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ZEIN: NOVEL NATURAL POLYMER FOR NANOPARTICLE- AND FILM- MEDIATED
GENE DELIVERY

By
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A THESIS

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Under the Supervision of Professor Angela K. Pannier

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ZEIN: NOVEL NATURAL POLYMER FOR NANOPARTICLE- AND FILM- MEDIATED GENE DELIVERY

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Gene delivery, the introduction of DNA into cells, is applicable to gene therapy, DNA vaccination, functional genomics and diagnostics, tissue engineering, and drug-eluting medical devices. Particulates incorporating DNA are promising vehicles for gene delivery and overcome some of the barriers that hinder successful gene transfer, with the ability to protect DNA and provide for controlled, localized, and sustained release and transfection. Furthermore, innovative new gene delivery strategies that incorporate DNA particulates or complexes within films or coatings for devices and scaffolds could further provide for controlled and sustained transfection at the site of implant. Zein, a hydrophobic protein from corn, has many unique properties that make it a promising candidate material for both particulate- and film-mediated gene delivery, including its ability to easily form both nanospheres and films. Zein/DNA nanospheres, formed through a simple coacervation process, demonstrated the ability to protect DNA against nucleases, displayed robust biocompatibility, and were able to be internalized by cells. In addition, zein films were developed for substrate-mediated gene delivery. Zein films were formed by evaporation- induced self-assembly and were comprised of particles that increased in size with zein concentration, up to 1.8 ± 0.11 nm in diameter. Films formed at different pH values (2-12) resulted in morphological differences, from a smooth surface at pH 2 to films composed of spheres that decreased in size as the pH of the zein solution used to form the film

increased. Zein films degraded minimally in PBS, and were able to adsorb DNA complexes formed with cationic lipids or cationic polymers, with 0.4 μg and 1.12 μg of total DNA on film surface for lipid/DNA and polymer/DNA complexes, respectively, which was independent of pH. However, transfection levels increased on films with increasing pH and highest gene expression was achieved on pH 9 films with lipid/DNA complexes. This increase in transfection on pH 9 films could be attributed to the decreased surface roughness and increased hydrophilicity of the film. Both the zein spheres and films investigated in this thesis display great potential for gene delivery applications, in particular for oral and intramuscular gene delivery and as a surface-coating for biomedical devices.

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LIST OF ABBREVIATIONS

ADA-SCID	Adenosine deaminase- severe combined immunodeficiency
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
Caco-2	Human colon carcinoma cells
CAD	Coronary heart disease
CpG	Cytosine—phosphate—guanine
CS	Chitosan
CTLs	Cytotoxic T lymphocytes
DD	Degree of deacetylation
ddH ₂ O	Double-distilled water
DES(s)	Drug-eluting stent(s)
DHA	Docosahexaenoic acid
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNases	Deoxyribonucleases
ECM	Extracellular Matrix
EISA	Evaporation induced self-assembly
EMEM	Eagle's minimum essential medium
EtOH	Ethanol
EVAc	ethylene-co-vinyl acetate

FBS	Fetal bovine serum
GES(s)	Gene-eluting stent(s)
GFP	Green fluorescent protein
GI tract	Gastro-intestinal tract
HEK-293T	Human embryonic kidney cells
HIV	Human immunodeficiency virus
IPA	Isopropyl alcohol
LF	Lipofectamine
LF2000	Lipofectamine2000
MI	Myocardial infarction
MSC	Mesenchymal stem cells
MW	Molecular weight
N/P ratio	Ratio of nitrogen to phosphorous atoms in a gene delivery complex
NLS	Nuclear localization signal
PBS	Phosphate buffered saline
PC	Phosphorylocholic
PCL	Poly (ϵ -caprolactone)
PdI	Polydispersity index
PDGF	Platelet-derived growth factor
pDNA	Plasmid DNA
pDsRed2-N1	Plasmid encoding for the red fluorescent protein, DsRed2

PEI	Polyethyleneimine
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PS	polystyrene
RLU(s)	Relative light unit(s)
SEM	Scanning electron microscopy
SEM	Standard error of the mean
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SMD	Substrate-mediated delivery
TBC	Tributyl citrate
TBS	Tris buffered saline
TCA	Transfected cell array
TE	Tissue Engineering
TE	Tris-EDTA buffer
TIMP	Tissue inhibitor of metalloproteinase
TPP	Triphosphosphate
VEGF	Vascular endothelial growth factor
WST-1	Water Soluble Tetrazolium
X-ALD	X-linked adrenoleukodystrophy
ZN/CS/DNA NP	Zein-coated chitosan/DNA nanoparticles
5-FU	5-fluorouracil

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CHAPTER 1: Introduction to Gene Delivery and Zein

1.1 Introduction

Nonviral gene delivery is the introduction of exogenous DNA into cells through the use of nonviral vectors for subsequent gene expression and is applicable to the fields of gene therapy, DNA vaccination, functional genomics and diagnostics, and biomedical devices. Nonviral gene delivery systems include naked DNA, chemical vectors and particulates, which are delivered typically through a bolus addition to cells or through substrate-mediated delivery, where DNA vectors are adsorbed to a biomaterial substrate. However, nonviral vectors are often inefficient and their lack of efficacy in nonviral systems can be attributed to a variety of barriers and prevents their use in widespread applications. Some of these barriers to gene delivery systems include protection of DNA against degradation from nucleases or enzymes, appropriate degradation rates of DNA vectors and carriers, and providing sustained delivery of DNA to the target. The use of particulates incorporating DNA, which can be delivered as distinct spheres or used to form particulate composite films, are promising vehicles for gene delivery, with the ability to protect DNA and provide for controlled, localized, and sustained release and transfection. Zein, a hydrophobic protein from corn, is biocompatible and has properties that make it a promising candidate material for particulate delivery, including its ability to form both nanospheres and films through coacervation or evaporation induced self-assembly, respectively. Due to the promise of this natural biomaterial for drug and gene delivery, the objectives of this thesis were to: 1) further investigate DNA-loaded zein nanospheres for their ability to protect cargo from nucleases, as well as their biocompatibility and the ability of cells to internalize the spheres, and 2) develop zein films for substrate-mediated

gene delivery. The zein spheres and films investigated in this thesis show great potential to revolutionize applications in oral and intramuscular gene delivery and as a surface-coating for biomedical devices, respectively.

1.2 Gene Delivery

Gene delivery is the introduction of exogenous DNA to host cells for subsequent gene expression and is applicable to the fields of gene therapy (Niidome and Huang 2002), DNA vaccination (Donnelly, Ulmer et al. 1997), functional genomics and diagnostics (Pannier, Ariazi et al. 2007), tissue engineering (Shea, Smiley et al. 1999) and medical implants (Zilberman, Kraitzer et al. 2010). Gene delivery methods are broadly classified into two categories, viral and nonviral. Viral methods have a known history of being effective by achieving high rates of transduction, as well as stable expression levels that have led to limited success in clinical trials (Thomas, Ehrhardt et al. 2003; Ginn, Alexander et al. 2013). However, several fundamental problems are associated with the use of viral vectors including toxicity, immunogenicity, endogenous virus recombination, oncogenic effects, limited transgene size, complexity of fabrication and lack of cell-specific targeting (Thomas, Ehrhardt et al. 2003; Gao, Kim et al. 2007). In addition, despite therapeutic effects, patient deaths have been reported from the use of viral gene delivery (Thomas, Ehrhardt et al. 2003). Hence, there is a need to investigate new vectors and methods of gene delivery. The development of nonviral gene vectors circumvents some of these safety concerns and technical drawbacks of viral vectors. Advantages of nonviral vectors include simplicity of preparation, easy scale-up for mass production, flexibility in transgene size capacity, low immunogenicity, biocompatibility, and cost

effectiveness (Niidome and Huang 2002; Wiethoff and Middaugh 2003). However, nonviral vectors exhibit significantly reduced transfection rates (expression of the delivered genes) when compared to viral delivery systems (Wiethoff and Middaugh 2003). Most researchers in the field would agree that the major challenge of nonviral gene delivery is the issue of efficient delivery.

