraperitoneal injection and found that oral administration resulted in lower but significant IgG titres, similar IgM titres and higher IgA titres (Jones, Clegg, & Farrar, 1998). The induction of immunity to measles using DNA encoding the measles virus nucleocapsid protein was evaluated by Fooks and coworkers (Fooks, Sharpe, Shallcross, Clegg, & Cranage, 2000). They found that the administration of plasmid DNA encapsulated in PLGA resulted in an increase in specific IgG antibody titers in three of ten mice, whereas viral oral vaccination had a 100% induction of IgG titer increase (Fooks, Sharpe, Shallcross, Clegg, & Cranage, 2000). These results indicated that there was much room for improvement in the efficacy of nonviral oral DNA vaccine vehicles. However, several other studies have shown more promising results. PLGA microparticles containing plasmid encoding hepatitis B virus HBsAg induced a long-lasting and stable antigen-specific antibody, in

both serum and intestinal IgA, and CTL responses in BALB/c mice (He et al., 2005). Mice immunized with microparticles encapsulating HIV protein encoding plasmid by oral vaccination showed a multifaceted envelope-specific immune response and higher resistance to mucosal viral transmission than mice injected intramuscularly with plasmid encoding the same protein (Kaneko et al., 2000). DNA vaccines encoding allergens like the dermatophgoides pteronyssinus (Der p) allergen from dust mites and a dominant peanut allergen have been used to prevent sensitization of IgE reactions in mice. DNA vaccination may be used to modify the ongoing Th2-type immune response in allergic diseases by inducing a dominant Th1 response (G. Li, Liu, Liao, & Zhong, 2009). Mice that received chitosan nanoparticles encapsulating a gene for a dominant peanut allergen were significantly less likely to suffer from allergen-induced anaphylaxis and had reduced levels of IgE, plasma histamine, and vascular leakage (Roy, Mao, Huang, & Leong, 1999). Li et al. found that oral delivery of chitosan/pDer p 2 nanoparticles resulted in the expression of Der p 2 in both the stomach and small intestine, prevention of sensitization of the Th2 cell-regulated specific IgE response, and the induction of Th1-type immune response (G. Li, Liu, Liao, & Zhong, 2009). These studies provide evidence of the promise of oral DNA vaccination in combating infection and in treating disorders of the immune system, making DNA vaccination a prospective application of DNA-encapsulating zein nano- and microspheres.

1.2.5 Materials for oral DNA delivery

Synthetic materials for DNA delivery offer a higher degree of control and uniformity of properties and generally provide a more sustained release than natural materials.

However, they also may require the use of harsh solvents and may have detrimental degradation products. The most commonly used synthetic polymer for oral gene therapy and vaccination applications is PLGA. It is approved by the FDA and has been used for internal sutures, implants, and drug and protein delivery with a documented history of safe use (Abbas, Donovan, & Salem, 2008; Jones, Clegg, & Farrar, 1998). It is a biodegradeable polymer that degrades by non-enzymatic hydrolysis to two normal metabolites, lactic and glycolic acids (Jones, Clegg, & Farrar, 1998). However the acidic nature of these degradation products can lead to the degradation of encapsulated DNA. This degradation can be avoided by forming PLGA nanospheres. Nanospheres have the advantage of a large surface area to volume ratio which promotes diffusion of lactic and glycolic acids away from the particle, thereby preventing DNA degradation (Agarwal & Mallapragada, 2008). Encapsulation of DNA in PLGA particles by these methods is complicated by the hydrophobic and hydrophilic natures of PLGA and DNA, respectively, and the large size of DNA (Agarwal & Mallapragada, 2008). Release profiles of encapsulated DNA can be affected by the composition and molecular weight of the PLGA polymers (Agarwal & Mallapragada, 2008; Jang & Shea, 2006). Various modifications can be made to improve the formulation and DNA delivery performance of PLGA particles including the use of surfactants and excipients, and covalent modifications like PEGylation and the addition of diethylaminopropylamine substituents (Abbas, Donovan, & Salem, 2008). Other synthetic polymers that have been used very limitedly to form DNA containing particles for oral delivery include polyanydride copolymers of fumaric and sebacic acid (Mathiowitz et al., 1997) and poly (ε-caprolactone)(PCL) (Bhavsar & Amiji, 2007).

Natural polymers have also been applied to drug/gene delivery. Natural materials have an advantage over synthetic biomaterials in that these natural biomaterials provide innate degradability, biocompatibility, and bioactivity (Dang & Leong, 2006). The most commonly used natural polymer for the formation of DNA-containing particles is chitosan. Chitosan is a polysaccharide obtained by between 66% and 95% deacetylation of chitin (abundantly available in marine crustaceans) and is biocompatible and biodegradable (Agnihotri, Mallikarjuna, & Aminabhavi, 2004). Chitosan contains many primary amine groups that endow it with properties that make it pharmaceutically useful (i.e., give it a positive charge and mucoadhesive properties) (Agnihotri, Mallikarjuna, & Aminabhavi, 2004). Chitosan has been intensively studied for oral delivery applications due to its ability to increase paracellular permeability in the intestinal epithelium by interfering with tight junctions (Bowman & Leong, 2006). The properties and transfection efficiency are affected by the ratio of nitrogens from chitosan to phosphoruses from DNA (N/P ratio), chitosan molecular weight, degree of acetylation, and modification of chitosan including attachment of targeting ligands and addition of endosomolytic compounds like polyethyleneimine (PEI) (Bowman & Leong, 2006). Other natural polymers that have been used for the oral delivery of DNA include collagen (Jun Wang et al., 2004), gelatin (Bhavsar & Amiji, 2007), and alginate (Mittal et al., 2001), however their application to oral DNA delivery is not common.

1.2.6 Investigating new materials

To date many advances have been made toward the development of therapies that utilize nonviral gene delivery, specifically in the realm of micro- or nanoparticles that

encapsulate and deliver plasmid DNA. However, no delivery system can meet the demands for therapeutic action in humans. There are a number of prerequisites for success in gene delivery applications including high DNA encapsulation efficiency and loading within the particles, stability of DNA in the particle, nontoxicity of the particle and its degradation products and the ability to facilitate transfection. Oral delivery further requires protection of the payload from the acidic environment and enzymes of the stomach, protection of the DNA from enzymes in the intestine, facilitation of transport of DNA across the mucous layer, and mediation of transfection of intestinal cells or passage through the intestinal epithelium into circulation and subsequent transfection. The investigation of new materials, and modifications thereof, which have the ability to meet these requirements promises to fuel more breakthroughs and bring nonviral gene delivery via oral administration closer to clinical application. Due to its demonstrated ability to deliver drugs orally, zein has been investigated in this thesis for the oral delivery of DNA.

1.3 Zein

Zein is the prolamin, or storage protein, of corn. Protein comprises 6-12% (db) of corn depending on the variety, and approximately 75% of that protein is found in the endosperm (Shukla & Cheryan, 2001). Zein, which accounts for approximately 45-60% of the total corn protein, is found exclusively in the endosperm (Anderson & Lamsal, 2011; Shukla & Cheryan, 2001). Protein bodies, membrane-bound subcellular compartments, typically around 1 μm in diameter, are formed within the endoplasmic reticulum as deposits of zein and are located in the cytoplasm between starch granules

(Duvick, 1961; Larkins & Hurkman, 1978). Zein serves as nitrogen sink, facilitating the transport of photosynthates into the kernel as the seed develops, and later is used by the germinating plant as a source of nitrogen (Mohammad & Esen, 1990; Tsai, Huber, & Warren, 1980). The properties of zein, as determined by its structure, vary widely depending upon protein fraction, processing, environmental conditions, chemical modifications and degree of degradation. The physicochemical properties of zein can be tailored to a number of applications as evidenced by its various past uses. Today zein is used in pharmaceutical tableting and coating, and is considered most promising for applications in edible and biodegradable packaging and coatings as well as biomedical applications (Lawton, 2002). Zein has shown great potential in the field of drug delivery as it has the potential for a wide range of potential modifications, excellent biocompatibility and biodegradability (undergoing enzymatic degradation) (S. Gong, Wang, Sun, Xue, & Wang, 2006; Mathiowitz, Bernstein, Morrel, & Schwaller, 1993; Sun, Dong, Lin, Yang, & Wang, 2005). In this thesis zein was used to for nano- and microspheres encapsulating DNA for the first time. A background on zein is presented below.

1.3.1 History

Zein was discovered and named by John Gorham in 1821, but it was not classified as a prolamin until Osborne did so in 1924 (Anderson & Lamsal, 2011; Lawton, 2002; Osborne, 1924).The first patent for extracting zein was issued to Osborne in 1891(Lawton, 2002; Osborne, 1891b), however, the first facility for the production of zein did not open until the late 1930's (Anderson & Lamsal, 2011; Lawton, 2002; Shukla

& Cheryan, 2001). At the peak of zein production, there was a total output of about 15 million pounds per year (Anonymous, 1978). From the late 1800's to today, numerous applications for zein have been identified including coatings, fibers, inks, adhesives and plastics (Bers, 1945; Coleman, 1939, 1941; Osborne, 1891a; Swallen, 1939). Zein has been supplanted in many of its former applications due to the development of synthetics with superior properties and lower costs. In many applications zein has been plagued by its prohibitive cost and its poor resistance to water (Lawton, 2002). Zein is currently used to coat materials that remain dry, namely, nuts and pharmaceuticals. Unless its hygroscopic nature can be overcome, zein will continue to be limited to applications where its change in moisture content with relative humidity will not affect its performance (Lawton, 2002). Today zein is manufactured commercially by four companies: Freeman Industries (Tuckahoe, NY), Showa Sangyo (Tokyo, Japan), POET Inc. (Sioux Falls, SD) and Prairie Gold Inc. (Bloomington, IL), with Freeman Industries and Showa Sangyo responsible for the bulk of zein production (Anderson & Lamsal, 2011). The current, relatively high price of zein, $10-40/kg (Anderson & Lamsal, 2011), compared to the estimated cost of extraction, $2-3/kg (Dickey, McAloon, Craig, & Parris, 1999), has been attributed to the lack of demand (Lawton, 2002). Shukla asserts however, that the high price is due to the large amount of organic solvents used and the energy-intensive processes, such as distillation and evaporation, needed in zein production (Shukla & Cheryan, 2001).

1.3.2 Fractions

Zein nomenclature is rather convoluted. Shukla and Cheryan counted at least five systems of naming the various zein fractions (Shukla & Cheryan, 2001). In this thesis Esen's system will be used. In Esen's system, which was proposed in 1987, α (75-85% of total zein, 21-25 kDa and 10 kDa), β (10-15% of total zein , 17-18 kDa), and γ (5-10% of total zein, 27 kDa) zein were separated based on their varying solubility in aqueous isopropyl alcohol and aqueous isopropyl alcohol/sodium acetate solutions (Esen, 1987). The system was later revised to include δ zein as the 10 kDa fraction (Esen, 1990). Zein consists of several fractions that vary in molecular size, charge and solubility. Although some zein fractions are proteins soluble in aqueous ethanol and a reducing agent rather than true prolamins, they are found in the protein bodies and are also considered to be zein (Wilson, 1991). Commercial zein primarily contains only one of these fractions, namely α zein, sometimes with traces of δ zein (Wilson, 1988). Lawton attributes the lack of other fractions in commercial zein to a combination of two factors. First, the starting material for commercially extracting zein has been treated with sulfur dioxide, reducing the disulfide bonds in β, γ, and δ zein which increases their water solubility. Second, the solvent, 86% isopropyl alcohol, used to extract commercial zein is not a good solvent for these fractions (Lawton, 2002). The lack of these minor fractions is beneficial to producers as β, γ, and δ zein are thought to cause gelling (Lawton, 2002). Commercial zein was used for the formation of zein/DNA nanospheres in this thesis.

1.3.3 Structure

Although many of zein's properties as they relate to solubility and phase separation can be explained by what is known about the protein's primary and secondary structures, the exact tertiary structure of zein, specifically for α zein, has not been definitively determined. The amino acid sequence of zein consists of more than 50% nonpolar amino acids, including high percentages of leucine, proline, and alanine, causing zein to be insoluble in water. Zein also has a high glutamine content which results in zein's insolubility in absolute alcohol (Padua & Wang, 2009; Pomes, 1971). The 19kDa and 22kDa α zein peptides consist of an eight residue long C-terminal end and a 35-37 residue long N-terminal end separated by either nine or ten repetitive sequences that range in length from 14 to 25 residues averaging 19-20 amino acids (Padua & Wang, 2009). This and other data have given rise to several possible models for the tertiary structure, but have not yet pointed to a definitive structure (Agros, Pedersen, Marks, & Larkins, 1982; Bugs et al., 2004; Garratt, Oliva, Caracelli, Leite, & Arruda, 1993; Matsuda et al., 1989; Padua & Wang, 2009).

1.3.4 Solubility

Zein is soluble in a number of primary, binary and ternary solvent systems, primarily determined by the balance between the polar/nonpolar groups in those systems. Because of zein's large proportion of hydrophobic residues it is not soluble in water at standard pH. It is however, soluble in water above pH 11 due to the ionization of the tyrosine phenolic groups and of the few carboxyl groups (Pomes, 1971). Evans and Manley found that zein was soluble in a number of primary solvents which were all hydroxyl

compounds, amines, amides or acids with the exception of pyridine (Evans & Manley, 1941). Zein is soluble in organic solvents with hydroxyl, carboxyl, amino, and other polar groups in proper ratio to nonpolar groups because of its large number of hydrocarbon side chains (Pomes, 1971). Binary solvents studied by Manley and Evans included systems of water in combination with a ketone, lower aliphatic alcohol, dioxane, or dioxolane or lower aliphatic alcohols in combination with a number of compounds including aldehydes, ketones, nitroparaffins, partially chlorinated hydrocarbons, cyclic ethers, aromatic hydrocarbons and glycols (Manley & Evans, 1943). Ternary solvents include systems containing water, a lower aliphatic alcohol and any one of a number of other compounds or systems containing water and two other compounds including glycols and butanediols (Evans & Manley, 1944; Shukla & Cheryan, 2001). Depending on the concentration of aqueous ethanol, the solubility of zein ranges from 2 to 60% (w/w) at constant temperature (Shukla & Cheryan, 2001). At temperatures below the boiling point of ethanol, zein is soluble in between 50% and 90% ethanol, with coacervation and precipitation taking place both above and below this range (Shukla & Cheryan, 2001), the former of which will be utilized later in this thesis.