The extracellular and intracellular barriers associated with these processes (Figure 1-1) that must be overcome for successful gene delivery are, for the most part, common to all delivery systems. Successful delivery of DNA by any system requires cellular internalization, endosomal escape, nuclear localization and production of the therapeutic protein encoded by the gene (Ledley 1995). Packaged DNA must first be internalized by endocytosis for complexes (Mintzer and Simanek 2009) or phagocytosis for microspheres (Agarwal and Mallapragada 2008). After internalization, the DNA must escape the endosome without being degraded by low pH, nucleases or enzymes and then navigate through the cytosol to the nucleus. Nuclear localization and entry into the nucleus are subsequently needed in order to lead to the production of the protein from the delivered gene (De Laporte, Cruz Rea et al. 2006). 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 (Pichon, Billiet et al. 2010). *In vivo* transfection is further complicated by instability of complexes under physiological conditions and adsorption of serum proteins prior to cellular internalization (Mintzer and Simanek 2009). Unlike viral vectors that have evolved to overcome most of these cellular barriers to gene delivery, nonviral vectors are still hindered by them

(Mintzer and Simanek 2009). In addition to these cellular barriers, even more barriers exist when gene delivery is considered in the context of whole organism for certain delivery routes, for example the different pH environments of the gastrointestinal tract for oral gene delivery. It is important to understand that one delivery vector cannot possibly be effective for all the different applications of gene delivery and thus there is a need for designing efficient and safe delivery systems that take into account these barriers and include tactics to overcome them.

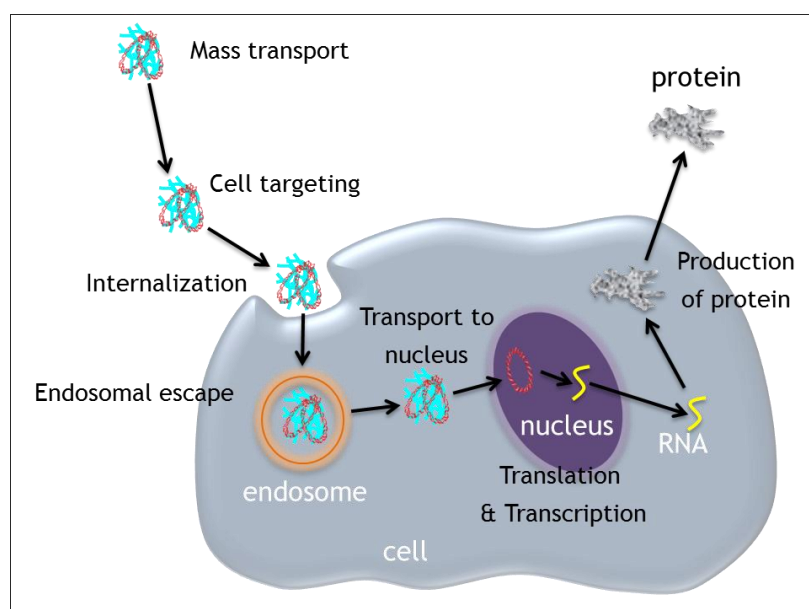


Figure 1-1. Barriers to nonviral gene delivery mechanism (courtesy of T. Kasputis).

1.3 Nonviral Delivery Systems

Nonviral delivery of DNA is typically accomplished by one of three delivery systems: naked DNA, chemical vectors and particulates. Each system has advantages and disadvantages depending on the intended application.

1.3.1 Naked DNA

The injection of naked DNA is perhaps the simplest and most non-toxic delivery system, but often requires addition of a physical approach such as electroporation (Rols, Delteil et al. 1998) or the use of a gene gun (Yang, Burkholder et al. 1990) to help the DNA penetrate the cell membrane before degradation by nucleases occurs (Niidome and Huang 2002). Successful delivery of naked DNA *in vivo* was first discovered by Wolff et al when plasmid DNA was injected into the muscle of a mouse and induced protein production (Wolff J.A 1990). Intramuscular injection of naked DNA has been used to introduce the dystrophin gene into animal models of Duchenne muscular dystrophy and yielded long-term gene expression in a limited number of muscle fibers and protection of those fibers against degeneration (Acsadi, Dickson et al. 1991; Danko, Fritz et al. 1994). However, the delivery of naked DNA achieves low levels of gene expression, mainly localized along the track of injection (Ledley 1995), compared to other forms of DNA delivery (Ledley 1996). This limited uptake and expression could be due to the large size of DNA and the repulsion of the negatively charged DNA and negatively charged cellular membranes (Segura and Shea 2001). Therefore this technique may be appropriate when limited transfection is acceptable such as DNA vaccination (Cotten and Wagner 1994). Approaches to enhance DNA delivery by not only overcoming the extracellular and intracellular barriers mentioned above but also that of stability, size, charge density and biodistribution of DNA have been investigated through the use of chemical vectors and particulates (Segura and Shea 2001). The remainder of this thesis will focus on the improvement and use of chemical vectors and particulates for DNA delivery.

1.3.2 Chemical Vectors

Nonviral chemical vectors deliver DNA by forming a DNA complex, which condenses the DNA and neutralizes the charge, typically by cationic polymers or lipids (Mintzer and Simanek 2009). Two commonly used chemical vectors for delivery of DNA include a cationic polymer, polyethylenimine (PEI) and a cationic lipid, Lipofectamine (LF) (Segura and Shea 2001). Cationic polymers, such as PEI, condense DNA by electrostatic interactions between the positively charged polymer and negatively charged DNA forming net positively charged polyplexes, which are less than 100 nm. PEI exists in either branched or linear conformation but branched PEI at a molecular weight of 25 kDa has become the most commonly used due to its high transfection efficiency. Some of the success of PEI has been attributed to its endosomal buffer capacity which aids the release of the polyplex into the cytosol (Mintzer and Simanek 2009). The efficiency of PEI/DNA complexes is influenced by PEI material properties such as molecular weight and degree of branching, but also the polyplex properties including the N/P ratio (ratio of nitrogen atoms of PEI to DNA phosphates), concentration of DNA, size and zeta potential (Lungwitz, Breunig et al. 2005). PEI/DNA complexes are known to be more toxic than the use of cationic lipids, such as LF, as a nonviral gene delivery vector.

Cationic lipids complex with DNA also via electrostatic interactions of the positively charged head of the lipid moiety and negatively charged phosphate backbone of the DNA to form lipoplexes of DNA and lipid (Segura and Shea 2001). This method condenses the DNA, resulting in a positively charged lipoplex that can interact with the negatively charged cell membrane, aiding in increased cellular uptake and expression levels (Segura

and Shea 2001). Lipofectamine2000 (LF2000) is a commercially available cationic lipid and widely used in many labs due to its effectiveness in producing high transfection efficiencies in certain cell lines when optimized (Plautz, Boanca et al. 2011).

Optimization of lipoplexes involves fine-tuning of the ratio of cationic lipid to DNA, amount of DNA, and delivery volume, which ultimately affects the size, charge and stability of the lipoplex. Cationic lipids have been shown to be more efficient at delivery of DNA than cationic polymers and used in several clinical trials for treatment of cancer (Nabel, Nabel et al. 1993) and cystic fibrosis (Caplen, Alton et al. 1995). Drawbacks of lipoplexes involve the physiochemical properties of the lipoplex in vivo due to the presence of salts and other molecules that could lead to aggregation and dissociation of lipoplexes and result in reduced bioavailability, cellular uptake and transfection efficiencies (Ledley 1995). Therefore, specific delivery strategies are needed to sustain the optimal properties while overcoming the barriers of the lipoplexes to enhance cellular uptake and transection. In this thesis, lipoplexes (using LF2000) and polyplexes (using PEI) were used to deliver DNA by substrate-mediated delivery (described below) on zein films.

1.3.3 Particulates

Particulates are an alternative to chemical vectors for DNA delivery, which consist of polymers that encapsulate DNA or DNA complexes within or throughout a particle, or through adsorbing/conjugating DNA to the surface of the particle (Panyam and Labhasetwar 2003). The use of particulates for DNA delivery provides numerous advantages such as protection of DNA against degradation, sustained DNA release, and

tissue targeting abilities (Panyam and Labhasetwar 2003; Agarwal and Mallapragada 2008). Using particulates to encapsulate DNA can shield it from the immune system, DNA nucleases, and degradation from different pH and enzymes, such as those found in the gastrointestinal (GI) tract, which can improve the *in vivo* response and transfection efficiency of the delivery system by increasing the residence time of the DNA-loaded particles (Agarwal and Mallapragada 2008). Particulates can be internalized by cells (nanoparticles) or phagocytized (microparticles), depending on their size and can serve as transgene depots inside the cell (Pannier and Shea 2004). Particles release their cargo through a combination of diffusion and degradation of the polymer matrix (Agarwal and Mallapragada 2008) and thus the delivery system can be tailored to a particular application through the polymer choice (Segura and Shea 2001). Both natural and synthetic polymers have been researched as nanocarriers for DNA. Natural polymers have the advantages of providing innate degradability and bioactivity (Dang and Leong 2006; Malafaya, Silva et al. 2007), but typically suffer from insufficient mechanical properties, poor water stability, limited ability to be processed, and a relatively short release period compared to synthetic polymers (Panyam and Labhasetwar 2003; Yannas 2004). However, some natural polymers derived from plants, including wheat gluten, glutenin, zein, soy protein, cellulose, and starch (Czaja, Young et al. 2006; Malafaya, Silva et al. 2007; Reddy and Yang 2011) overcome some of the shortcomings of other natural polymers. Among the various plant proteins, zein, the prolamin or storage protein from corn, has properties that make it a promising candidate material for particulate delivery and was investigated in this thesis for application in gene therapy or DNA vaccination.

1.4 Delivery Strategies for *in Vitro* Transfection

Controlled and efficient delivery of DNA complexes or particulates loaded with DNA is an essential aspect in both basic and advanced applications gene delivery. In addition to choice of vector or particulate material, the delivery strategy is an important factor involved in enhancing gene expression. Currently, there are two main strategies for delivering DNA to cells, conventional bolus delivery and substrate-mediated delivery (SMD). The most common strategy is bolus delivery, where the nonviral DNA complexes or particulates are employed *in vitro* as a “bolus” addition to the media (Tseng, Haselton et al. 1997; Varga, Hong et al. 2001). Bolus delivery, which is also analogous to intravenous (IV) *in vivo* delivery, has been widely utilized to study gene function and screen for efficient delivery vehicles (Pannier and Segura 2013). However, a major limitation is in mass transport of DNA to the cells, leading to the use of a large amounts of DNA vectors, which cause toxicity (Luo and Saltzman 2000). Also bolus delivery cannot be used to control which cells ultimately express the gene (without the use of targeting ligands) and may not result in efficient gene delivery for all cell types (Pannier and Segura 2013).

In the process of developing controlled and efficient gene delivery systems and strategies, it has been shown that increased transfection can be achieved through increasing the concentration of DNA in the cellular environment (Varga, Hong et al. 2001). Therefore, an alternative technique of delivering DNA to cells, known as SMD (Segura and Shea 2002), reverse transfection (Ziauddin and Sabatini 2001), or solid-phase delivery (Bielinska, Yen et al. 2000) involves the immobilization and maintenance of DNA or

DNA complexes to the surface of a substrate, onto which cells are seeded. This mechanism has shown to increase the concentration of DNA in the cellular environment resulting in enhanced gene delivery and increased cell viability (Bengali, Pannier et al. 2005). SMD limits aggregation of DNA complexes by their immobilization and places DNA vectors in direct contact with the cell to reduce mass transport limitations (Segura and Shea 2002; Segura, Volk et al. 2003; Bengali, Pannier et al. 2005; Bengali and Shea 2005; Pannier, Anderson et al. 2005; Jang, Bengali et al. 2006; Bengali, Rea et al. 2007; Pannier, Wieland et al. 2008; Bengali, Rea et al. 2009; De Laporte, Lei Yan et al. 2009). SMD also allows for local and patterned delivery (Pannier, Anderson et al. 2005; Houchin-Ray, Whittlesey et al. 2007). Furthermore, the adsorption of extracellular matrix (ECM) proteins, such as fibronectin, onto substrates prior to the addition of DNA complexes can increase DNA complex immobilization and transgene expression (Bengali, Rea et al. 2007). SMD is a rising technique in the field of gene delivery and has great potential in applications such tissue engineering, biomedical devices, and transfected cell arrays. Thus, the use of zein was investigated in Chapter 3 in this thesis, as a potential surface-coating material for substrate-mediated gene delivery on biomedical devices and scaffolds.

1.5 Applications of Gene Delivery

Once delivery systems and strategies are fully optimized and result in high levels of transfection *in vitro*, they then can be utilized for various gene delivery routes and clinical applications. Here, the oral and intramuscular routes will be discussed as they are most applicable to the zein spheres developed in this thesis for applications in gene

therapy and DNA vaccination. While zein films were investigated in this thesis for local gene delivery in applications such as functional genomics and diagnostics, tissue engineering and drug-eluting biomedical devices.

1.5.1 Gene Therapy

The ultimate goal of gene therapy is to produce the stable expression of a correct functioning gene (Drugan A 1987), in cases where the normal gene is deficient and the therapy may be accomplished via one of three approaches: the modification of existing genetic material, removal of genetic material or addition of genetic material. Gene therapy was first perceived as a treatment or cure for hereditary single-gene defects (monogenic disorders) like cystic fibrosis and hemophilia (Kay and Woo 1994), but has expanded its potential to include acquired diseases such as cancer (Lowenstein 1997), cardiovascular disease (Isner 2002), neurodegenerative disorders (Baekelandt, De Strooper et al. 2000) and infectious disease (Bunnell and Morgan 1998). Currently, there have been over 1800 gene therapy trials worldwide, either completed, ongoing or approved, and 81.5% of those trials address cancer (64.4%), cardiovascular disease (8.7%) and inherited monogenic disease (8.4%) (Ginn, Alexander et al. 2013). Also, of those 1800 trials, 66.8% used viral vectors and the two most popular nonviral vectors used were naked plasmid (18.3%) and lipofection (5.9%) (Ginn, Alexander et al. 2013). The two most promising results in the clinic have been targeting diseases in the hematopoietic system, adenosine deaminase- severe combined immunodeficiency (ADA-SCID) and X-linked adrenoleukodystrophy (X-ALD), with over 30 patients treated with ADA-SCID (Gaspar, Aiuti et al. 2009; Ferrua, Brigida et al. 2010) and two young

patients treated with X-ALD (Cartier, Hacein-Bey-Abina et al. 2009). Viral vectors were used for both of these clinical trials. These numbers highlight the exciting therapeutic progress that is being made in the field of gene therapy but also the need to develop more efficient delivery systems. In addition, the diversity of targets for treatment of the numerous diseases elucidates the notion that a single vector, viral or nonviral, cannot be appropriate for all applications. Thus there is need to investigate new vectors, specifically nonviral vectors due to their advantages mentioned above, in order to move the field of gene therapy into clinical reality. New nonviral vectors are commonly investigated for DNA delivery through the oral or intramuscular route.

1.5.1.1 Oral route

Delivery of DNA via the oral route is an appealing delivery method due to its associated high patient compliance, with convenience in both non-invasiveness and in control of dosing (frequency and strength) (Shahiwala 2011). The oral route has the additional advantages of presenting a large surface area intestinal epithelium for transfection and allowing treatment of regional disorders by providing access to the luminal side of the intestine (Bhavsar and Amiji 2007). Oral gene delivery has the potential to treat diseases associated with the gastro-intestinal tract (GI-tract) such as colon cancer, cystic fibrosis, inflammatory bowel disease and Crohn's disease (Page and Cudmore 2001) as well as systemic diseases (Rothman, Tseng et al. 2005). However, efficient and consistent gene expression mediated by oral DNA delivery is complicated by a number of physiological barriers associated with GI-tract, including low pH in the stomach and DNases in the intestine (Loretz, Foger et al. 2006), which can degrade unprotected DNA. Particulates

are considered a viable tool for the protection of DNA from the harsh environment of both the stomach and intestine (Chen and 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 (Jones, Clegg et al. 1998; Roy, Mao et al. 1999; Agarwal and Mallapragada 2008; Bowman, Sarkar et al. 2008). 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 (He, Wang et al. 2005; Bowman, Sarkar et al. 2008). Furthermore, although these materials have provided a valuable proof of concept, their lack of sufficient efficacy suggests that new polymers need to be investigated to overcome the multiple barriers of oral gene delivery. Hence, Chapter 2 focuses on the use of zein as a DNA particulate for applications in oral gene delivery.

1.5.1.2 Intramuscular route

Skeletal muscle comprises 40% of total body mass and skeletal myocytes have a long half-life *in vivo*, thus the readily availability and stable platform of muscle creates a promising target tissue for gene delivery (Marshall and Leiden 1998). The direct injection of DNA delivery systems into muscle has been investigated for gene therapy to treat serum-protein deficiencies, muscular dystrophies and chronic ischemic limb syndromes (Tripathy, Svensson et al. 1996). However, as for oral delivery of DNA-loaded particulates, lack of sufficient efficacy with intramuscular administration suggests that new polymers need to be investigated for DNA delivery. Thus, zein was investigated

in Chapter 2 as a DNA-loaded particulate for application in intramuscular delivery, in addition to its potential use in oral delivery.