1.3.5 Crosslinking

Crosslinking is a modification of zein often investigated to improve its physical properties. Crosslinking typically affects the tensile strength, elongation and Young's modulus of zein fibers and films as well as their solubility in solvent systems that normally dissolve zein and degree of swelling when soaked in an aqueous solution (Jiang, Reddy, & Yang, 2010; Kim, Sessa, & Lawton, 2004; Parris & Coffin, 1997; Reddy &

Yang, 2009; Xu, Karst, Yang, & Yang, 2008; Yao, Li, & Song, 2007). Zein can be crosslinked by many compounds including glyoxal, hexamethylene diisoyanate, polymeric dialdehyde starch, 1-[3-dimethylaminopropyl]-3-ethyl-carbodiimide hydrochloride, formaldehyde, *N*-hydroxysuccinimide, glutaraldehyde, epichlorohydrin, citric acid and butanetetracarboxylic acid (Kim, Sessa, & Lawton, 2004; Parris & Coffin, 1997; Yang, Wang, & Li, 1996; Yao, Li, & Song, 2007). Zein can also be crosslinked enzymatically using the enzymes transglutaminase or protein disulfide isomerase (Mathiowitz, Bernstein, Morrel, & Schwaller, 1993). Zein nano- and microspheres have the potential to be crosslinked using the same compounds that are used in films and fibers (Mathiowitz, Bernstein, Morrel, & Schwaller, 1993). In forming zein microspheres, glutaraldehyde and trimethylsilyl chloride have been used to modify zein with certain drugs and likely resulted in additional crosslinking of the zein (Matsuda et al., 1989; Suzuki et al., 1989). Although nano- and microsphere crosslinking has not been well investigated, it is conceivable that this type of modification would be useful in altering properties such as the degradation rate of zein particles. Crosslinking may be required for the improvement of the stability and release profiles of DNA-loaded zein particles produced in this thesis.

1.3.6 Other chemical modifications

Zein has also been modified by derivitization of surfaces, forming copolymers, and deaminization or phosphorylation of its amino acids (Biswas, Selling, Woods, & Evans; Mathiowitz, Bernstein, Morrel, & Schwaller, 1993; Q. Wu, Yoshino, Sakabe, Zhang, & Isobe, 2003). Esterification of zein with fatty alcohols or acylation with fatty anhidrides,

which can alter the acid or base sensitivity of the protein, are other options for chemical modification of zein. Acylation with fatty anhydrides was demonstrated by adding the fatty anhydrides to zein containing solution under reactive conditions. The modified zein was later used to form microspheres encapsulating insulin with different fatty acid modifications resulting in different release profiles (Mathiowitz, Bernstein, Morrel, & Schwaller, 1993). By deaminating or phosphorylating the amino acids of zein, the characteristics, e.g. hydrophobicity, of the protein can be altered (Casella & Whitaker, 1990; Mathiowitz, Bernstein, Morrel, & Schwaller, 1993). Deamination and phosphorylation of whole zein and zein hydrolysate increased the peptides' solubility in water in certain pH ranges. These modifications also affected the emulsifying activities and stabilities of some of the peptides (Casella & Whitaker, 1990). Mathiowitz et al. demonstrated that zein could be deamidated and further modified with fatty acids, hexanoic anhydride, octanoic anhydride, decanoic anhydride, or lauric anhydride. Zein modified thusly and zein that was deaminated but not modified with a fatty anhydrides were then used to form microspheres encapsulating insulin, with the various modifications, again, resulting in differing release profiles (Mathiowitz, Bernstein, Morrel, & Schwaller, 1993). These studies give examples of the ways in which zein can be modified and offer evidence of the usefulness of modified zein. Zein microspheres may require one or more of these modifications to be efficient as gene delivering vehicles.

1.3.7 Degradation

Zein is degraded by many enzymes including pepsin, trypsin, thermolysin, collagenase, milezyme, alcalase, pronase, papain and chymotrypsin (Hurtado-Lopez & Murdan, 2006b; Kong & Xiong, 2006; Mannheim & Cheryan, 1993; Mathiowitz, Bernstein, Morrel, & Schwaller, 1993; Miyoshi et al., 1991; Sun, Dong, Lin, Yang, & Wang, 2005). Hurtado-Lopez and Murdan found that while zein was extremely resistant to degradation in the absence of enzymes, i.e. in chloride buffer (pH 2), acetate buffer (pH 5) and PBS (pH 7.4), zein was degraded much more quickly by pepsin and pancreatin enzymes found in gastric and intestinal fluids, respectively, than it was by solutions free of enzymes (Hurtado-Lopez & Murdan, 2006b). Zein was also hydrolyzed by collagenase, trypsin, or pepsin and its degradation products were found to have a significant positive effect on cell proliferation for HL-7702 liver cells and HUVECs with an optimal concentration of about 0.3 mg/mL (Sun, Dong, Lin, Yang, & Wang, 2005; H.-J. Wang, Lin, Liu, Sheng, & Wang, 2005). The results of these studies suggest that zein nano- and microspheres encapsulating DNA will be degraded by enzymes and that their degradation products will not adversely affect cells.

1.3.8 Biocompatibility

Zein, a natural polymer, has been shown to be very biocompatible. Cell proliferation on films and electrospun mats composed of zein has been compared to that of cells on a number of other surfaces composed of compounds commonly used in tissue engineering and biomaterials. Notably, zein surfaces have consistently outperformed poly(lactic acid) (PLA) and Corning plates in regard to supporting cell proliferation (Dong, Sun, & Wang,

2004; Jiang, Reddy, & Yang, 2010; Sun, Dong, Lin, Yang, & Wang, 2005). Cell-biomaterial interactions for zein substrates can be improved with appropriate modifications. As previously mentioned, crosslinked electrospun zein fiber mats supported superior cell attachment, spreading and proliferation than uncrosslinked fibers (Jiang, Reddy, & Yang, 2010). The ability of mesenchymal stem cells (MSCs) to adhere, grow and proliferate on zein scaffolds was demonstrated with significantly higher proliferation on zein scaffolds containing stearic acid as a plasticizer compared to unmodified zein and zein with other plasticizers (H.-J. Wang et al., 2007). Also, the differentiation of MSCs to osteoblasts on porous zein scaffolds was improved by coating the scaffold in hydroxyapatite (Qu et al., 2008). Because of zein's excellent biocompatibility which can be further improved upon, it is a promising biomaterial for gene delivery applications. This property as well as the mode of degradation of zein and its potential for modification make zein nanospheres tools for gene delivery with the ability to be fine-tuned and tailored to specific applications.

1.3.9 Micro- and nanoparticles

Zein nano- and microspheres have been investigated by several groups for nutraceutical and drug delivery, usually administered orally. There are several methods for forming zein micro- and nanospheres, the most common of which is coacervation, or phase separation. Coacervation is defined as the separation of colloidal systems into two liquid phases (Gander, Blanco-Prieto, Thomasin, Wandrey, & Hunkeler, 2006). This separation involves the development of one phase rich in polymer (the coacervate) and another lacking polymer brought about by the partial desolvation of a previously dissolved

polymer (Arshady, 1990; Gander, Blanco-Prieto, Thomasin, Wandrey, & Hunkeler, 2006; Madan, 1978). This change in solubility may result from a change in temperature, pH or electrolyte concentration or from polymer-polymer interactions (Gander, Blanco-Prieto, Thomasin, Wandrey, & Hunkeler, 2006; Madan, 1978). As opposed to precipitation, this process results in a colloidally stable system, although flocculation may occur making it difficult to distinguish from precipitation (Gander, Blanco-Prieto, Thomasin, Wandrey, & Hunkeler, 2006; Madan, 1978). In the case of zein, the process typically begins with zein dissolved in a solvent, e.g. aqueous ethanol, and proceeds with the addition of a nonsolvent, namely an aqueous solution. The resultant decrease in ethanol concentration causes the necessary partial desolvation of zein.

When using the traditional coacervation technique, size, encapsulation efficiency, loading, and release profiles of encapsulated drugs have been shown to be dependent on several parameters of zein microsphere formation and characteristics of compounds incorporated. Increasing the zein to encapsulated drug ratio typically results in increased encapsulation efficiency but decreased loading (Fu, Wang, Zhou, & Wang, 2009b; H.-J. Wang, Lin, Liu, Sheng, & Wang, 2005). Zein microspheres formed by coacervation generally release their payload relatively quickly for approximately the first 24 hours (possibly due to surface-associated drug) followed by a slower sustained release for the rest of the study, with the released compound maintaining its activity.

There are several examples of the utility of zein nano- and microspheres formed by the common coacervation technique. These examples include spheres and films comprised

of spheres. Several studies have shown the potential of these spheres and films to provide sustained and effective release of the encapsulated compound. Fu et al. formed zein microspheres and microsphere films encapsulating the antibiotic, ciprofloxacin, for possible application in films on implanted devices. The loading and encapsulation efficiencies were relatively low, (size, encapsulation efficiency and loading data for zein microspheres loaded with nutraceuticals, essential oils, and drugs can be found in Appendix A.) probably due to the solubility of the drug (relatively hydrophilic). *In vitro* release from microsphere films in trypsin-containing solution was observed for up to 28 days and was correlated to the degradation of the films. Bacterial adhesion was significantly reduced by the release of the antibiotic (Fu, Wang, Zhou, & Wang, 2009a). Sustained release was also demonstrated for zein microsphere films encapsulating heparin over 20 days. The released heparin significantly increased the time of clotting for at least ten days (H.-J. Wang, Lin, Liu, Sheng, & Wang, 2005). In forming zein microspheres encapsulating polysaccharide-K (PS-K) by first conjugating PS-K to zein, Matsuda and coworkers found that using appropriate amounts of catalyst, using glutaraldehyde as the crosslinker, and rapid addition of aqueous solution containing polyvinylpyrrolidone (PVP) as a stabilizer were crucial for forming mono-disperse microspheres less than one micrometer in diameter. They also found that release was dependent on the zein to PS-K ratio as well as the nature of the release media, i.e. pH and concentration of enzyme (Matsuda et al., 1989). The release profile of zein microspheres can be further controlled by tabletting. Tabletting zein microspheres encapsulating the hydrophobic drug, ivermectin, significantly slowed release and resulted in zero order

release over eleven days and degradation over fourteen days in pepsin containing buffer (Liu, Sun, Wang, Zhang, & Wang, 2005).

One of the drawbacks of the simple coacervation technique is that the loading of hydrophilic drugs is often low. However this hurdle to efficient encapsulation has previously been overcome. Spheres composed of conjugates of zein with antitumor drugs mitomycin , daunomycin hydrochloride, and peplomycin sulfate were formed using three methods. Encapsulation of the most hydrophobic drug was relatively straightforward, while the efficient encapsulation of the moderately and highly hydrophilic drugs required protocol modifications but resulted in loadings as high as 31.3% and 19.0%, respectively (Suzuki et al., 1989).

Zein has been shown to have great potential for the oral delivery of compounds, as it is capable of protecting its payload through the stomach. The possibility of oral delivery of compounds to the intestine was explored by Parris and coworkers who found that less than half of the zein in the nanospheres that they formed was degraded after four hours in pepsin-containing solution. This slow degradation would allow the majority of the spheres to pass through the stomach undegraded and to deliver their payload to the intestine. Incubation of zein with swine manure solids indicated that digestion would occur rapidly, possibly within minutes, in the intestine (Parris, Cooke, & Hicks, 2005). Release of encapsulated essential oils in phosphate buffered saline (PBS) resulted in an initially rapid release, up to approximately 40-60% over the first ten hours, followed by a slower phase of release that reached a peak at 60-80% after which no more release was

detected (Parris, Cooke, & Hicks, 2005). Based on their ability to release encapsulated drugs in a sustained manner, to maintain drug activity, to be tabletted to alter release, to encapsulate hydrophilic drugs with the proper modifications, and to deliver their payload to the intestine, spheres formed using the traditional coacervation technique (the method used in this thesis) are of particular interest.

Another embodiment of coacervation involves the addition of zein solution to an aqueous non-solvent. In 1991 Stark and Gross received a patent for their invention entailing microsphere formation by the addition of hydrophobic protein solution to an aqueous solution with agitation. They found that microspheres size could be altered by varying parameters including the concentration of the protein solution, rate of agitation, and temperature (Stark & Gross, 1991). Zhong and Jin proposed three competing mechanisms for sphere formation by this method. First is the breakup of the zein solution into droplets by the shear force, leading to the second mechanism, the solidification of zein during solvent removal to the aqueous phase. And third, the coalescence of droplets that have not yet solidified (Zhong & Jin, 2009b). Using this method, zein was dissolved in various concentrations of ethanol and this solution was sheared into water with a homogenizer. Diameter was affected by zein concentration, ethanol concentration, shear rate and homogenizer speed. The minimum sphere diameter was obtained at an ethanol concentration of 90% according to scanning electron microscopy (SEM) and the trend of dynamic light scattering (DLS) measurements, at a zein concentration of 0.67% (w/v), and at a homogenizer speed of 10,000 rpm according to DLS and 15,000 rpm according to SEM measurements. DLS measurements of the same samples were slightly larger than

SEM measurements as is the case with the data presented in this thesis. Using the Searle setup of the rheometer, the effect of the shear rate was assessed, demonstrating a minimum sphere diameter of 200 nm at a shear rate of 3000 s^{-1} (Zhong & Jin, 2009b). This method of sphere formation offers a potential alternative to traditional coacervation with different means of affecting sphere size.

Another form of coacervation used to form zein micro-nanospheres involves the removal of ethanol, typically by evaporation, bringing about a similar decrease in ethanol concentration and subsequently sphere formation (Hurtado-Lopez & Murdan, 2005). Hurtado-Lopez and Murdan performed a series of studies investigating the potential of zein as a drug and vaccine carrier. They first extensively characterized the effects of the ratio of zein to albumin, the ratio of stabilizers, and the concentration of sodium hydroxide as these factors had the strongest impact on sphere size and degree of sphere formation (Hurtado-Lopez & Murdan, 2005). Evidence of the potential of sphere property modulation, release by slow erosion, and the maintained integrity of the loaded albumin (Hurtado-Lopez & Murdan, 2005) led to subsequent studies investigating the degradation, adjuvanticity and immunogenicity of these spheres. Degradation studies in the absence of enzymes explained the slow release of ovalbumin previously seen in PBS as zein was very resistant to degradation; however in the presence of pepsin and pancreatin degradation was very much accelerated. Blank spheres and ovalbumin loaded spheres were administered intramuscularly and blank spheres were also administered orally to two sets of mice. Sera antibodies in mice dosed with blank spheres indicated that zein elicited the productions of anti-zein antibodies that cross-reacted with the

ovalbumin antigen preventing the assessment of zein's ability to act as an adjuvant. Oral administration led to an increase in intestinal IgA levels following booster doses, showing that mucosal tolerance had not been achieved (Hurtado-Lopez & Murdan, 2006a). The results of the degradation study led Hurtado-Lopez and Murdan to conclude that zein microspheres were better suited for intra-muscular administration than administration via the oral route, and their research into the immunogenicity of zein led them to question the applicability of these spheres via either route (Hurtado-Lopez & Murdan, 2006a, 2006b). However another study has shown *in vivo* biocompatibility of implanted zein scaffolds (H.-J. Wang et al., 2007), and as will be demonstrated below, clinical results show that concerns about the biocompatibility and immunogenicity of zein appear to have been unfounded as adverse effects were not seen in dogs and humans (DiBiase & Morrel, 1997; S.-J. Gong et al., 2010).