1.5.2 DNA Vaccination

Vaccination is the introduction of antigenic material designed to provoke an individual's immune response and generate immunity to a pathogen (Gurunathan, Klinman et al. 2000). The introduction of vaccinations against smallpox, diphtheria, pertussis, tetanus, polio, measles, rubella, and mumps have had a large impact on human health, decreasing mortality and morbidity rates from infectious diseases (O'Hagan and Rappuoli 2004). Success from these vaccinations is from the induction of long-lived antibody response through humoral immunity (Gurunathan, Klinman et al. 2000), which is typically mediated by recognition of specific antigens and antibody production from B lymphocytes. Nevertheless, vaccinations against intracellular organisms that require cell-mediated immunity are not available or are inefficient for clinical use. Cell-mediated immunity does not involve antibodies but activates cytotoxic T-lymphocytes (CTL). Therefore, there is a need to develop new vaccines against infectious diseases such as tuberculosis, malaria, and human immunodeficiency virus (HIV) that induce cellular immunity response. Research into vaccination approaches has included the use of attenuated live vaccines that employ recombinant viruses or bacteria, and genetic vaccines which consist of DNA (Leitner, Ying et al. 1999). In contrast to other vaccine types, genetic vaccinations are plasmid DNA vectors encoding genes for antigens that will produce an immune response. DNA vaccines hold great potential not only because of the safety profile but also due to ease of manufacturing and purifying, and low cost of

production (O'Hagan, Singh et al. 2004). The direct injection of DNA delivery systems into muscle has been investigated for DNA vaccination (O'Hagan and Rappuoli 2004). In addition, DNA vaccines have demonstrated the induction of both humoral and cellular immune responses in various animal models (Ulmer, Donnelly et al. 1993; Donnelly, Ulmer et al. 1997; Gurunathan, Klinman et al. 2000). However, DNA vaccine potency in large animal and human systems is lacking due to the inefficient delivery of DNA to effectively invade antigen presenting cells (APC), resist degradation by extracellular nucleases and self-replicate (Leitner, Ying et al. 1999) .

An approach to overcome these delivery barriers of DNA vaccines is the use of biodegradable polymeric microparticles to entrap the DNA. Microparticles are taken up efficiently *in vivo* and *in vitro* by APC and have also been shown to exert an adjuvant (improve immunogenicity of antigen) effect for the induction of cell-mediated immunity through CTL responses (O'Hagan and Rappuoli 2004; O'Hagan, Singh et al. 2004). Injection of DNA-loaded nanoparticles or microparticles into muscle primarily results in local transfection because the particles do not readily diffuse from the tissue, but can serve as a depot releasing the encoded protein into circulation (Tripathy, Svensson 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 (Wang, Zhang et al. 2002; Agarwal and Mallapragada 2008). A commonly used polymer for DNA vaccination has been PLGA, which can be delivered systemically or to mucosal surfaces such as GI- or respiratory- tract (O'Hagan, Singh et al. 2004). However, progress in clinical evaluation of DNA microparticle

delivery has been limited by a variety of problems in areas such as maintenance of DNA integrity during encapsulation, ability for controlled release, and low DNA encapsulation and loading (Dubensky, Liu et al. 2000; Gurunathan, Klinman et al. 2000; O'Hagan and Rappuoli 2004; O'Hagan, Singh et al. 2004). Hence, the development of efficient delivery systems through the consideration of new polymers to overcome the current drawbacks of microparticle DNA vaccines should be explored. Zein nanoparticles capable of transfection would be a potential DNA vaccine delivery system, and were investigated in this thesis.

1.5.3 Functional Genomics & Diagnostics

The goal of functional genomics and resulting diagnostics is to understand the relationship between an organism's genome and phenotype through the dynamic aspects of gene transcription, translation and protein-protein interactions. Identifying targets for the treatment of human disease requires functional characterization of the products encoded by genes (Bailey, Wu et al. 2002). The use of transfected cell arrays (TCA) is a powerful method that has allowed for high-throughput of genomic data through transfection by several thousand different DNA molecules in microarray format. TCAs involve the immobilization of DNA probes onto specific sites of a substrate, then cells are seeded on top of the microarray and express defined gene products (Ziauddin and Sabatini 2001; Bellis, Peñalver-Bernabé et al. 2011). Advantages of TCAs allow for scaled down quantities of reagents and samples, long-term storage before transfection, and analysis of individual cells (Ziauddin and Sabatini 2001). Conversely, limitations of TCAs involve essential processes of transfection, such as internalization and subsequent

expression of DNA. Transfection on microarrays has only been achieved with HEK and COS cell lines due to the size of the array spot (Bailey, Wu et al. 2002). Thus, new approaches need to be investigated to increase transfection efficiencies for more cell types on microarrays. Ziauddin and Sabatini used SMD to transfect hundreds of cells on a DNA/gelatin printed microarray and discovered gene products that alter cellular physiology (Ziauddin and Sabatini 2001). Further development of using the SMD approach in cell arrays requires the investigation of a cost-effective delivery system that efficiently transfects a wide variety of primary cell lines and provides for spatially controlled DNA within different domains (Ziauddin and Sabatini 2001; Pannier and Shea 2004). This need indicates exploring different immobilization techniques and materials for an efficient interaction of the DNA probe with the microarray for improved performance. Zein, a low-cost and abundant protein from corn, has been shown to interact with DNA (Regier, Taylor et al. 2012), promote cell proliferation (Sun, Dong et al. 2005), and has been used as a coating in the food and pharmaceutical industry (Shukla and Cheryan 2001). Therefore, zein would be a potential candidate for use in microarray technology to adsorb DNA complexes, support cell adhesion and be fabricated and stored for future use.

1.5.4 Tissue Engineering

Tissue engineering (TE) is defined as “an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain or improve tissue function or a whole organ” (Langer and Vacanti 1993). The integration of gene delivery and structural support in TE 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 and also serves as a depot for sustained gene delivery and controls the local environment of gene transfer (Agarwal and Mallapragada 2008). Using this controlled manner of transfection, direction of cell function can be achieved. TE scaffolds have been formed from both synthetic and natural polymers. Natural polymers have the advantages of providing innate degradability and bioactivity, (Dang and Leong 2006; Malafaya, Silva et al. 2007) but typically suffer from low mechanical stability, limited ability to be processed, and a relatively short release period compared to synthetic polymers (Panyam and Labhasetwar 2003; Yannas 2004). Natural polymers such as collagen, gelatin and chitosan have been extensively used as TE scaffolds. However, these materials are often chemically treated to improve biological stability and mechanical properties, which can result in future problems. Therefore, new materials should be investigated for their potential in TE scaffolds and tailored for tissue-specific considerations.

Another major challenge in the design and engineering of tissue, is the temporal and spatial combination of signals to produce efficient gene delivery that is required for the formation of functional tissues (De Laporte and Shea 2007), which is based on the interaction of the scaffold and vector. DNA incorporated into TE scaffolds can be either encapsulated in the scaffold material by DNA-loaded microspheres, or blended with the polymer prior to scaffold formation (Jang, Rives et al. 2005) or immobilized to the scaffold surface (Jang, Bengali et al. 2006). Jang and Shea formed a TE scaffold through

the fusion of DNA-loaded PLGA microspheres using a gas foaming and particulate leaching process, and demonstrated sustained release of DNA but the degradation products of PLGA negatively affected the stability and integrity of the DNA (Jang and Shea 2003). Another study showed plasmid encoding vascular endothelial growth factor (VEGF) incorporated into porous PLGA scaffolds that were subcutaneously implanted into mice, not only induced transgene expression for 105 days but increased the blood vessel density and size of vessels at the implantation site (Jang, Rives et al. 2005). The alternative approach, immobilizing the DNA to the surface of the scaffold after fabrication, can overcome degradation or deactivation problems associated with the scaffold processing technique, such as high temperatures and harsh solvents.

Polyethylenimine (PEI)/DNA complexes were immobilized on a PLGA scaffold surface and *in vitro* transfection studies employing the surface-immobilized complexes reported increased number of transfected cells and higher level of gene expression than traditional bolus delivery, with the use of lower amounts of DNA (Jang, Bengali et al. 2006). These studies highlight the strategies that are possible for controlled DNA delivery from TE scaffolds but do not provide an optimal delivery system for guiding tissue formation. Thus, engineering a controlled and efficient DNA-loaded tissue scaffold is still needed and new polymers should be explored. Recently, DNA-loaded zein nanoparticles were formed and demonstrated stability of the DNA encapsulated and released from zein particles (Regier, Taylor et al. 2012), indicating their potential use in fabrication of TE scaffolds for sustained gene delivery and improved performance of tissue engineering constructs (Shea, Smiley et al. 1999). These DNA-loaded zein nanoparticles were further

investigated in Chapter 2 of this thesis for resistance to DNase degradation, cytocompatibility and cellular internalization for gene delivery applications.

1.5.5 Drug-Eluting Biomedical Devices

Controlled delivery of therapeutic agents from biomedical devices can improve their safety and efficacy *in vivo* by reducing the foreign body response of the host, such as infiltration of inflammatory cells and development of a dense layer of fibrotic connective tissue (Anderson 2001). This dense layer of tissue shields the biomedical device from the immune system and isolates it from the surrounding tissues (Rolfe, Mooney et al. 2011). Numerous biomedical devices that incorporate drug delivery including vascular stents and wound dressings (Zilberman, Kraitzer et al. 2010), will be discussed in more detail below as both stand to benefit the most from incorporation of gene delivery.

Drugs are typically released from medical devices through surface coatings of a continuous film or a film of nanoparticles encapsulating the drug (Lo, Van Tassel et al. 2010). Biocompatible coatings are used due to their ability to mask the underlying surface of the implanted biomedical device by producing a hydrophilic interface between the device surface and the tissue fluids, which minimizes tissue reactions induced by device implantation (Göpferich 1996). The incorporation of drugs within the coating improves the device/host tissue interactions (Morais, Papadimitrakopoulos et al. 2010), as seen with drug eluting vascular stents and wound dressings (Zilberman, Kraitzer et al. 2010).

1.5.5.1 Drug-eluting stents

Coronary artery disease (CAD), the narrowing of the arteries of the heart, is the most common cause of death in the world (Hoyert DL 2012). The technique of balloon angioplasty, the augmentation of the artery with the use of a stent, was a great milestone to combat this disease, but the re-narrowing of arteries, known as restenosis, was seen in 30-60% of patients within 6 months of surgery (Holmes, Vlietstra et al. 1984; Fischman, Leon et al. 1994; Serruys, de Jaegere et al. 1994). This high percentage of restenosis led to the development of drug-eluting stents (DESs), where drugs that inhibit platelet activation, acute inflammation, smooth muscle cell proliferation, extracellular matrix production, angiogenesis, and vascular remodeling (Sousa, Serruys et al. 2003) are incorporated onto the surface of the stent, by covalently attaching the drug (Joung, Kim et al. 2003) or infusing the drug into a polymer coating (Holmes Jr, Camrud et al. 1994). Currently, there are a few commercial DESs on the market, but a drawback of DESs is an increased rate of myocardial infarction (MI) due to late stent thrombosis (Camenzind 2006; Aziz, Morris et al. 2007; Camenzind, Steg et al. 2007). Thus, there still remains a need to investigate delivery of therapeutic compounds from stents.

Recent investigation of the incorporation of DNA onto stents has become the forefront of the field as local delivery of DNA allows for smaller, more efficient and prolonged dose of therapeutic agents (i.e. genes) as compared to the transient delivery of drugs (Takahashi, Letourneur et al. 2007; Ganly, Hynes et al. 2013). Early work of gene-eluting stents (GESs) used polymer coatings with viral vectors and showed limited success in animal studies with both reporter and therapeutic gene delivery (Fishbein, Alferiev et al.

2006). Both the polymer coating and gene vector have been explored to improve efficiency of the gene-eluting stents in reducing restenosis, inflammation and late thrombosis (Takahashi, Letourneur et al. 2007). The majority of these studies have used viral vectors because of their high intrinsic transfection efficiency. Ye et al reported the use of adenovirus encoding β -galactosidase using a polyactide/polycaprolactone coating for stent-based delivery (Ye, Landau et al. 1998). Adenoviruses have also successfully delivered a green fluorescent protein (GFP) as a reporter gene, and a tissue inhibitor of metalloproteinase (TIMP) as a therapeutic gene against restenosis, from collagen-coated stents and phosphorylocholic (PC)-coated- stents, respectively (Klugherz, Song et al. 2002; Johnson, Wu et al. 2005).

However, due to the safety and technical concerns discussed with use of viral vectors above, recent studies have focused on using nonviral vectors for GESs. For example, lipo(poly)plex-based gene delivery from PLGA/gelatin-coated meshes has been reported in a rabbit iliac artery restenosis model with substantial GFP expression at early time points; however, limitations were encountered due to rapid dissolution of the coatings *in vivo* (Brito, Chandrasekhar et al. 2010). Overall, the level of transfection achieved with nonviral vectors remains low in GESs (Leclerc, Gal et al. 1992), and some polymer coatings to deliver the DNA have shown to be problematic because of harmful properties of the polymer coatings, such as the degradation products of poly(lactide-co-glycolide) (PLGA) (vanderGiessen, Lincoff et al. 1996; Carter, Aggarwal et al. 2004). Thus, there is a need for investigating new polymeric coating materials with the ability to support delivery of nonviral gene vectors to improve efficacy of GESs. In this thesis, zein was

studied as a potential surface coating material for substrate-mediated gene delivery, with potential application in GESs.

1.5.5.2 Wound dressings

Wound dressings are another type of drug-eluting medical devices that have great potential in medicine, in particular for revolutionizing treatment of wounds including burns, diabetic ulcer and pressure sores, are drug-eluting wound dressings. In all of these types of wounds, skin is damaged, which is an important organ for serving as a physical barrier for infections, providing sensation, regulating temperature and managing excretion of waste, salt and sweat (Proksch, Brandner et al. 2008). The major complication in healing wounds is infection, which has been estimated to cause 75% of the mortality following burn injuries (Revathi, Puri et al. 1998). In contrast to drug-eluting stents that deliver anti-proliferative agents, wound dressings are loaded with antibacterial agents and growth factors to reduce bacterial infection and promote healing, respectively. Antibacterial agents are highly water-soluble and thus fast release from the dressing is a major issue and must be addressed to improve the performance of the dressing (Zilberman, Kraitzer et al. 2010). Along with reducing infiltration of bacteria, promoting new tissue growth is essential to successful wound healing and involves a cascade of sequential but overlapping molecular and cellular events including inflammatory response, re-epithelialization, granulation tissue phase and finally remodeling of the healed wound (Andreadis and Geer 2006). This complex process is difficult to emulate, with most research focusing on different biomaterials and therapeutic

molecules to achieve successful wound healing by inhibiting infection and simultaneously promoting tissue growth (Andreadis and Geer 2006).

For wound healing, biomaterials must possess enough elasticity to conform to the size and shape of the wound, endow temporary mechanical support to withstand *in vivo* forces, provide some level of bioactivity to accommodate cellular attachment and migration, act as reservoirs for the controlled delivery of wound healing factors, and, finally, should be non-immunogenic and absorbed by the body once tissue regeneration is complete (Andreadis and Geer 2006). Natural and synthetic biomaterials have been used in wound healing such as collagen and PLGA, respectively (Ishikawa, Terai et al. 2003; Said, El-Halfawy et al. 2012). For synthetic biomaterials, a significant limitation is the need to change dressings throughout the wound healing process, which could increase the likelihood of infection in addition to causing unnecessary pain. This drawback leads to the investigation of natural biomaterials for wound healing due to innate biocompatibility and degradability with collagen being the most commonly used natural material.

However, despite its limited success in wound healing, collagen is susceptible to rapid biodegradation and to bacterial invasion (Sela, Kohavi et al. 2003). Therefore, there is a need to investigate new biomaterials that can improve the efficiency of wound healing.

Administration of antibacterial agents and growth factors has shown success in wound healing applications. Nevertheless, the lack of efficient delivery of growth factors is due to the short half-lives and susceptibility from harsh proteolytic environments (Lee, Silva et al. 2011). Thus, gene delivery has recently been explored as an alternative to growth

factor delivery since infiltrating cells internalize the genes and produce the therapeutic protein(s) in the local environment continuously. For example, collagen dressings embedded with DNA encoding for platelet-derived growth factor (PDGF-A or - B) was shown to increase granulation tissue, re-epithelialization and wound closure in an ischemic rabbit ear model (Tyrone, Mogford et al. 2000). Poly(lactide-co-glycolide) (PLGA) matrices loaded with DNA encoding PDGF were implanted subcutaneously in Lewis rats, and resulted in significantly increased vascularization and granulation tissue formation up to four weeks (Shea, Smiley et al. 1999). However, with the use of nonviral delivery systems, the DNA transfer efficacy in these previous studies were too low for translation to the clinic and thus new delivery systems along with new biomaterials need to be investigated for controlled released of DNA and promotion of tissue growth for optimal wound healing. Due to zein's ability to form films composed of nanospheres with properties that would be beneficial to wound healing, such as resistance to microbial attack, flexibility and capability of sustained DNA release from zein spheres, make it a promising candidate for wound dressings (Shukla and Cheryan 2001; Zhong, Jin et al. 2009; Gong, Sun et al. 2011). In this thesis, zein, a hydrophobic protein from corn, was used to form a film for substrate-mediated gene delivery for potential application in gene-eluting biomedical devices.