The solvent extraction/evaporation or emulsion method is also used to form zein microspheres. This method differs from the solvent removal coacervation technique in that the formation of microdroplets is not entirely dependent on the removal of the solvent but rather depends on an emulsification step in a nonsolvent. The formation of droplets by a method such as mixing is followed by solvent evaporation (Freitas, Merkle, & Gander, 2005). Zein microspheres encapsulating abamectin, an avermectin, were formed by this method using acetic acid, which was later extracted, as the solvent of the dispersed phase and lecithin in mineral oil as the continuous phase. These spheres effectively protected the encapsulated chemical from photodegradation (Demchak & Dybas, 1997). Mathiowitz and coworkers received a patent using this technique to

fabricate protein microspheres and coatings. Specifically, they dissolved zein in the first solvent, 90% ethanol, and added the compound to be incorporated. They then dispersed this solution in the immiscible continuous phase, corn oil, with agitation, and finally removed the solvent by evaporation. This method was used to encapsulate solid zinc insulin, soluble insulin or rhodamine B in zein microspheres. This technique was also applicable to modified zein, namely fatty anhydride modified and deaminated zein as described previously (Mathiowitz, Bernstein, Morrel, & Schwaller, 1993). Blood glucose levels in diabetic rats following subcutaneous injection of zein microspheres encapsulating insulin compared to injection of unencapsulated insulin indicated that the microsphere provided a longer period of bioactivity. Release of insulin from deaminated and fatty anhydride modified zein spheres also produced a reduction in blood glucose levels for an extended period of time (Mathiowitz, Bernstein, Morrel, & Schwaller, 1993). This process is similar to coacervation in that it does not involve harsh chemicals or high temperatures that might degrade bioactive compounds to be encapsulated or the zein itself, and the spheres have the potential to consist of only natural compounds and the drug (Mathiowitz, Bernstein, Morrel, & Schwaller, 1993).

Production of zein microparticles by the supercritical anti-solvent process and spray-drying has also been demonstrated. Using the supercritical anti-solvent process in which zein solution was atomized into supercritical CO_2, thereby removing the solvent and precipitating the zein, microcapsule containing continuous matrices with internal voids were formed. The encapsulated lysozyme was released over 36 days indicating that this system could be used to enhance antimicrobial efficacy during the shelf-life of foods

(Zhong, Jin, Davidson, & Zivanovic, 2009). Spray-drying zein with thymol yielded wrinkled microparticles on the micron scale. When these particles encapsulated lysozyme, release was highly dependent on pH and nanoscalar matrix structure. The formulation with a continuous matrix structure showed sustained release of lysozyme at over 49 days at pH 6 (Zhong & Jin, 2009a).

The combination of zein with other materials, usually other natural polymers, provides a useful method of altering the spheres' properties. Soy potein isolate (SPI)/zein spheres, formed by an emulsification/internal gelation technique, showed sustained release profiles of the encapsulated riboflavin in simulated gastric fluid and simulated intestinal fluid that were dependent on the zein:SPI ratio (L. Chen & Subirade, 2009). When fed into an artificial digestive system consisting of gastric, duodenal, jejunal and ileal compartments, SPI/zein microspheres achieved near-zero order absorption profiles in the jejunum and ileum. Administering the microspheres with yogurt delayed release, likely allowing the riboflavin to reach the intestine with preserved activity (L. Chen, Hebrard, Beyssac, Denis, & Subirade, 2010). In another study Fourier transform infrared spectroscopy (FT-IR) and thermal analysis provided proof that chitosan could be incorporated into zein-based microspheres by adding aqueous chitosan solution to zein solution (Muller et al., 2011). Zein/pectin microspheres were formed by adding pectin solution to solutions containing zein, piroxicam, bivalent metal salts, and alcohol. The properties of the resultant spheres (loading, swelling, and yield) depended on the polymer ratio, bivalent ion, and the molecular mass of the pectin (Mukhidinov et al., 2011). Amoxicillin or indomethacin loaded PLGA/zein microspheres that released the drug at

sufficient levels for antimicrobial activity over six days were prepared by spray-drying. Again, varying the ratio of the polymers resulted in alteration in the antibiotic release as did the hydrophilic/hydrophobic nature of the encapsulated drug (Sousa, Luzardo-Alvarez, Perez-Estevez, Seoane-Prado, & Blanco-Mendez, 2010). The studies discussed above give examples of the ways in which zein can be combined with other compounds to yield spheres with altered properties, specifically changes in release kinetics. In the future, combining zein with another polymer may provide better release profiles and transfection efficiencies for zein/DNA nanospheres.

Zein microspheres have not been limited to *in vitro* studies, but have successfully been applied *in vivo* and in clinical applications. In the early 1990's a zein microsphere-based system for protein delivery called OraLease® was produced by Alkermes (DiBiase & Morrel, 1997). Several peptides and proteins were successfully encapsulated including calcitonin, erythropoietin, desmopressin (dDAVP), vasopressin, and insulin. The efficacy of the system was based on the properties of zein that allowed the system to protect the payload from the pH and enzymes of the stomach and small intestine, increase the residence time of drugs in the GI tract, and improve the transport of drugs from the lumen of the GI tract into the body. The dDAVP formulation was evaluated in a Phase I clinical trial. Results showed that the drug delivery system was well tolerated, safe and physiologically effective with a dose-dependent response (DiBiase & Morrel, 1997). More recently, tablets composed of compressed zein microspheres encapsulating ivermectin were evaluated in dogs receiving treatment for demodicidosis. These tablets had similar pharmacokinetic parameters to a commercially available treatment

formulation, with an improved bioavailability factor. The administration of a full dose or higher of ivermectin in zein microsphere tablets resulted in a 100% curative ratio, the half dose had a curative ratio of 93%, and the commercially available formulation had a curative ratio of 92%. Dogs given a triple dosage of ivermectin in zein tablets showed no adverse side effects indicating a high degree of safety and low toxicity (S.-J. Gong et al., 2010). These studies demonstrate that the efficacy of zein nano- and microspheres, specifically for oral administration, predicted by *in vitro* studies does in fact translate to *in vivo* administration. Based on the clinical trial of a zein microsphere delivery system for dDAVP it appears that zein/DNA particles possess much promise of efficacy *in vivo* and even in humans.

1.4 Objectives of thesis

The objectives of this thesis are to fabricate and characterize pDNA loaded zein nanospheres as well as to preliminarily evaluate their potential for application in oral gene delivery. Nano- and microsphere fabrication was achieved by the coacervation technique. Characterization entailed the measurement of sphere size and zeta potential in PBS and water, the observation of sphere stability at various salt concentrations, the quantification of encapsulation efficiency and loading of DNA, and the quantification of cytotoxicity. The assessment of the nanospheres' suitability for oral DNA delivery involved release studies performed in pepsin and pancreatin containing solutions. Chapter 2 describes the purpose, materials and methods, results, and conclusions of this project, while Chapter 3 describes additional studies and possible future directions for this project. These studies revealed that, as had been hypothesized, zein spheres

encapsulating DNA can be formed and have the potential to be used in multiple gene delivery applications.

Chapter 2

Zein nanospheres for DNA delivery

Abstract

Particulates incorporating DNA are promising vehicles for nonviral gene delivery as they have the ability to protect DNA and provide for sustained release and transfection. Zein is a natural, hydrophobic protein from corn that is biocompatible and capable of forming drug encapsulating nanospheres that release their payload in a controlled, enzyme mediated manner. Zein nanospheres encapsulating DNA were fabricated using a coacervation technique yielding particles that ranged from 157.8 ± 3.9 nm to 396.8 ± 16.1 nm and from -21.8 ± 4.2 mV to -46.6 ± 1.6 mV for hydrodynamic diameter and zeta potential measured in water, respectively. Spheres of 20:1 and 40:1 zein to DNA ratios flocculated in PBS while spheres formed with a higher zein to DNA ratios demonstrated limited stability in PBS. DNA encapsulation efficiency was as high as 65.3 ± 1.9% with a maximum loading of 6.1 ± 0.2 mg DNA/g zein. Encapsulated DNA maintained a primarily supercoiled conformation as did DNA released from the spheres. The cumulative release of DNA from these nanospheres in PBS was 17.8 ± 0.2% over seven days with near zero order release for the last six days of the study. These particles released approximately 20% of their payload in the first 30 minutes of incubation in pepsin containing media and released over 70% of the encapsulated DNA within two hours. In simulated intestinal fluid, release was practically instantaneous. Release studies indicate that degradation of these spheres is primarily enzymatic. Zein/DNA nanospheres demonstrated similar biocompatibility at high and low concentrations in two cell lines. Spheres showed cellular association and there was evidence of internalization

and transfection. These results indicate that nanospheres fabricated as described here are promising particles for gene delivery in gene therapy, DNA vaccination and tissue engineering applications.

2.1 Introduction

Gene delivery, the introduction of exogenous DNA into cells with subsequent expression, is applicable to the fields of gene therapy (Niidome & Huang, 2002), DNA vaccination (Donnelly, Ulmer, Shiver, & Liu, 1997), functional genomics and diagnostics (Pannier et al., 2007), and tissue engineering (Shea, Smiley, Bonadio, & Mooney, 1999). Because of the technical and safety issues associated with viral gene delivery, the use of plasmid DNA (pDNA) , which has lower immunogenicity, more flexibility in transgene capacity, and has the potential for industrial production, is an appealing alternative for gene transfer (Mintzer & Simanek, 2009). Delivery of pDNA can result in the expression of a therapeutic gene or induction of protective immunity (Donnelly, Ulmer, Shiver, & Liu, 1997; S. Li & Huang, 2000). Although the injection of naked DNA can lead to transgene expression, the level and localization of expression are limited by rapid degradation by nucleases in the serum and clearance by the mononuclear phagocyte system (Niidome & Huang, 2002). Encapsulation of DNA has the potential to improve *in vivo* response and transfection by shielding the CpG methylation patterns of plasmid DNA from the immune system, shielding the plasmid from degradation by pH and enzymes, increasing residence time, and providing a controlled release (Agarwal & Mallapragada, 2008). Nano- and microspheres incorporating DNA, which administer DNA in a controlled,

sustained, and localized/targeted manner through the use of polymer systems that entrap DNA and release through hydrolytic or enzymatic mechanisms, have been applied to gene therapy, DNA vaccination, and tissue engineering (Agarwal & Mallapragada, 2008; Panyam & Labhasetwar, 2003). When designing such a DNA delivery system an important consideration that must be made is which route of administration will be used.

Among the various routes of administration, the oral route is perhaps the most appealing due to its associated high patient compliance and convenience. The oral route has the additional advantages of presenting a large surface area of intestinal epithelium for transfection and allowing treatment of regional disorders by providing access to the luminal side of the intestine (Bhavsar & Amiji, 2007). Oral gene delivery has the potential to treat diseases associated with the gastro-intestinal tract (GI-tract) (Page & Cudmore, 2001) as well as systemic diseases (Rothman, Tseng, & Goldfine, 2005), and can provide for systemic and mucosal immunity (Jones, Corris, McDonald, Clegg, & Farrar, 1997). However, oral delivery of DNA is complicated by low pH in the stomach and DNases in the GI-tract (Loretz, Foger, Werle, & Bernkop-Schnurch, 2006), which degrade unprotected DNA. Particulates are considered a viable tool for the protection of DNA from the harsh environment of the stomach and intestine (H. Chen & Langer, 1998). Previous studies have focused on poly(lactide-co-glycolide) (PLGA) and chitosan to form DNA-loaded nano- and microspheres for oral gene delivery (Agarwal & Mallapragada, 2008; Bowman, Sarkar, Raut, & Leong, 2008; Jones, Clegg, & Farrar, 1998; Roy, Mao, Huang, & Leong, 1999). While these delivery vehicles have been shown to elicit the production of a therapeutic or immune response inducing protein, the

level of protein produced is often modest with high variability (Bowman, Sarkar, Raut, & Leong, 2008; He et al., 2005). These materials have provided a valuable proof of concept, but due to their lack of sufficient efficacy, new polymers need to be investigated.

Natural polymers have been applied to drug and gene delivery and tissue engineering, and have the advantages of providing innate degradability, and bioactivity (Dang & Leong, 2006; Malafaya, Silva, & Reis, 2007), but typically suffer from low mechanical stability, limited ability to be processed, and a relatively short release period compared to synthetic polymers (Panyam & Labhasetwar, 2003; Yannas, 2004). Zein, the prolamin or storage protein from corn, has properties that make it a promising candidate material for particulate gene delivery. Zein's hydrophobic nature makes it insoluble under physiological conditions, providing sustained release of encapsulated compounds (Hurtado-Lopez & Murdan, 2006b). Degradation of zein occurs very slowly by hydrolysis but is accelerated by the action of enzymes (Hurtado-Lopez & Murdan, 2006b) and has been shown to be especially well suited for oral delivery (DiBiase & Morrel, 1997; S.-J. Gong et al., 2010; Parris, Cooke, & Hicks, 2005). Zein has been shown to be biocompatible and to have degradation products that can enhance cell proliferation (Sun, Dong, Lin, Yang, & Wang, 2005). The functional groups available on the side chains of zein's residues provide sites where a number of modifications, including deamination and phosphorylation, can be made (Mathiowitz, Bernstein, Morrel, & Schwaller, 1993). Nano- and microspheres composed of zein can be fabricated using a simple coacervation technique, which involves no harsh solvents or high temperatures.

Zein microparticles have successfully been used to orally deliver ivermectin in a canine model (S.-J. Gong et al., 2010) and desmopressin in a Phase I clinical trial (DiBiase & Morrel, 1997). Due to the promise of this natural biomaterial for drug delivery, the objective of this study was to formulate and characterize zein nanospheres encapsulating DNA as the therapeutic compound, and to perform preliminary investigations into the degradation of these particles, using gastric and intestinal-specific release studies, as well as to characterize cell cytoxicity and association.

2.2 Methods

2.2.1 Plasmid preparation

All experiments used pDsRed2-N1 (Clontech, Mountain View, CA), a plasmid encoding for the red fluorescent protein, DsRed2. The plasmid was purified from bacteria culture using Qiagen (Valencia, CA) reagents and stored in Tris-EDTA (TE) buffer solution (10mM Tris, 1mM EDTA, pH 7.4) at -20^{o}C. Only plasmids with purity of 1.8 or better measured by 260/280 ratio (Nanodrop 2000 Spectrophotometer, Thermo Scientific, Waltham, MA) were used.

2.2.2 Sphere preparation

Zein spheres were formed by a coacervation technique. Zein (Freeman Industries LLC, Tuckahoe, New York) was first dissolved in 70% ethanol at pH 3 (pH adjusted with 1M hydrochloric acid) forming a 1% w/v zein solution. Subsequently, 1 mL of 100% ethanol pH 3 was added to 1 mL of the 1% zein solution. A total of 1 mL of DsRed plasmid DNA (1 mg/mL in TE buffer) and TE buffer were added followed by the addition of 10mL

ddH_2O (18.2 mΩ) drop by drop while vortexing. The zein to DNA ratio was determined by the amount of DNA solution added and ranged from 20:1 to 250:1. Blank spheres were formed without DNA (TE buffer substituted for DNA solution). The pH of the resultant sphere suspension was increased to 10 by the addition of 1M NaOH. Spheres were pelleted by centrifugation at 10,000 g for 1 hr at room temperature. The supernatant was removed and spheres were resuspended in ddH_2O.