1.6 Zein: General Information

A major objective of this thesis is to investigate a new biomaterial, zein, for its ability to be used in gene delivery strategies for applications in gene therapy, DNA vaccination, functional genomics and diagnostics, and biomedical devices. Zein, the major storage

protein from corn, was first isolated in 1821 by John Gorham and first extracted in 1902 by Osborne (Osborne 1891; Lawton 2002; Anderson and Lamsal 2011). The majority of zein is found in the endosperm of the corn kernel and contributes to the hardness of the endosperm (Shukla, Cheryan et al. 2000; Anderson and Lamsal 2011). Since zein is a natural protein, it is heterogeneous in makeup and is actually a mixture of different peptides of various molecular weights (Mw) and solubility (Pomes 1971; Esen 1987; Lawton 2002; Anderson and Lamsal 2011). There have been numerous systems of nomenclature identifying zein fractions but the classifications proposed by Esen have become the standard for understanding the various polypeptides. Esen fractionated whole zein into three main fractions of zein, α , β and γ , determined by their molecular weight and solubility in isopropyl alcohol (IPA). The major fraction, α -zein, accounts for 75-85% of total zein content and is soluble in 50-95% IPA, with a Mw of 21-25 kDa and 10kDa. β -zein (Mw of 17-18 kDa) accounts for 10-15% of total zein content and is soluble in 30-95% IPA with a reducing agent. γ -zein (Mw of 27 kDa) accounts for 5-10% of zein content and is soluble in 0-80% IPA with addition of a reducing agent (Esen 1987).

Zein is an amphiphilic protein, possessing both hydrophobic and hydrophilic properties, with more than 50% of its 225 amino acid residues (Geraghty, Peifer et al. 1981) being hydrophobic (Cabra, Arregun et al. 2005), including high percentages of leucine (20%), proline (10%) and alanine (10%), but zein also has a high glutamine (hydrophilic) content (21-26%) (Pomes 1971; Padua Graciela and Wang 2009). Zein's solubility is attributed to its amino acid composition and therefore due to the high content of nonpolar amino acid

residues, zein is insoluble in water and alcohol but soluble in an aqueous alcohol solution. Also, due to its deficiency in the essential amino acids, lysine and tryptophan, zein is of poor nutritional quality. (Pomes 1971).

The molecular structure of zein is controversial due to the presence of different fractions. However, a structural model of zein in 70% methanol from CD spectra was proposed in 1982 by Argos et al. as a helical wheel with nine homologous repeating units arranged in an anti-parallel form stabilized by hydrogen bonds resulting in a slight asymmetric protein structure (Argos, Pedersen et al. 1982). Later, Matsushima updated the model through data obtained by X-ray scattering, stating that α -zein exists as asymmetric particles of 13 nm in length, had an elongated molecular structure (axial ratio of 6:1) and the helices, which make up 50-60% of zein's molecular structure, were connected at each end by glutamine bridges as seen in Figure 1-2 (Matsushima, Danno et al. 1997). This new model leads to the understanding of zein being amphiphilic, with a hydrophobic outer surface and hydrophilic top and bottom surfaces containing the glutamine-rich bridges (Wang and Padua 2012).

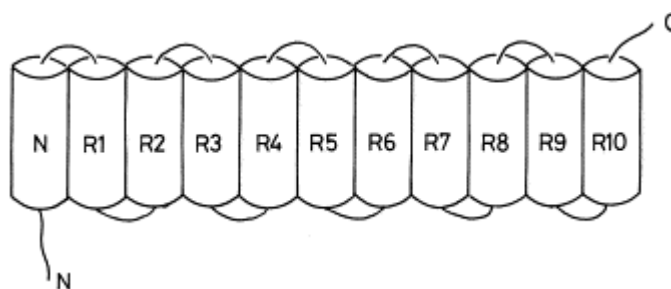


Figure 1-2. A possible structural model for α -zeins (Z22). Each of the tandem repeat units formed by a single α -helix is presented by the cylinder and glutamine-rich 'turns' or loops joining them by the curve. The anti-parallel helices of tandem repeats stack linearly in the direction perpendicular to the helical axis (the *c*-axis) (Reprinted from Matsushima, Danno et al. 1997).

With its amphiphilic character (Wang, Wang et al. 2004), the hydrophobic regions of zein can cause aggregation into colloidal particles, and the polar side chains allow for interaction with drugs or molecules to be encapsulated, including DNA as presented in Chapter 2 of this thesis. Also due to zein's amphiphilicity, it has the ability to self-assemble into a film through a mechanism known as evaporation-induced self-assembly (EISA). This mechanism is a process that involves the preferential evaporation of one of the solvents of a binary or tertiary solvent, causing a polarity change in the solution, which drives the self-assembly of zein (Wang and Padua 2010; Wang and Padua 2012). Zein films formed using EISA are presented in Chapter 3 of this Thesis.

Since the isolation of zein in 1821, it has been extensively researched due to its natural film-forming and solubility properties. Goldsmith was one of the first to promote the use of zein. He demonstrated that mixing zein with formaldehyde could produce a tougher and more stable composite for possible use as a plastic substitute (Lawton 2002). However, it was not until the mid-1930's that the use of zein expanded in research, which may be due to the start of operation of a commercial zein plant in 1938 (Lawton 2002; Anderson and Lamsal 2011). Today, zein is used in numerous industrial and consumer applications, described below, and suggest its potential for use in biomedical applications, including gene delivery systems.

1.6.1 Zein Films & Scaffolds

As described above, zein can form films, with a smooth or particle comprised surface, using simple fabrication techniques, and thus the first major commercial uses of zein

involved the coating of various objects during World War II (Lawton 2002). Zein films are tough, glossy, hydrophobic, greaseproof, biodegradable and resistant to microbial attack (Shukla and Cheryan 2001). These unique properties of zein led to its use as a protective coating for: floors for steamship engine rooms, carton stocks of doughnuts, crackers, cookies, and paper that wrapped airplane and machine parts for shipment (Lawton 2002). However, the use of zein for grease-proofing died out by 1954, but this did not stop exploration of zein's potential in other areas, such as food and pharmaceutical industries.

The food industry first utilized zein as a protective coating on fortified rice in 1955 (Mickus 1955). This application opened the door for zein films to be used for preserving edible products due to its biodegradability, water-resistance and excellent gas barrier (Lawton 2002). Zein has been used to coat nuts to protect against rancidity and demonstrated increased storage time (Cosler 1958). Tomatoes coated with zein showed delayed color change, maintained firmness, and extended shelf-life by 6 days (Park, Chinnan et al. 1994).

By the mid-1950s, the pharmaceutical industry started using zein instead of sugar for tablet coating (Winters and Deardorff 1958). Using zein to coat tablets instead of sugar allowed for quicker coating procedures, resistance to heat, abrasion and humidity as well as the ability to conceal taste and odors of original tablets (Winters and Deardorff 1958; Lawton 2002). More recently, zein has been investigated for its potential in controlled release applications in the pharmaceutical industry (Shukla and Cheryan 2001). Due to its

water insolubility, zein allows for the sustained release of cargo (Hurtado-Lopez and Murdan 2006). Rosenthal was the first to claim sustained release using zein as a matrix polymer with the drug dispersed throughout to extrude pellets for tableting (Fritz 1959). O'Donnell et al. retarded the release of acetaminophen tablets by the application of an aqueous pseudolatex of zein (O'Donnell, Wu et al. 1997). Zein served as the coating for pectin hydrogel beads for drug delivery to the colon, since pectin remains intact in the upper GI tract and is degraded specifically in colon. The zein coating increased the water resistance of the pectin beads as well as protected the drug from the acidic environment of the stomach (Liu, Fishman et al. 2006).

Other studies on zein films suggest its potential use for SMD. Patterned hydrophobic and hydrophilic zein films were formed by adsorbing zein on different hydrophilic and hydrophobic surfaces. Spatial distribution of cells was confined to the areas of zein adsorbed on hydrophilic surfaces. This study suggests that zein could be used to control spatial growth of cells (Wang, Xian et al. 2008) for applications in tissue engineering or transfected cells arrays. Zein films composed of microspheres have been investigated as a drug-eluting coating implanted devices. Controllable heparin- loaded zein films produced superior anticoagulation and inhibited of platelet adhesion for application in cardiovascular devices, such as a stent (Wang, Lin et al. 2005). Zein microspheres displayed sustained release of ciprofloxacin, an antibiotic, for the prevention of bacterial infection, which could have potential in wound healing applications (Fu, Wang et al. 2009). The wide use and success of zein films suggests its potential in SMD for gene delivery applications.

The success of zein films in sustained delivery of an active compound has led to the investigation of zein use in tissue engineering applications. Jin-Ye Wang and colleagues evaluated the surface morphology and biocompatibility of two different methods of preparing zein films, using HL-7702 liver cells and NIH3T3 mouse fibroblast cells (Dong, Sun et al. 2004). The two zein films were comprised of particles and differed by particle sizes ranging from 100-500 to 500-2500nm. The film composed of smaller particles at the lowest concentration showed the best proliferation of cells. Wang and colleagues also measured the degradation of zein films by trypsin and collagenase and showed that the resulting degradation products of zein can enhance cell viability at certain concentrations (Sun, Dong et al. 2005). These studies established the biocompatibility and degradation of zein films for *in vitro* studies (Sun, Dong et al. 2005) but also paved the way for the use of zein in tissue engineering and gene delivery applications. Further investigation of zein scaffolds for possible bone substitute implants demonstrated adequate mechanical properties including a Young's modulus of 28-87 MPa and compressive strength of 1.5-11.8 MPa, which is comparable to cancellous bone (Gong, Wang et al. 2006). Mesenchymal stem cells (MSC) displayed good adhesion, proliferation and differentiation into osteoblasts on the porous zein scaffolds in the presence of dexamethasone (Gong, Wang et al. 2006). Wang also demonstrated that the mechanical properties (tensile and flexural) of the porous zein scaffold could be improved by the addition of the fatty acid, oleic acid (Wang, Gong et al. 2007). These findings add to the already many unique properties of zein and strengthens its use in not only tissue engineering but other medical applications, including gene delivery.

1.6.2 Zein Nano- and Microspheres

In addition to films, zein has been used as particulates encapsulating molecules for sustained release and zein particles are most commonly formed by phase separation, also known as coacervation, which is the separation of colloidal systems into two liquid phases (Gander, Blanco-Príeto et al.). Zein particles formed by coacervation have been used for a variety of molecules for a wide range of applications. For example, zein microspheres encapsulating abamectin, a natural pesticide that degrades rapidly in air and sunlight, was shown to have enhanced photostability when encapsulated in zein microspheres (Demchak and Dybas 1997).

Furthermore, the success of zein to provide sustained release has translated to its use in drug delivery. Zein microspheres encapsulating ivermectin, an agent used to destroy parasites, were used for oral and subcutaneous administration in dogs to protect against parasites (Liu, Sun et al. 2005; Gong, Sun et al. 2011). Biocompatible fluorescent zein nanoparticles were also synthesized for simultaneous bioimaging and drug delivery of 5-fluorouracil (5-FU). This study displayed good biocompatibility of nanoparticles with L929 and MCF-7 cell lines even at high concentrations (Ravindran Girija, Balasubramanian et al. 2012). Zein microspheres have also been used as carriers for antigens, such as ovalbumin, to be delivered orally, intramuscularly and vaginally (Hurtado-Lopez and Murdan 2005). Zein microspheres demonstrated sustained release of Gitoxin, a cardiotonic glycoside used for treating cardiac arrhythmia, and were effective in suppressing platelet adhesion (Muthuselvi and Dhathathreyan 2006). Hollow zein nanoparticles loaded with metformin, an anti-diabetic drug, displayed high loading

capacity, sustained and controlled release, and cellular internalization within an hour of incubation (Xu, Jiang et al. 2011). Recently in our lab, zein has been used to encapsulate DNA and demonstrated plasmid integrity during the fabrication process, high loading and encapsulation, and sustained release (Regier 2011; Regier, Taylor et al. 2012). These studies demonstrate zein's potential in providing sustained and effective release of the encapsulated compound for the intended application. The DNA-loaded zein spheres previously formed in our lab were further investigated in Chapter 2 of this thesis for applications in oral and intramuscular gene delivery.

1.7 Objective of thesis

The objective of this thesis was to investigate the potential of zein as a carrier for DNA, in both nanosphere and film form, to improve gene delivery strategies. Chapter 2 discusses the development of zein nanospheres encapsulating DNA, with demonstrated ability to resist DNase degradation, promote cellular internalization and robust biocompatibility. Chapter 3 describes preliminary studies on the fabrication of zein films for SMD of DNA. Finally, Chapter 4 presents future directions for zein nanosphere and films in the realm of gene delivery.

CHAPTER 2: DNA-loaded Zein Nanospheres

2.1 Background

Gene delivery, the introduction of exogenous DNA into cells with subsequent expression, is applicable to the fields of gene therapy (Niidome and Huang 2002), DNA vaccination (Donnelly, Ulmer et al. 1997) functional genomics and diagnostics (Pannier, Ariazi et al. 2007), tissue engineering (Shea, Smiley et al. 1999), and drug-eluting medical implants (Zilberman, Kraitzer et al. 2010). Because of the technical and safety issues associated with viral gene delivery, the use of plasmid DNA, which has lower immunogenicity, more flexibility in transgene capacity, and potential for industrial production, is an appealing alternative for gene transfer (Mintzer and Simanek 2009). Delivery of DNA can result in the expression of a therapeutic gene or induction of protective immunity (Donnelly, Ulmer et al. 1997; Li and 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 and Huang 2002). Encapsulation of DNA has the potential to improve *in vivo* response and transfection by shielding the CpG methylation patterns of DNA from the immune system, shielding the plasmid from degradation by enzymes and low pH, increasing residence time, and providing a controlled release (Agarwal and 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 it through hydrolytic or enzymatic mechanisms, have been applied to gene therapy, DNA vaccination, and tissue engineering (Panyam and Labhasetwar 2003; Agarwal and Mallapragada 2008).

For gene therapy and DNA vaccination applications, several routes of administration for DNA delivery systems are possible, including oral delivery and intramuscular injection. 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 and Amiji 2007). Oral gene delivery has the potential to treat diseases associated with the gastro-intestinal tract (GI-tract) (Page and Cudmore 2001) as well as systemic diseases (Rothman, Tseng et al. 2005), and can provide for systemic and mucosal immunity (Jones, Corris et al. 1997). However, oral delivery of DNA is complicated by low pH in the stomach and DNases in the GI-tract (Loretz, Foger et al. 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 (Chen and 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 (Jones, Clegg et al. 1998; Roy, Mao et al. 1999; Agarwal and Mallapragada 2008; Bowman, Sarkar et al. 2008). 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 (He, Wang et al. 2005; Bowman, Sarkar et al. 2008). Furthermore, although these materials have provided a valuable proof of concept, their lack of sufficient efficacy suggests that new polymers need to be investigated.

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 and Rappuoli 2004) and gene therapy (Tripathy, Svensson et al. 1996). Injection of DNA-loaded nanoparticles and microparticles into 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 and Rappuoli 2004), can result in a physiological change in the injected muscle (Shyu, Manor et al. 1998), or can serve as a depot releasing the encoded protein into circulation (Tripathy, Svensson 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 (Wang, Zhang et al. 2002; Agarwal and Mallapragada 2008). However, as for oral delivery of DNA-loaded particulates, lack of sufficient efficacy with intramuscular administration suggests that new polymers need to be investigated for DNA delivery.