2.2.3 Sphere characterization

2.2.3.1 Field-emission scanning electron microscopy

Scanning electron microscopy (SEM, S4700 Field-Emission SEM, Hitachi, Japan) was used to image and subsequently analyze sphere size and morphology. For SEM imaging spheres were resusupended in 2 mL of ddH_2O and lyophilized. The lyophilized powder was placed in a thin layer on carbon tape (Electron Microscopy Sciences, Hatfield, PA) and mounted for imaging. Spheres were then sputter coated with chromium and imaged at 10 kV and varying magnifications. Three micrographs for each ratio were analyzed using Image J (NIH, Bethesda, MD) to determine average sphere diameter.

2.2.3.2 Dynamic light scattering

A dynamic light scattering (DLS) analyzer (Zetasizer Nano ZS90, Malvern Instruments Ltd, UK) was used to measure the mean diameter and particle size distribution of spheres after dilution in either 1X Dulbecco's phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA) or ddH_2O. For dilution in PBS, spheres were first resuspended in 2 mL of water and then diluted further with 1X PBS due to the inability of spheres to be

completely resuspended in PBS. Intensity mean diameter was used to express average particle size. The same instrument was used to measure the zeta potential of the spheres by a combination of laser Doppler velocimetry and phase analysis light scattering. Sphere suspensions above were likewise diluted in 1X PBS or ddH_2O before measurement. Size, zeta potential and the polydispersity index (PdI) were measured directly after resuspension and 3 hours after resuspension for each sample to assess stability.

2.2.3.3 Aggregation

To determine the cause of aggregation and flocculation of the spheres in PBS and the inability of pelleted spheres to be resuspended in PBS, sphere size and zeta potential were similarly measured by DLS at various salt concentrations. The spheres were resuspended and diluted in ddH_2O with NaCl concentrations of 300, 150, 50 and 10 mM or 1X PBS. Size and zeta potential were measured before centrifugation and again after centrifugation and resuspension. The degree of resuspension was noted for all conditions.

2.2.3.4 Encapsulation & loading

DNA encapsulation within the spheres was quantified using a standard phenol/chloroform extraction followed by fluorescent staining with Hoechst 33258 (Invitrogen). After sphere preparation and centrifugation, the supernatant was removed to a separate conical test tube and pelleted spheres were resuspended in 5 mL ddH_2O. An equal volume of phenol/chloroform/isoamyl alcohol 25:24:1 (Fisher BioReagents, Fair

Lawn, NJ) was added to both the sphere and the supernatant solutions and these solutions were vortexed. The solutions were then centrifuged at 10,000 g for 15 minutes at 4°C and then the top, aqueous layer was removed to separate tubes to which 0.5 volume of chloroform (Fisher Chemical, Fair Lawn, NJ) was added followed by vortexing. Solutions were again centrifuged at 10,000 g for 15 minutes at 4°C and the top, aqueous layer was then removed to separate tubes which were centrifuged at 10,000 g for 15 minutes at 4°C to separate any residual chloroform from the aqueous layer. Any organic layer present was removed from the bottom of the tubes. The volumes of the aqueous DNA solutions were measured. A Hoechst standard curve was produced by graphing raw fluorescence as measured by a fluorometer (Modulus Luminometer/Fluorometer 9200-001, Turner Biosystems, Sunnyvale, CA) versus DNA concentration for various dilutions of the stock DNA solution. Samples extracted from spheres and supernatants were diluted appropriately in 1X TNE buffer (10mM Tris; 0.2M NaCl; 1mM EDTA; pH 7.4) and measured after 5 minutes incubation at room temperature with one volume Hoechst dye solution (200 ng/mL). The standard curve was then used to quantify the amount of DNA in the sphere and supernatant extraction solutions. The mass balance for DNA was closed between 80% and 101% as measured by recovery:

$$\text{Recovery} = \frac{\text{Mass of DNA in Sphere Extract} + \text{Mass of DNA in Supernatant Extract}}{\text{Mass of DNA Added}} \times 100\%$$

Eq. 1

Percent encapsulation was calculated as

$$\text{Percent Encapsulation} = \frac{\text{Mass of DNA in Sphere Extact}}{\text{Mass of DNA Added}} \times 100\%$$

Eq. 2

For each sphere preparation ratio of zein to DNA, the mass of DNA in spheres per mass of spheres was computed, using measurements of DNA encapsulation described above and mass of lyophilized spheres for each ratio, as

$$\text{Loading} = \frac{\text{Mass of DNA in Sphere Extract (mg)}}{\text{Mass of Spheres (g)}} \qquad \text{Eq. 3}$$

2.2.3.5 DNA integrity

The integrity of the encapsulated plasmid was analyzed by agarose (1%) gel electrophoresis. Sphere extraction solutions and release samples (as described below) were analyzed for plasmid integrity. DNA was detected using ethidium bromide (Fisher BioReagents). A Kodak gel documentation system (EDAS 290, Kodak, *Rochester, NY)* was used to capture digital images of the gels.

2.2.4 Release studies

For release studies, pelleted spheres were resuspended in 2 mL ddH_2O. Separate samples for each time point were prepared by diluting 150 µL of sphere suspension in 3 mL of release media. These samples were incubated in a humidified 37°C chamber for varying times. At predetermined time points, an entire sample was removed from the incubator and spheres were separated from the supernatant by centrifugation. The spheres and the supernatant were extracted with phenol/chloroform and DNA was quantified by Hoechst assay, both as described above.

DNA release from spheres into 1X PBS was measured over 7 days. Samples for loaded and blank spheres were centrifuged at 0, 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, and 168 hours. At each time point, samples were centrifuged at 10,000 g for 30 minutes at room temperature to separate spheres from the supernatant and analyzed as described above. Release was calculated:

$$\text{PBS Release} = \frac{\text{Mass of DNA in Supernatant (at Time = t)}}{\text{Total Mass of DNA (at Time = 0)}} \times 100\% \qquad \text{Eq. 4}$$

For enzyme release studies, either pepsin or pancreatin were used. To simulate gastric release, pepsin (MP Biomedicals, Solon, OH) in 0.01 M KH_2PO_4-citrate buffer, pH 3.5, as previously described (Parris, Cooke, & Hicks, 2005) with the omission of Tween 20, was prepared and added to zein sphere aliquots as described above(final 10:1 w zein/w pepsin). Entire samples were removed from the incubator at 0, 20, 40, 60, 80, 100, 120, 180 and 240 minutes. Samples were then centrifuged at 10,000 g for 15 minutes at room temperature and analyzed. Because DNA was degraded in the release media, release was calculated according to the mass of DNA remaining in the spheres with the mass of DNA in a 150 μL sphere suspension aliquot as the denominator:

$$\text{Pepsin Release} = \left(1 - \frac{\text{Mass of DNA in Spheres (at Time = t)}}{\text{Mass of DNA in Prerelease Aliquot}}\right) \times 100\% \qquad \text{Eq.5}$$

Pancreatin (MP Biomedicals)-containing release media was made according to the USP XXIX protocol for simulated intestinal fluid (SIF). Briefly, 3.4 g of monobasic potassium phosphate (MP Biomedicals) was dissolved in 125 mL ddH_2O water, to which 38.5 mL of 0.2 N NaOH, 250 mL water, and 5 g of pancreatin were added. The pH of the solution was raised to 6.8 with 0.2 N NaOH and the volume was increased to 500 mL.

A volume of 3 mL of SIF was added to the sphere suspension aliquots as described above. Aliquots were removed at 0, 20, 40, 60, 90, 120, 150, 180, 240, 300, and 360 minutes. Suspensions were centrifuged for 15 minutes at 10,000 g and room temperature and analyzed. Release was calculated in the same manner as PBS release:

$$\text{Pancreatin Release} = \frac{\text{Mass of DNA in Supernatant (at Time = t)}}{\text{Mass of DNA in Spheres} + \text{Mass of DNA in Supernatant (at Time = 0)}} \times 100\%$$

Eq.6

Because enzymatic degradation by pepsin or pancreatin was not stopped prior to centrifugation of the spheres, time of centrifugation was included in the time on the release graph.

2.2.5 Cellular response

2.2.5.1 Cell culture

Human embryonic epithelial kidney cells, HEK 293T (ATCC, Manassas, VA), were cultured in T-75 flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Carlsbad, CA) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). For seeding, cells were counted using a hemocytometer and trypan blue staining for viable cells after being dissociated with 1mM EDTA. Human colon carcinoma cells, Caco-2, were obtained from ATCC and were cultured in T-75 flasks in Eagle's minimum essential medium (EMEM, ATCC) supplemented with 20% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). For seeding, cells were dissociated with 0.05% trysin/EDTA (Gibco). For both cell lines, cells were seeded into 48 well

plates or 8 well Nunc Lab-Tek chambered coverglass slides (Thermo Scientific) at a density of 30,000-33,000 cells per well for HEK 293-T cells and 25,000 cells per well for Caco-2 cells.

2.2.5.2 Cytotoxicity

Cytoxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (Invitrogen). Cells were seeded in 48 well plates as described above and after 18 hours of culture, zein nanospheres containing DNA were added to the culture media. Nanospheres were formed at a zein to DNA ratio of 80:1 and resuspended in 1 mL ddH_2O prior to delivery to cells. Spheres containing 0.05, 0.1, 0.25, 0.5, 1.0 or 2.0 μg of DNA were then diluted in OptiMem (GIbco) to a final volume of 75 μL and were added to triplicate wells. Cells were imaged to observe cell morphology (Leica DMI 3000B, Bannockburn, IL) and the MTT assay was conducted 24 hours after the addition of zein nanospheres. The assay kit was used according to the manufacture's protocol scaled up appropriately for 48 well plates. Assays were performed in triplicate on duplicate days.

2.2.5.3 Confocal microscopy

To verify that confocal microscopy could be used to image zein/DNA nanospheres without labeling, the autofluorescence of zein was investigated. The autofluorescence of various zein concentrations in 70% ethanol was measured in a fluorometer with ultraviolet and blue modules. Confocal microscopy was used to assess cellular morphology in response to the zein nanospheres, as well as to analyze cellular association

of the spheres. Caco-2 and HEK 293T cells cultured with 80:1 or 250:1 nanospheres were imaged using a confocal microscope (Olympus IX 81, Olympus, Center Valley, PA). Cells were seeded as described above into 8 well coverslides. Zein spheres, which exhibit autofluorescence were visualized with an excitation wavelength of (405 nm) and an emission wavelength of (590 nm) and these images were overlayed with corresponding phase images.

2.2.6 Statistics

All experiments were performed between three and six times. Comparative analyses were completed using a student's t-test or one-way ANOVA followed by Tukey's multiple comparison test for multiple data points, both at a 95% confidence level using Prism software (GraphPad Prism 5, LaJolla, CA). Mean values with standard error of the mean (SEM) are reported for all data.

2.3 Results & Discussion

2.3.1 Sphere formation

Coacervation, the separation of solutions into colloidal systems with two liquid phases (Gander, Blanco-Prieto, Thomasin, Wandrey, & Hunkeler, 2006), was used to form spheres. This separation involves the formation of one phase rich in polymer (the coacervate) and another lacking polymer, which is brought about by the partial desolvation of a previously dissolved polymer (Arshady, 1990; Gander, Blanco-Prieto, Thomasin, Wandrey, & Hunkeler, 2006; Madan, 1978). Decreasing the ethanol concentration of dissolved zein solutions by the addition of aqueous solutions results in

the necessary desolvation and the formation of a zein-rich nanosphere phase (Hurtado-Lopez & Murdan, 2005). This method has been previously used to encapsulate a variety of drugs (Fu, Wang, Zhou, & Wang, 2009a; Liu, Sun, Wang, Zhang, & Wang, 2005; Suzuki et al., 1989; H.-J. Wang, Lin, Liu, Sheng, & Wang, 2005), however, to the best of our knowledge, no studies have reported on the encapsulation of DNA in zein. In this study, DNA was included with the aqueous solution, which resulted in the encapsulation of DNA within the zein nanosphere phase formed through coacervation, thus resulting in spheres that incorporated DNA. Encapsulation of hydrophilic DNA within the hydrophobic zein spheres was enhanced by lowering the pH of the initial ethanolic zein solution, which resulted in a net positive charge on the protein due to its higher isoelectric point (Cabra et al., 2005). This change in charge promoted the electrostatic interaction of the zein and the negatively charged plasmid DNA. Zein nanospheres formed by coacervation and encapsulating DNA formed a suspension free of visible aggregates during formation while blank spheres aggregated slightly (data not shown). These observations indicate that the DNA may act as a stabilizer for zein nano- and microsphere dispersions. Zein nanospheres centrifuged at a pH less than 9 could not be resuspended (data not shown). By raising the pH to above 9, in this study the pH was raised to 10, the surface charge of the spheres was lowered to ~-70 mV (a value sufficiently far from zero) resulting in sufficient repulsive forces between spheres to prevent irreversibly aggregation of the pelleted spheres. This sphere formation procedure allowed for the simple and repeatable production of DNA-loaded nanospheres which were then characterized.

2.3.2 Characterization

2.3.2.1 SEM

The morphology and size were characterized for zein/DNA nanospheres using SEM images. SEM images confirmed the spherical nature of the nanospheres with smooth surfaces (Figure 2-1) at all ratios of zein to DNA. Average sizes measured from SEM images were 73.0 ± 0.8 nm (Figure 2-1A), 95.4 ± 1.0 nm (Figure 2-1B), 133.2 ± 1.5 nm (Figure 2-1C), 196.1 ± 2.6 nm (Figure 2-1D), and 270 ± 4.5 (Figure 2-1E) for 20:1, 40:1, 80:1, 160:1 and 250:1 spheres respectively (Figure 2-1F). The spheres prepared in this study were relatively small compared to zein nanospheres encapsulating various compounds prepared by similar methods (Fu, Wang, Zhou, & Wang, 2009a; Liu, Sun, Wang, Zhang, & Wang, 2005; Parris, Cooke, & Hicks, 2005; H.-J. Wang, Lin, Liu, Sheng, & Wang, 2005). Sphere size increased linearly as the zein to DNA ratio increased. This increase could be due to a lower mass of DNA associated with the surface which may result in an increase in interfacial tension between the hydrophobic sphere surface and the aqueous medium, leading to a greater radius of curvature (Hurtado-Lopez & Murdan, 2005). The increase in sphere size with an increase of zein to incorporated compound has been reported elsewhere (Hurtado-Lopez & Murdan, 2005). This data suggests that the amount of DNA present affects the nucleation and growth of zein/DNA nanospheres and that size can be controlled by adjusting the zein to DNA ratio.

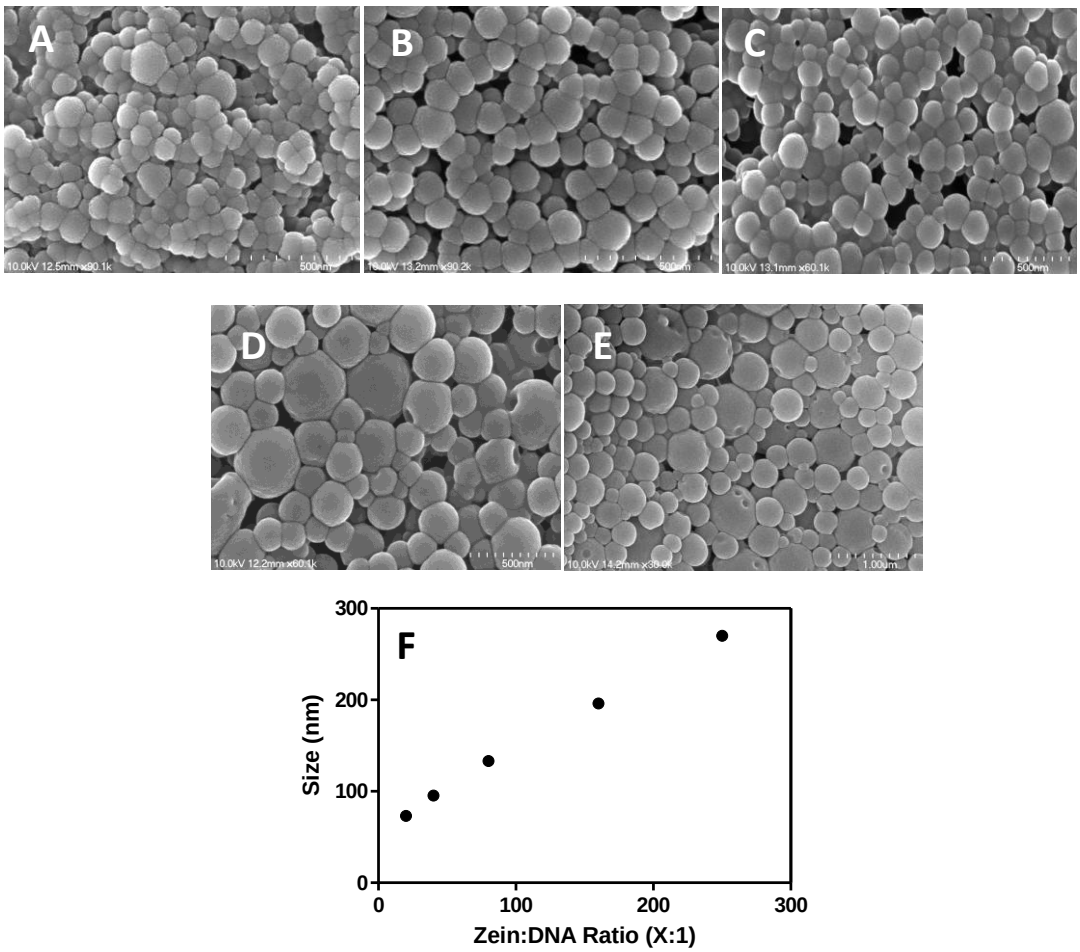

Figure 2-1: SEM of zein-DNA nanospheres formed at 20:1 (A, 90k magnification), 40:1 (B, 90k), 80:1 (C, 60k), 160:1(D, 60k), 250:1 (E, 30k) zein:DNA ratios; average diameter for each ratio measured from SEM images (F), reported as mean ± SEM.

2.3.2.2 DLS

Size and zeta potential were measured to further characterize and assess the stability of zein/DNA nanospheres. Immediately after resuspension, the hydrodynamic diameters of zein/DNA nanospheres measured in water increased with increasing zein:DNA ratio for

40:1, 80:1, and 160:1 zein: DNA ratios (157.8 ± 3.9 nm, 266.5 ± 28.2 nm, and 385.6 ± 25.0 nm, respectively), similar to the trend observed in SEM images, however there were not significant differences in size between 20:1 (178.9 ± 10.9 nm) and 40:1 and between 160:1 and 250:1 (396.8 ± 16.1 nm) for spheres measures by DLS (Figure 2-2A). The average measured diameter from DLS analysis was found to be greater than the corresponding diameter measured by SEM for all ratios (Zhong & Jin, 2009b), which may be attributed to the presence of residual solvent in the spheres that had not been dried, and/or swelling, which has been reported for zein fibers in an aqueous medium (Jiang & Yang, 2011) due to zein's hygroscopic character (Gennadios & Weller, 1994). Zein nano- and microspheres were, for the most part, stable in water over three hours, although 20:1 and 40:1 zein:DNA nanospheres exhibited a significant increase in diameter over the three hours of incubation in water, indicating the possibility of limited aggregation at these ratios. For all zein:DNA ratios PdIs varied between 0.2 and 0.1 demonstrating uniformity of the spheres (Figure 2-2C).

Size and PdI measurements, over 10 μm and 0.5, respectively, for 20:1 and 40:1 zein:DNA spheres in PBS align with the observation of flocculation at these ratios (Figure 2-2B, 2-2D). For 80:1 and higher zein: DNA ratios, spheres did not flocculate and measured sizes were much lower than those of 20:1 and 40:1 particles in PBS, but were still higher than those measured in water. Larger sizes, between 740 and 1120 nm, measured in PBS were attributed to salt induced aggregation (Everett, 1988), which was subsequently verified. Spheres formulated at the higher zein:DNA ratios were found to be relatively stable over three hours in water and PBS. The zeta potentials of the spheres

formed at the various zein to DNA ratios were relatively uniform in both water and PBS, with values distributed between -20 and -50 mV (Figure 2-2E,2-2F). Negative values were expected as zein has an isoelectric point of 6.8 (Cabra et al., 2005) and DNA is also negatively charged at physiological pH.

Overall, the characteristics of the zein/DNA nanospheres formulated in this study suggest that the spheres are well suited for oral administration, particularly for DNA vaccination, as particles less than 10 μm in diameter are taken up transparacellularly by Peyer's patches in the intestine (O'Hagan, 1996), a target for DNA encoding antigens because of their proximity to gut-associated lymphoid tissue (Clark, Jepson, & Hirst, 2001). Stabilization of the 20:1 and 40:1 particles could lend them to oral administration for gene therapy where widespread transfection is desired since particles less than 200 nm in diameter are taken up by endocytosis by intestinal enterocytes (O'Hagan, 1996). While the negative surface charge measured for the zein/DNA spheres may prevent favorable interactions with the negatively charged cell membrane, a negative surface charge may impart mucoadhesive properties to the particles, which could increase intestinal residence time (Hombach & Bernkop-Schnurch, 2010), further enhancing the ability of these particles to be used for oral administration of DNA therapeutics. Although the efficacy of the zein/DNA nanospheres formulated as described in this study could possibly benefit from stabilization under physiological conditions and alteration of the surface charge, the spheres have appropriate size and uniformity for oral delivery of genes.

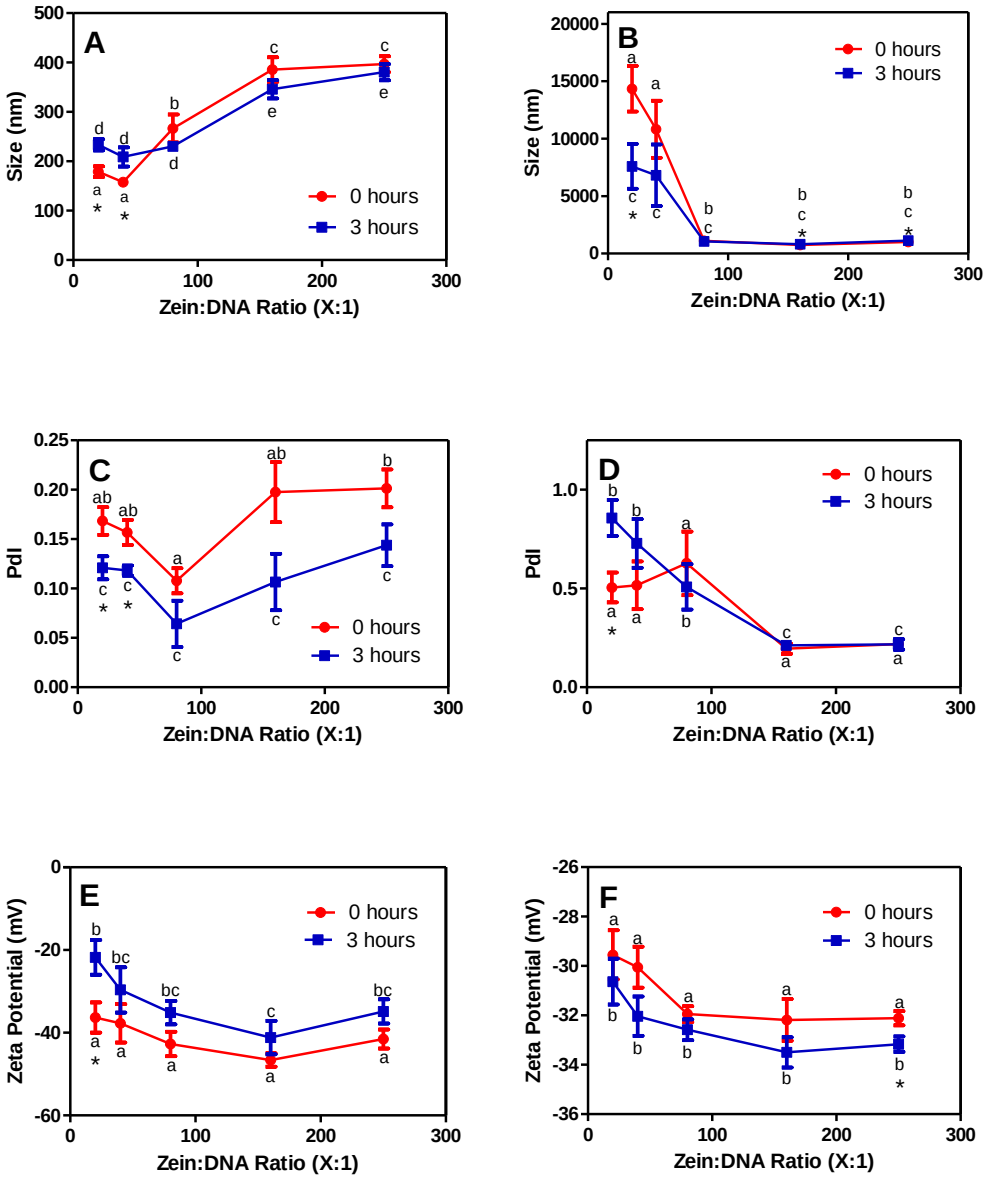

Figure 2-2: Size, PdI, and zeta potential for zein/DNA nanospheres formed at various zein to DNA ratios measured directly after resuspension (0 hours, ●) and three hours after resuspension (3 hours, ■) in water (A, C, E) or PBS (B, D, F). Data points labeled with the same letter are not significantly different, while those labeled with asterisks vary significantly between the zero hour and 3 hour measurements. All data points are reported as mean ± SEM, with n=6

2.3.2.3 Aggregation

Since aggregation in PBS of the zein/DNA particles was observed at all zein:DNA ratios and flocculation took place at 20:1 and 40:1 zein:DNA ratios, the cause of this instability was investigated. It is known that the salt concentration of the suspension solution can affect the stability of a colloidal system (Everett, 1988), so this variable was investigated. When resuspended in water (pH 7-8) with varying concentrations of sodium chloride, the degree of sphere resuspension observed varied. The 80:1 zein to DNA ratio was selected for the investigation of the effect of salt concentration because this ratio did not flocculate, which can cause the size reading to exceed the limit of the DLS instrument. As with PBS, most of the spheres remained pelleted in 300 mM and 150 mM NaCl, while most but not all of the pellet was resuspended in 50 mM NaCl, and spheres were completely resuspended in 10 mM NaCl. The size of the spheres that were able to resuspend in PBS, 300 mM, 150 mM, and 50 mM NaCl was significantly larger than their initial size indicating aggregation (Figure 2-3A). Also the PdI, which can sometimes serve as an indicator of aggregation, was high for spheres resuspended in PBS and 300 mM NaCl (Figure 2-3B). Although the measured zeta potentials were not different for the various salt concentrations (Figure 2-3C), it appears that electrostatic shielding due to salt concentration resulted in the destabilization and increase in the measured size of 80:1-250:1 particles (Figure 2B) and flocculation of 20:1 and 40:1 particles (Y. Wu, Guo, Yang, Wang, & Fu, 2006). Salt concentration dependent flocculation of zein particles was previously noted (Patel, Bouwens, & Velikov, 2010). Based on these results, it was concluded that the salt concentration of PBS caused the

observed aggregation or flocculation of the spheres formulated at various zein:DNA ratios.

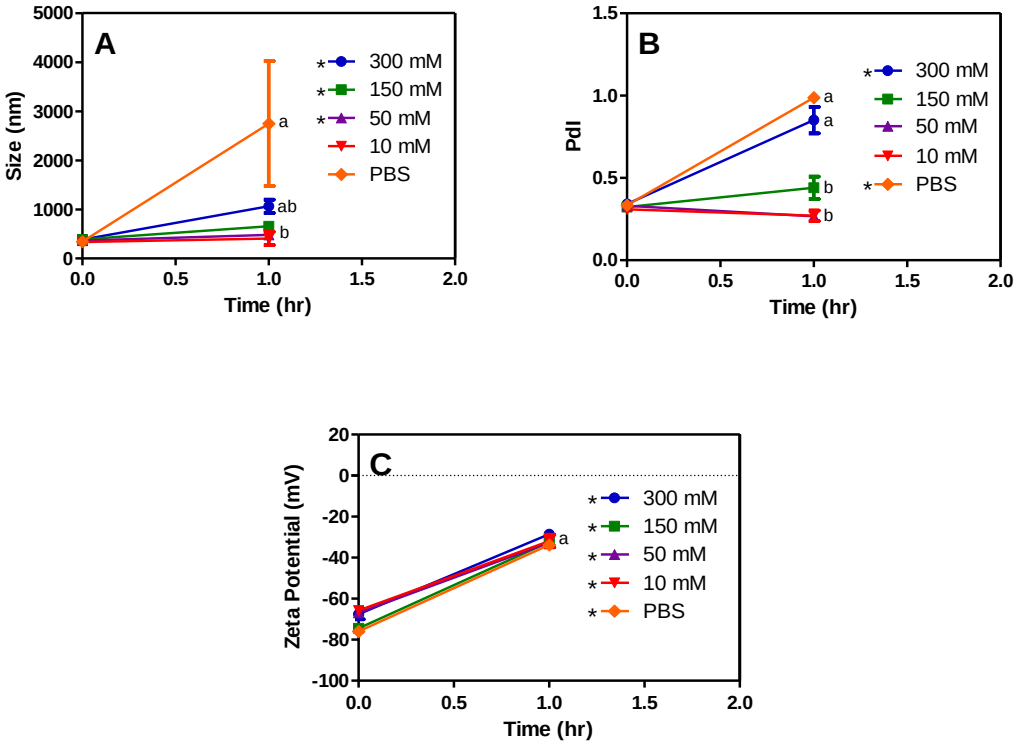

Figure 2-3: Size, PdI, and zeta potential for nanospheres resuspended at various salt concentrations (A, B, C respectively). One hour data points labeled with the same letter do not vary significantly while asterisks in the legend denote a significant difference between zero and one hour measurements. All data points are reported as mean ± SEM, with n=3.

2.3.2.4 Encapsulation & loading

To determine the efficiency of DNA encapsulation for the method of sphere production used in this study, percent encapsulation and loading were measured. Similar to sphere

size, encapsulation and loading of DNA in zein nanospheres was dependent on the ratio of zein to DNA (Figure 2-4). Percent of DNA encapsulation increased with the ratio of zein:DNA up to 160:1 where it reached its maximum of 65.3% ± 1.9%. Loading was highest with 6.1 ± 0.2 mg DNA/g zein for spheres with a zein:DNA ratio of 40:1. The encapsulation of hydrophilic drugs in zein is more difficult to achieve than the encapsulation of hydrophobic drugs presumably due to hydrophilic/hydrophobic interactions. The DNA loading reported here is typical for the encapsulation of relatively hydrophilic drugs in zein nano- and microspheres (Fu, Wang, Zhou, & Wang, 2009a; H.-J. Wang, Lin, Liu, Sheng, & Wang, 2005) while higher loading (up to 190 mg drug/g zein) has been attained by conjugating the hydrophilic drug to zein prior to particle formation (Suzuki et al., 1989). The loading achieved in this study is also comparable to that achieved with most PLGA nano- and microspheres (Astete & Sabliov, 2006), but is much lower than DNA loading into chitosan/DNA nanoparticles (Mao et al., 2001). Because nanospheres formed at a zein to DNA ratio of 80:1 did not flocculate in PBS and had relatively high encapsulation efficiency and loading, this ratio was chosen for further characterization and cell studies.

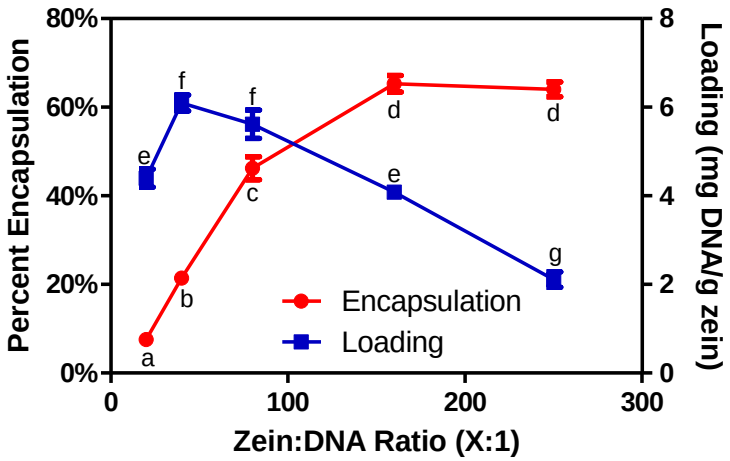

Figure 2-4: Percent encapsulation (●)and loading (■)of nanospheres prepared at various zein to DNA ratios. Data points labeled with the same letter are not significantly different. Data is reported as mean ± SEM with n=6.

2.3.2.5 DNA integrity

DNA integrity is necessary for efficient gene delivery, specifically maintenance of the supercoiled conformation; thus integrity measurements were made to ensure that encapsulation of the DNA within the zein spheres did not damage the plasmid DNA structure. Agarose gel electrophoresis revealed that DNA was not damaged by encapsulation under the conditions used in this study (Figure 2-5A). DNA integrity was also maintained in spheres during seven days of incubation in PBS for the release studies, as indicated by a supercoiled to nicked ratio similar to that of stock DNA (Figure 2-5B). Although the nicked band appears to be larger in release samples than in the stock DNA, there does not appear to be an increase in the ratio of nicked to supercoiled DNA over the

seven day span of the release study (Figure 2-5C). While the stability of the DNA in and released from zein particles is critical for oral delivery applications, it also suggests that these zein/DNA nanospheres could be used for the fabrication of scaffolds with sustained gene delivery in tissue engineering (Shea, Smiley, Bonadio, & Mooney, 1999), in that this stability is superior to that of DNA encapsulated in PLGA microspheres, which produce degradation products that lower the local pH and can degrade DNA (Jang & Shea, 2003).

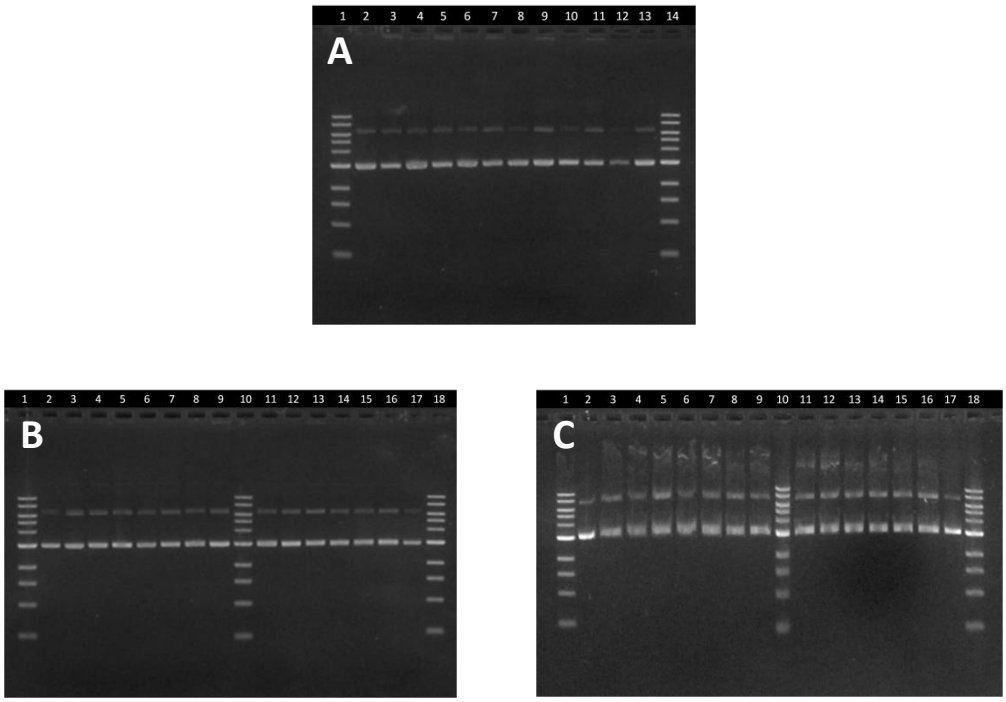

Figure 2-5: Agarose gel electrophoresis image of extracted samples for spheres made at various zein to DNA ratios (A): lane 1, ladder; lane 2, stock DNA; lane 3, 20:1 spheres; lane 4, 20:1 supernatant; lane 5, 40:1 spheres; lane 6, 40:1 supernatant; lane 7, 80:1 spheres; lane 8, 80:1 supernatant; lane 9, 160:1 spheres; lane 10, 160:1 supernatant; lane 11, 250:1 spheres; lane 12, 250:1 supernatant; lane 13, stock DNA; lane 14 ladder. Agarose gel image of DNA extracted from spheres (B) and supernatants (C) at various time points in the PBS release study: lane 1, ladder; lane 2, stock DNA; lane 3, 0 hr; lane 4, 1 hr; lane 5, 3 hr; lane 6, 6 hr; lane 7, 9 hr; lane 8, 12 hr; lane 9, 24 hr; lane 10, ladder; lane 11, 48 hr; lane 12, 72 hr; lane 13, 96 hr; lane 14, 120 hr; lane 15, 144 hr; lane 16, 168 hr; lane 17, stock DNA; lane 18, ladder.

2.3.3 Release studies

DNA release from zein nanospheres was measured in a standard PBS buffer as well as solutions designed to mimic gastric and intestinal fluids. Nanospheres formulated at the

80:1 zein to DNA ratio released encapsulated DNA relatively rapidly for the first 12 hours of the PBS release study (Figure 2-6). This period of fast release was followed by a near zero order release profile for the remainder of the study. After seven days in PBS only 17.8 ± 0.2% of the encapsulated DNA was released. Sustained release of encapsulated drug in PBS has previously been demonstrated for zein nano- and microspheres (Hurtado-Lopez & Murdan, 2005). These results indicate that if breakdown occurs by hydrolysis, it does so slowly, allowing for the release of DNA in a sustained manner over at least one week and likely much longer. This sustained release also indicates that DNA is encapsulated within the particles rather than being absorbed to the surface.

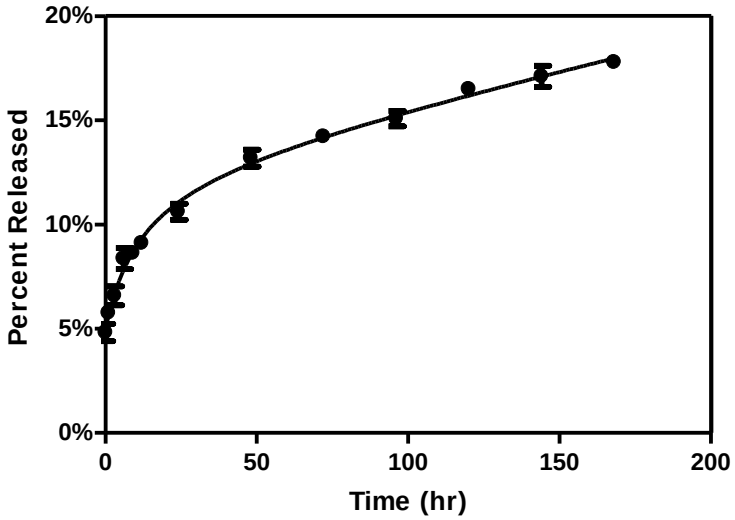

Figure 2-6: Release of DNA from 80:1 zein spheres incubated in PBS at 37°C over 7 days. Data reported as mean ± SEM, with n = 5.

The ability of zein nano/microspheres to protect and deliver DNA to the stomach and intestine was investigated by performing release studies in pepsin-containing medium and simulated intestinal fluid containing pancreatin, respectively. Release in pepsin solution began slowly but accelerated in the first 55 minutes (Figure 2-7), possibly due to the lag in heating of the samples as pepsin has an optimum activity temperature of 50°C (Hirano & Miura, 1979). This acceleration was followed by near zero order release that plateaued at 115 minutes. The cumulative release after 255 minutes was 77.5 ± 0.6% of encapsulated DNA. Because typical residence time in the stomach is approximately two hours for post-prandial administration (Klein, Wunderlich, Stippler, & Dressman, 2005), and the nanospheres investigated here released over 70% of their payload in this time, modifications may be necessary to achieve efficient oral delivery via this method of administration. However, since pre-prandial residence time is shorter, at approximately 30 minutes (Klein, Wunderlich, Stippler, & Dressman, 2005), and at this time point about 80% of the DNA remained encapsulated, these spheres have the necessary enzyme resistance to transport the bulk of their payload through the stomach and into the intestine.

In simulated intestinal fluid, release was nearly instantaneous (Figure 7), indicating that release of the DNA from the spheres was mediated by enzymes found within the intestine. Spheres formulated as described in this study would likely deliver DNA to the lumen of the intestine. While transfection by free DNA delivered to a patent rat duodenum has been reported (Rothman, Tseng, & Goldfine, 2005), it is unlikely that a high level of transfection would be achieved by free DNA in the intestine due to the

presence of DNases and inefficient uptake of free DNA. If these spheres are intended to be taken up intact they must first be modified, possibly by crosslinking, to reduce their rate of degradation by the enzymes in pancreatin. The results of the release studies reported here are supported by release and degradation studies for zein nanospheres encapsulating essential oils. Release was monitored in pepsin containing media, demonstrating sustained release, and the degradation of zein incubated with swine manure solids was measured, indicating rapid degradation (Parris, Cooke, & Hicks, 2005). The release studies performed in this study demonstrate that upon oral administration, degradation of zein/DNA nanospheres will take place by enzyme-mediated mechanisms rather than hydrolysis. Synthetic biodegradable polymers like PLGA are often degraded by hydrolysis or oxidation reactions and not by enzymes (van Dijkhuizen-Radersma, Moroni, van Apeldoorn, Zhang, & Grijpma, 2008). Zein/DNA nanospheres are degraded to varying degrees by enzymes in the GI-tract, which would likely result in the delivery of the majority of their payload to the intestine upon oral administration.

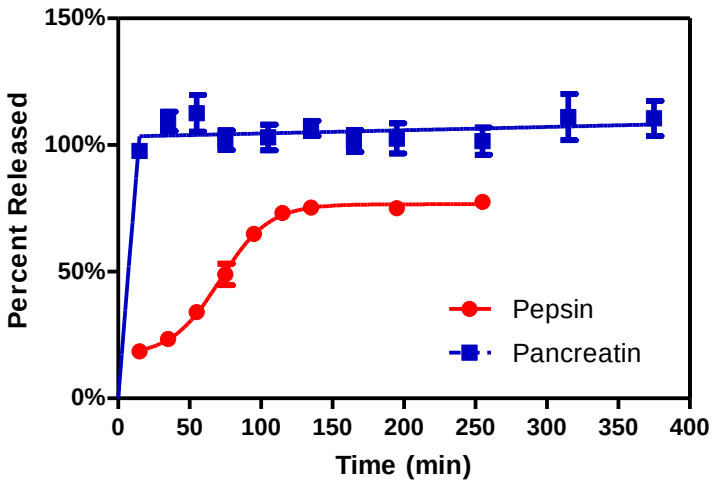

Figure 2-7: Release of DNA from spheres in pepsin (●) or pancreatin (■) containing solution. Data reported as mean ± SEM with n = 3 for pepsin mediated release and n=6 for pancreatin mediated release.

2.3.4 Cell studies

Cell studies were conducted to determine zein/DNA particles cytotoxicity and cellular association/internalization. For MTT assays, absorbance values at 570 nm normalized to control cells (without particles) showed that these particles were mildly cytotoxic to Caco-2 cells and more cytotoxic to HEK 293T cells at all concentrations (Figure 2-8). Chitosan/DNA nanoparticles were also shown to be more cyctotoxic to HEK 293T cells than other cell lines (Corsi, Chellat, Yahia, & Fernandes, 2003). For both cell types there was a lack of a dose response indicating that the presence of particles affected proliferation to an extent, but the quantity of particles did not determine the cellular response. Although the MTT data indicates cytotoxicity in the HEK 293T cells,

morphology appeared normal for both cell types incubated with zein/DNA nanospheres (data not shown). It should be noted that this was a short term study and that proliferation levels may increase at later time points, especially since zein degradation products have been shown to enhance cellular viability (Sun, Dong, Lin, Yang, & Wang, 2005). This type of cytotoxicity has not previously been reported for zein, possibly because cells were seeded onto zein films rather than having zein placed on top of the cells (Dong, Sun, & Wang, 2004; Sun, Dong, Lin, Yang, & Wang, 2005; H.-J. Wang, Lin, Liu, Sheng, & Wang, 2005).

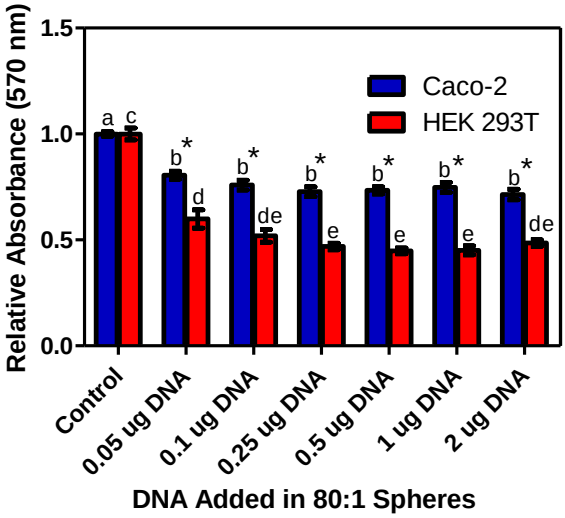

Figure 2-8: Cytotoxicity of zein-DNA nanospheres quantified by MTT assay for Caco-2 and HEK 293T. Data bars labeled with the same letter do not vary significantly, and columns labeled with asterisks vary significantly between HEK 293T and Caco-2 values. Data reported as mean ± SEM with n=6.

For confocal imaging nanospheres were not labeled as zein was observed to autofluoresce at multiple wavelengths of excitation and emission. For example, there was a linear

relationship between zein concentration and fluorescence for excitation wavelengths of 365-395 nm and 465-485 nm, indicating that zein autofluoresces at under these conditions (Figure 2-9).

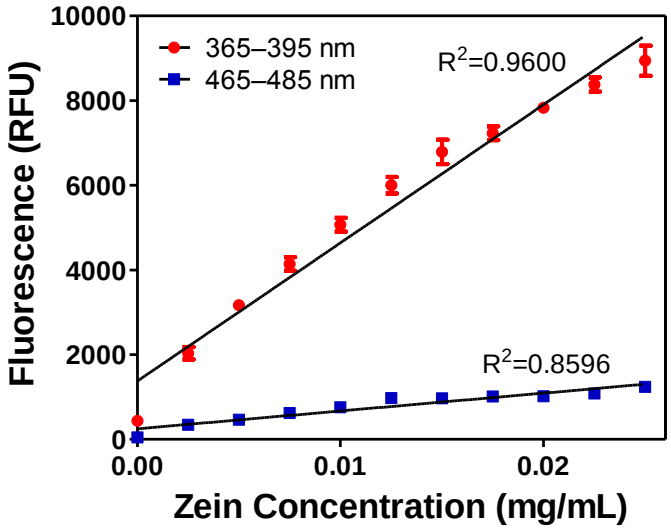

Figure 2-9: Autofluorescence of zein at various concentrations using ultraviolet (365-395 nm) and blue (465-485 nm) modules.

Confocal images of HEK 293T and Caco-2 cells with added particles revealed a high degree of particle association with both cell types (Figure 2-10). Nanospheres were found to concentrate on cells more than in the intercellular spaces of the culture slides. Z-series images showed that many of the spheres were located in the same area and same plane as many cells rather than on top of cells (data not shown) indicating that particles were likely internalized. There also appeared to be limited transfection in some images acquired using the fluorescence microscope (data not shown) suggesting that these nanospheres have the potential to be used to deliver DNA when properly optimized.

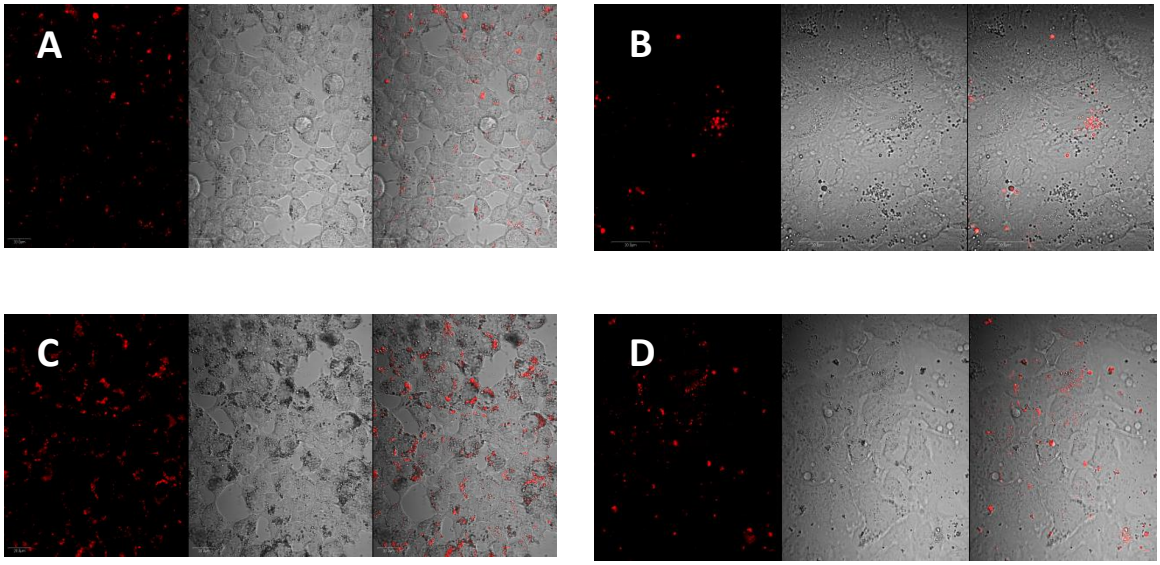

Figure 2-10: Confocal images of HEK 293T cells with associated 80:1 spheres (A, 60X magnification, 0.1 ug DNA/well) and 250:1 spheres (C, 60X, 0.1 ug DNA/well), and Caco-2 cells with associated 80:1 spheres (B, 60X, 0.1 ug DNA/well) and 250:1 spheres (D, 60X, 0.1 ug DNA/well). It should be noted that not all of the particles fluoresced in the images as some were out of the focal plane of the microscope.

2.4 Conclusions

Zein, a natural protein, was investigated as a possible material for the formation of spheres encapsulating DNA for oral gene delivery. Nanospheres were formed using a simple coacervation method, without the use of harsh solvents or temperatures, resulting in the preservation of DNA integrity. Results from this study indicate that particles formulated as described here were of the proper size for oral administration and the fabrication of tissue engineering scaffolds, with tunable sizes and surface charges that may promote intestinal retention. DNA encapsulation efficiencies were maximized to acceptable levels at the higher zein to DNA ratios, while loading was comparable to that

of other hydrophilic compounds encapsulated in zein and that of DNA incorporated into PLGA nano- and microspheres. Release studies indicate enzyme-mediated DNA release, with sustained release in the absence of enzymes. Nanospheres also showed good biocompatibility with the Caco-2 cell line. Microscopy of cells with nano/microspheres indicated that the particles were highly associated with cells and were possibly internalized with limited transfection. Future work should include the modification of these spheres to improve the resistance to degradation by intestinal enzymes so that intact spheres can be taken up. Modifications improving cellular uptake and transfection should also be investigated. Zein appears to be an excellent potential tool for the delivery of DNA with the ability to be fine tuned for specific applications including oral gene delivery, DNA vaccination, and tissue engineering.

Chapter 3

3.1 Introduction

In Chapter 2 zein/DNA nanospheres were formed using a simple coacervation method and were found to be of the proper size for oral administration and the fabrication of tissue engineering scaffolds. DNA encapsulation efficiencies were maximized to about 65% at the higher zein to DNA ratios, while loading, about 6 mg DNA/g zein, was comparable to that of other hydrophilic compounds encapsulated in zein and that of DNA incorporated into PLGA particles (Astete & Sabliov, 2006). Release studies indicated that release of DNA from these spheres takes place much more quickly by enzymatic degradation than by hydrolysis. Cell association was demonstrated by microscopy as well as possible internalization and isolated transfection, and nanospheres showed good biocompatibility with an intestinal cell line. Although the potential of DNA-loaded zein nanospheres was demonstrated in Chapter 2, there is much room for improvement in the properties of these particles to optimize for applications including not only oral delivery, as described in Chapter 2, but also intramuscular delivery and tissue engineering applications, as described below. DNA loading should be increased so that a lower mass of particles can be used to deliver a sufficient dose of DNA. Current levels of encapsulation efficiency, DNA activity, and biocompatibility should be maintained or improved upon in the process of improving loading. Also zein/DNA spheres need to remain stabilized against aggregation under physiological conditions. The resistance of the particles to degradation by gastric and especially intestinal enzymes will likely need to be enhanced to achieve sufficient *in vivo* transfection to be therapeutic without the administration of an excessive amount of DNA. Internalization should be better

characterized and possibly increased *in vitro* to better facilitate transfection. A strategy for nuclear localization may also be needed to achieve significant levels of transfection. A Caco-2 monolayer model should be used to assess the intestinal uptake and transport of particles *in vitro,* and *in vivo* administration and efficacy should then be assessed in an animal model. Applications in tissue engineering and intramuscular injection should also be explored. These improvements to the zein/DNA nanospheres formulated in this thesis and the investigation of new applications should render them more useful and increase the likelihood of their clinical application.

3.2 Improving loading

The current mechanism of promoting DNA incorporation into zein nanospheres namely, the use of low pH during the first steps of fabrication to facilitate electrostatic interactions between zein and DNA, has resulted in limited success. The maximum loading achieved was 6.1 ± 0.2 mg DNA/g zein, and could be improved upon. These results do not mean that the current strategy should be abandoned. Instead it may be possible to achieve significantly higher levels of loading by optimizing the pH to improve the affinity of zein and DNA for each other. For example Figure 3-1 shows that in a preliminary experiment using 85% ethanol at pH 3 rather than 70% and 100% ethanol, both at pH 3, as described in Chapter 2 to form 40:1 spheres, encapsulation efficiency was significantly improved, corresponding to an estimated loading of 11.5 mg DNA/g zein. This difference in encapsulation likely resulted from the unreliability of reading the pH of absolute ethanol with a standard pH meter, meaning it is possible that the combination of 70% ethanol at pH 3 and 100% ethanol at pH 3 did not result in a final solution with a pH of 3.

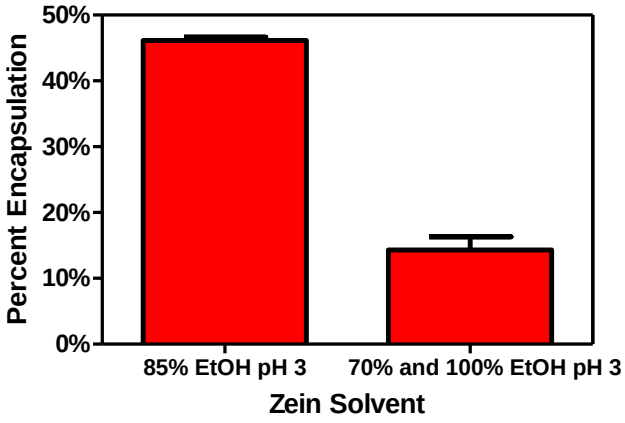

Figure 3-1: Encapsulation efficiencies of 40:1 spheres formed from zein dissolved in 85% ethanol at pH 3 or equal parts of 70% and 100% ethanol, both pH 3. Reported as mean ± SEM with n=3.

It is likely that the optimal conditions will involve the maintenance of the pH of the sphere forming solution between the isoelectric points of DNA and zein. This pH range should result in opposing net charges on zein and DNA. Optimization of loading in this way may also involve altering the pH of the zein solution and the water added to induce coacervation. This parameter was not optimized as the primary goals were to form zein/DNA spheres and to determine how to characterize them.

If the pH-induced electrostatic interaction technique cannot be optimized to levels of DNA loading high enough for efficient gene delivery, other chemical modifications may be necessary. It may be possible to mimic the complexation of DNA with commonly used cationic polymers and lipids by modifying the charge of zein using other chemical

methods. However, it may be difficult to modify zein while maintaining its solubility properties, which make coacervation possible, as well as its biocompatibility (Futami, Kitazoe, Murata, & Yamada, 2007). Another method of improving loading may be the conjugation of DNA to zein prior to sphere formation. This technique has been used to achieve higher levels of loading for other hydrophilic drugs in zein particles (Suzuki et al., 1989). However, this method may not be feasible as the activity of DNA modified in this way may not be maintained. The improvement of DNA loading by a method such as those described above should increase the efficacy of zein/DNA nanospheres at achieving significant and sustained transfection.

3.3 Stabilization under physiological conditions

All of the nanospheres described in this thesis would likely need some sort of modification endowing them with stability under physiological conditions to be effective gene delivery vehicles *in vivo.* Particularly nanospheres produced at the 20:1 and 40:1 zein to DNA ratios need stabilization under physiological conditions. Stability of colloids can be achieved through repulsion of particles based on surface charge or through steric stabilization (Myers, 1999). Because electrolytes reduce the electrostatic repulsion between particles, it may be best to sterically stabilize the zein/DNA particles. It may be possible to stabilize the sphere using an adsorbed surfactant or a surface grafted polymer such as poly(ethylene glycol) and polyethyleneimine (Qi, Colfen, & Antonietti, 2001), the latter of which could have the added benefit of DNA complexation (Patil, Rhodes, & Burgess, 2005). The use of surface modifications for stabilization will have to be balanced with the effects of the modifications on encapsulation efficiency,

biocompatibility and transfection efficiency. For example, particles may be coated with a cationic polymer to improve internalization (Shmueli, Anderson, & Green, 2010).

3.4 Resistance to degradation

As release studies in Chapter 2 showed, zein/DNA nanospheres are susceptible to enzymatic degradation in the stomach and particularly in the intestine. Because successful oral delivery of DNA will likely result from the cellular uptake of a DNA delivery system rather than free DNA (Rothman, Tseng, & Goldfine, 2005), in this case a particulate delivery system, it is preferred for the particles to remain intact long enough to be taken up by the cells lining the intestine. Although the effects of crosslinking on the enzymatic degradation of zein have not been reported, it is conceivable that this type of modification could slow the rate of zein degradation. Citric acid should be investigated first as a crosslinking agent, as such crosslinking may also be beneficial from a biocompatibility standpoint. In addition to decreasing the rate of zein degradation, this treatment may also enhance biocompatibility as fibers crosslinked with citric acid have shown better biocompatibility than uncrosslinked zein fibers (Jiang, Reddy, & Yang, 2010). Crosslinking of zein/DNA nanospheres with citric acid was attempted but not verified. The effect of this treatment on degradation was not, but should be, assessed. Alternatively, encapsulating zein in or blending zein with another polymer, natural or synthetic, with resistance to degradation in the gastrointestinal tract (GI-tract) could possibly delay release of DNA until after the particles have been internalized (L. Chen & Subirade, 2009). Conversely, zein may be useful as a coating for other DNA delivery systems designed to be released in the intestine.

3.5 Characterization & improvement of internalization

Although confocal images indicate internalization of zein nanosphreres containing DNA by Caco-2 cells, internalization should be more definitively characterized. In Figure 3-2A, a confocal image taken in a plane between the top and bottom of cells, spheres appear to be internalized and located within the cytoplasm around the nucleus of the cell encircled in the oval. Figure 3-2B shows a plane above the cells, where if spheres were not internalized one would expect distribution as is seen in the rectangle, indicating the presence of spheres above the nucleus. However, this is not the case in the oval where very little fluorescence is seen in the plane above the cell with almost none above the nucleus. Staining of the cell membrane would provide conclusive evidence of internalization. Internalization, once verified, could possibly be quantified by flow cytometry.

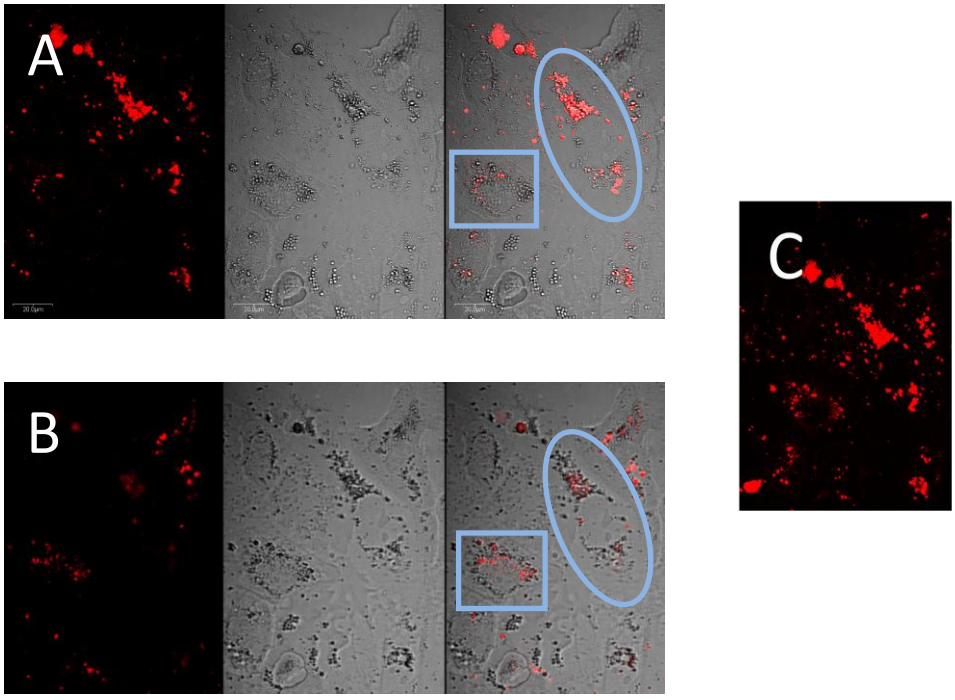

Figure 3-2: Confocal images taken in the plane of the Caco-2 cells (A) and above them (B). Also shown is a merged image of all of the fluorescence images from the planes from the bottom to the top of cells (C).

Strategies to improve internalization, if needed, could include surface cationization, addition of target moieties targeting cell surface receptors, and reduction in sphere size. Surface cationization could be achieved through a number of chemical modifications including adsorption of a cationic surfactant or surface conjugation of a cationic polymer as previously discussed. The targeting of cell surface receptors would likely entail the immobilization of a receptor specific ligand or a nonspecific membrane transduction sequence on the sphere surface. Ligands including lectins and the B subunit from cholera toxin have been investigated as possible GI-tract targeting ligands (Page & Cudmore,

2001). Sphere size may be reduced by altering sphere formation parameters such as the zein to DNA ratio as shown in Chapter 2 or by reducing the concentration of the initial zein solution as shown in Figure 3-3 (preliminary results). The reduction of the concentration of the initial zein solution from 1% to 0.33% or 0.2% w/v resulted in a significant decrease in the nanosphere size.

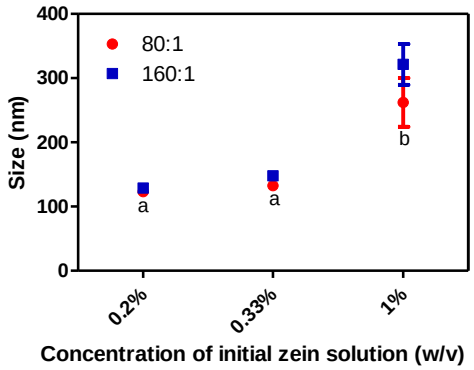

Figure 3-3: The effect of the initial zein concentration on sphere size for spheres formed at 80:1 and 160:1 zein to DNA ratios. Data points labeled with the same letter are not significantly different $p>0.05$. Reported as mean ± SEM with n=6.

If sphere internalization is determined to result in transport into the cytoplasm but not nuclear internalization as is seen in Figure 3-2A, it may be necessary to include a nuclear localization signal (NLS) or karyophilic protein to enhance transfection efficiency (Zanta, Belguise-Valladier, & Behr, 1999). A variety of NLSs have been used to enhance nonviral gene delivery (Cartier & Reszka, 2002).

3.6 *In vitro* & *in vivo* models

The Caco-2 monolayer is a commonly used model for investigating the intestinal uptake of and transport of particles. To model the single cell layer of the intestinal epithelium, Caco-2 cells are typically seeded onto a transwell membrane and allowed to proliferate to the point of forming a continuous monolayer (Artursson, Palm, & Luthman, 2001). For an M-cell model, Caco-2 cells grown on a transwell membrane are co-cultured with lymphocytes isolated from Peyer's patches. The presence of these cells in the co-culture system has been shown to cause the Caco-2 cells to undergo further differentiation to M-cells (Kerneis, Bogdanova, Kraehenbuhl, & Pringault, 1997). Like most *in vitro* models of *in vivo* tissue, these are far from perfect but have been demonstrated to have merit in predicting the behavior of at least part of the intestine. Several studies have shown varying correlation between the apparent permeability of the Caco-2 monolayer and the percent of absorption in humans after oral administration (Artursson, Palm, & Luthman, 2001). Because of its extensive use in predicting the intestinal absorption of pharmaceuticals, the Caco-2 monolayer is an obvious choice for the preliminary assessment of intestinal uptake and transfection. The M-cell model is likewise a logical *in vitro* choice for predicting the efficacy of oral delivery systems for DNA vaccination, as M-cells are common targets for oral DNA vaccination.

In vivo studies are particularly important for the validation of this gene delivery system. Despite *in vitro* results, positive or negative, *in vivo* studies may result in quite different transfection efficiencies than that seen in cultured cells. For example, Saeki and coworkers found a reciprocal relationship between the anionic or cationic nature of

liposomes and their efficacy *in vitro* and *in vivo* (Saeki et al., 1997). The selection of an animal model is an important factor determining the outcome of the study. For example, rabbits have significantly more Peyer's patch M-cells than rodents (H. Chen & Langer, 1998) making them an appealing model for oral DNA vaccination. However, the most commonly used models for oral gene delivery are mice. It is unclear which of the available animal models is best for predicting the intestinal absorption of particles in humans (H. Chen & Langer, 1998). While *in vivo* transfection can be quantified by measuring the expression of a reporter gene like luciferase (normalized to tissue mass) (Rothman, Tseng, & Goldfine, 2005), the telling indicator of successful transfection is a physiological change in a local disease, e.g. acute colitis (Bhavsar & Amiji, 2008), model or a systemic disease, e.g. diabetes (Rothman, Tseng, & Goldfine, 2005).

3.7 Intramuscular administration & applications

The oral route of administration is not the only route of interest for gene therapy and DNA vaccination. The direct injection of DNA delivery systems into muscle has been investigated for DNA vaccination (O'Hagan, Singh, & Ulmer, 2004) and gene therapy (Tripathy et al., 1996). As is the case with intramuscular injection of naked DNA, injection of DNA-loaded nanoparticles and microparticle into the muscle primarily results in local transfection because the particles do not readily diffuse from the tissue. Transfection of muscle cells can elicit an immune response (O'Hagan, Singh, & Ulmer, 2004), can result in a physiological change in the injected muscle (Shyu, Manor, Magner, Yancopoulos, & Isner, 1998), or can serve as a depot releasing the encoded protein into circulation (Tripathy et al., 1996). Particulate DNA delivery vehicles have been shown to

increase the magnitude and duration of transgene expression compared to other delivery vehicles or naked DNA administered intramuscularly. Compared to DNA complexed with lipofectamine, intramuscular delivery of nanospheres produced expression of plasmid DNA that was one or two orders of magnitude higher after seven days and was more sustained (Agarwal & Mallapragada, 2008). Intramuscular administration of DNA encapsulated in poly(2-aminoethyl propylene phosphate) resulted in enhanced transfection relative to naked DNA (J Wang, Zhang, Mao, & Leong, 2002). Similarly zein nanoparticles that have been stabilized under physiological conditions and that can facilitate uptake should be good DNA delivery vehicles via this route of administration.

3.8 Tissue engineering & applications

Tissue engineering is the application of engineering and biology to the development of functional substitutes for damaged or diseased tissues (Langer & Vacanti, 1993). Constructs for tissue engineering can consist of three components, namely the scaffold (the physical structure), cells (which make up the new tissue), and bioactive signals (which direct cellular function)(Langer & Vacanti, 1993). The integration of gene delivery and structural support into tissue engineering scaffolds has the potential to greatly enhance tissue repair in the case of injury or disease. The physical structure of the scaffold allows for the creation and maintenance of space for cell adhesion and migration, serves as a depot for sustained gene delivery, and controls the cellular environment of gene transfer (Agarwal & Mallapragada, 2008). Using this controlled manner of transfection, direction of cell function can be achieved. DNA can be

incorporated into tissue engineering scaffolds through encapsulation within the scaffold material or immobilized to the scaffold surface (De Laporte & Shea, 2007).

Gong and coworkers formed zein scaffolds by particle leaching in an 85°C water bath, resulting in scaffolds that ranged in porosity from 75.3% to 79.0% and varied in mechanical properties with a compressive Young's modulus of 28.2-86.6 MPa and compressive strength of 2.5-11.8 MPa. These properties were found to be suitable for application in non-load bearing bone tissue engineering (S. Gong, Wang, Sun, Xue, & Wang, 2006). If this technique cannot be used with zein/DNA nanospheres, a gas foaming, particulate leaching technique similar to that used for the fabrication of DNA-loaded PLGA microsphere scaffolds is another possibility for scaffold formation (Jang & Shea, 2003). It is possible to improve the mechanical properties of zein scaffolds, specifically their brittleness by the addition of plasticizers such as oleic acid (H.-J. Wang et al., 2007). Similarly, the bioactivity of the scaffold can be improved upon. For example, zein scaffolds were coated with hydroxyapatite by submerging them in simulated body fluid. These coated scaffolds resulted in higher alkaline phosphatase activity in seeded human bone marrow stroma cells, indicating a greater degree of differentiation to bone (Qu et al., 2008). Zein scaffolds (not coated in hydroxyapatite) were later implanted ectopically in mice and in critical-sized bone defects. Scaffolds seeded with MSCs showed markedly more bone formation in both cases than scaffolds without MSCs and the control (Tu et al., 2009). These studies indicate the promise of zein scaffolds in tissue engineering. The use of DNA-loaded zein nanospheres as the

building blocks of a similar scaffold could further improve the bioactivity and thus the performance of tissue engineering constructs.

3.9 Conclusions

In this thesis, a technology for gene delivery consisting of zein nanospheres encapsulating DNA has been established. These particles show great promise for oral DNA delivery, but could use modification to improve several properties pertaining to stability, uptake and transfection *in vitro* and in the GI-tract *in vivo*. Additionally applications in tissue engineering and intramuscular injection should be investigated as zein/DNA particles may also prove to be an effective tool for transfection in these applications.

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Appendix A:

Encapsulation and Size Data for Zein Microspheres Encapsulating Compounds

Material	Compound Loaded	Preparation Methods	Size	Encapsulation Efficiency (% w/w)	Loading (% w/w)	Source
Zein	riboflavin	emulsification	22.9 µm	22.9	9.2	Chen & Subirade, 2009
Soy protein isolate/Zein	riboflavin	emulsification/ internal gelation	20.8-25.5 µm	79.3-87.9	8.9-9.8	Chen & Subirade, 2009
Zein	abamectin	emulsification	< 70 µm	35	NR	Demchak & Dybas, 1997
Zein	ciprofloxacin	coacervation	0.5-2 µm	4.97-8.29	0.87-2.41	Fu et al., 2009
Zein	none	coacervation	1.36 µm	N/A	N/A	Hurtado-Lopez & Murdan, 2005
Zein	ovalbumin	coacervation	210-1882.9 nm	53.9	23.9	Hurtado-Lopez & Murdan, 2005
Zein	ivermectin	coacervation	0.3 - 1.2 µm	24.73-68.51	4.05-17.16	Liu et al., 2005
Zein	solid zinc insulin	solvent evaporation/extraction	3.2 µm	NR	4.8 or 9	Mathiowitz et al., 1993
Zein	insulin	solvent evaporation/extraction	NR	NR	17-42	Mathiowitz et al., 1993
Modified Zein *	insulin	solvent evaporation/extraction	NR	NR	17	Mathiowitz et al., 1993
Zein	polysaccharide-K	conjugation and coacervation	< 1 µm	4.2-34.5	NR	Matsuda et al., 1989
Pectin/Zein	piroxicam	gelation/coacervation	NR	9.8-95.2	NR	Mukhidinov et al.,2011
Zein	gitoxin	coacervation	1-1.7 µm	1.77-20.98	0.31-3.17	Muthuselvi & Dhathathreyan, 2006
Zein	essential oils	coacervation	~100 nm	NR	20 or 13	Parris et al., 2005
Zein	amoxicillin	spray-drying	9.4 µm	NR	NR	Sousa et al., 2010
Zein	indomethacin	spray-drying	5.6 µm	99	5.8	Sousa et al., 2010
PLGA/Zein	amoxicillin	spray-drying	28.05 or 38.3 µm	45.3 or 38.7	2.4 or 2.1	Sousa et al., 2010
PLGA/Zein	indomethacin	spray-drying	18.5 or 38.5 µm	91.8 or 85.8	5.4 or 5.1	Sousa et al., 2010
Zein	mitomycin C	conjugation and coacervation	< 1 µm	NR	16.4 or 30.0	Suzuki et al., 1989
Zein	daunomycin hydrochloride	conjugation and coacervation	NR	NR	31.3	Suzuki et al., 1989
Zein	peplomycin sulfate	conjugation and coacervation	NR	NR	7.6-19.0	Suzuki et al., 1989
Zein	heparin	coacervation	40-100 nm	1.97-22.77	0.34-3.67	Wang et al., 2005
Zein	lysozyme	supercritical anti-solvent	< 50 µm	46.5	NR	Zhong et al., 2009
Zein	lysozyme	spray-drying	NR	35.4-49.1	NR	Zhong et al., 2009