Natural polymers have been applied to drug and gene delivery and tissue engineering applications, and have the advantages of providing innate degradability and bioactivity (Dang and Leong 2006; Malafaya, Silva et al. 2007), but typically suffer from low mechanical stability, limited ability to be processed, and a relatively short release period compared to synthetic polymers (Panyam and Labhasetwar 2003; Yannas 2004). Zein, the prolamin or storage protein from corn, has properties that make it a promising candidate material for particulate delivery. Zein is composed of three fractions, which are defined by their molecular weight and solubility, including 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 (Esen 1987). More than 50% of the 225 amino acid residues (Geraghty, Peifer et al. 1981) of zein are hydrophobic (Cabra, Arregun et al. 2005), including high percentages of leucine, proline, and alanine, which renders it insoluble under physiological conditions and capable of sustained release of encapsulated compounds (Hurtado-Lopez and Murdan 2006). Zein also has a high glutamine content (Pomes 1971; Padua Graciela and Wang 2009), contributing polar, protonable side chains. With its amphiphilic character (Wang, Wang et al. 2004), the hydrophobic regions of zein can cause aggregation into colloidal particles, and the polar side chains allow for interaction with DNA. The surface charge of zein varies with the pH of the environment (Deo, Jockusch et al. 2003), with an isoelectric point of α -zein at pH 6.8 (Cabra, Arregun et al. 2005). Degradation of zein occurs very slowly by hydrolysis but is accelerated by the action of enzymes (Hurtado-Lopez and Murdan 2006) and has been shown to be especially well-suited for oral delivery (DiBiase and Morrel 1997; Parris, Cooke et al. 2005; Gong, Sun et al. 2011) Zein has been shown to be biocompatible and to have degradation products that can enhance cell proliferation (Sun, Dong et al. 2005).

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 (Gong, Sun et al. 2011) and desmopressin in a Phase I clinical trial (DiBiase and Morrel 1997).

However, to the best of our knowledge, there have been no reports on the fabrication of zein nanospheres encapsulating a large, charged and hydrophilic molecule, like DNA.

Due to the promise of this natural biomaterial for drug delivery, including its biocompatibility, promotion of cell proliferation, capability for sustained release, interaction with cell membranes and its versatility to interact, encapsulate and protect cargo, zein has recently been investigated as a particulate DNA delivery system.

Previous work in the Pannier Laboratory has shown zein's ability to form DNA- loaded nanospheres, prepared using a coacervation process that was capable of preserving the DNA during the encapsulation process and resulted in DNA loading maximized at 6.1 ± 0.2 mg DNA/g zein (Regier 2011; Regier, Taylor et al. 2012), which is comparable to that achieved by most PLGA particulates (Astete and Sabliov 2006). In addition, the fabrication technique allowed for the capability of controlling the size of spheres via the zein to DNA ratio; sphere sizes ranged from 157.8 ± 3.9 nm to 396.8 ± 16.1 nm.

Optimized spheres (80:1 zein to DNA ratio) demonstrated adequate stability in water and PBS and had a sustained release of DNA in PBS over a period of seven days. This work laid the foundation for the potential of zein to be used as a natural polymer for particulate gene delivery. However, the potential of these zein/DNA nanospheres to protect their DNA cargo from DNA nucleases found throughout the body, as well as the interactions of these DNA-loaded zein spheres with cells, remained uncharacterized. Therefore, the objective of this study was to investigate the ability of DNA-loaded zein nanospheres to resist DNase degradation and characterize cellular internalization of the spheres and cytotoxicity, to more fully demonstrate zein's potential in future clinical applications of gene delivery.

2.2. Materials and 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°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 the 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 DsRed2 plasmid DNA (1 mg/mL in TE buffer) and TE buffer were added, followed by the dropwise addition of 10mL ddH₂O (18.2 mΩ-cm) while vortexing. Spheres were formed by coacervation during the addition of the ddH₂O. 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 so that the spheres could be resuspended, as this pH induced sufficient surface charge on the spheres to prevent aggregation during centrifugation (Demetriades, Coupland et al. 1997; Gan, Wang et al. 2005; Lai and Guo 2011). Spheres were then pelleted by centrifugation at

10,000 g for 1 hour at room temperature. The supernatant was removed and spheres were resuspended in ddH₂O.

2.2.3 DNA integrity

DNA was extracted from 80:1 zein:DNA ratio spheres using a standard phenol/chloroform extraction followed by gel electrophoresis. After sphere preparation and centrifugation, the supernatant was removed to a separate conical tube and pelleted spheres were resuspended in 5 mL ddH₂O. 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 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 and were used for gel electrophoresis, as described below.

For PBS release study, pelleted spheres were resuspended in 2 mL ddH₂O and DNA release from spheres into 1X PBS was measured over 7 days. Separate samples for each time point were prepared by diluting 150 µL of sphere suspension (containing approximately 750 µg of zein and 4.3 µg of DNA) in 3 mL of PBS. 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, as described above, and DNA was analyzed through gel electrophoresis.

Sphere extraction solutions and release samples (as described above) were analyzed for plasmid integrity, as was the ability of the spheres to protect encapsulated DNA from endonucleases. For the latter, naked DNA (10 µg) and optimized 80:1 zein/DNA spheres (equivalent to 10 µg of DNA) were treated with 7.5 U of DNase I (Promega, Madison, WI) and incubated for 15 min at 37°C. The reaction was stopped by adding 50 mM Ethylenediaminetetraacetic acid (EDTA) and incubating at 65°C for 10 min. The integrity of the DNA in the sphere extraction solutions, release samples and DNaseI degradation study was all analyzed by agarose (1%) gel electrophoresis. 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 Cellular response

2.2.4.1 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% trypsin/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.

2.2.4.2 Cytotoxicity

Cytotoxicity was assessed using a Water Soluble Tetrazolium (WST-1) salts cell proliferation assay kit (Roche, Indianapolis, IN). For the WST-1 assay, nanospheres containing 0.1, 0.25, 0.5, 1.0 or 2.0 μg of DNA were formed at a zein to DNA ratio of 80:1, resuspended in 1 mL ddH₂O, and then diluted in OptiMem (Gibco) to a final volume of 75 μL . Cells were seeded in 48-well plates as described above and after 24 hours of culture, zein/DNA nanospheres were added to the culture media in triplicate wells. Cells were imaged to observe cell morphology (Leica DMI 3000B, Bannockburn, IL) and the WST-1 assay was conducted according to the manufacturer's protocol at 24, 48, and 72 hours after the addition of zein nanospheres. Briefly, cells were washed with PBS, and incubated at 37°C with the WST-1 solution (10 vol.% WST-1 reagent in phenol-free DMEM, 400 μL /well). After incubation for 3 hours, absorbance values of WST-1 solution (100 μL from each well) were measured on an Epoch Microplate

spectrophotometer (BioTek, Winooski, VT) at a wavelength of 430 nm. Assays were performed in triplicate on duplicate days.

2.2.4.3 Confocal microscopy

To verify that confocal microscopy could be used to image zein/DNA nanospheres without labeling, the autofluorescence of zein was investigated (Regier 2011). The autofluorescence of various zein concentrations in 70% ethanol was measured in a fluorometer with ultraviolet and blue modules. Once autofluorescence was confirmed (Regier 2011), 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 were visualized with an excitation wavelength of (405 nm) and an emission wavelength of (590 nm) and these images were overlaid with corresponding phase images.

2.2.4.4 Sphere internalization

To assess internalization of zein nanospheres, Caco-2 cells were seeded onto 8-well coverslides described above and after 36 hours of culture, media was replaced and zein/DNA nanospheres containing 0.1 μ g DNA at a zein to DNA ratio of 80:1 were added to the fresh culture media. Twelve hours after the media change and addition of spheres, the cells were washed and then membranes were stained using Vybrant DiO cell-labeling solution (Molecular Probes/Life Technologies, Carlsbad, CA) for 25 minutes at

37° C according to the manufacturer's protocol. Cells were then washed with PBS and fixed with 10% buffered formalin for 10 minutes. Following staining, confocal microscopy was performed, with zein and DiO sequentially excited using dual excitation of 405 and 543 nm for zein and 488 nm for DiO. A series of 15 optical sections in the Z-plane were acquired at intervals of 1 μ m for at least two different locations per well and images were processed using Olympus FluoView software (v.5.0) to determine location of the spheres relative to cellular membranes.

2.2.5 Statistics

All experiments were performed between three and six times (noted in figure legends). 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 are reported for all data.

2.3 Results & Discussion

Previously, it was shown that DNA-loaded zein spheres, formed via a coacervation technique, demonstrated tunable sizes that ranged from 157.8 ± 3.9 nm to 396.8 ± 16.1 nm depending on zein to DNA ratio, and maximized DNA encapsulation efficiency and loading of $65.3 \pm 1.9\%$ and 6.1 ± 0.2 mg DNA/g, respectively. Zein/DNA spheres also exhibited sustained DNA release in PBS over 7 days with a minimal burst release during initial phase of release (Regier 2011; Regier, Taylor et al. 2012). Yet, the potential of these zein/DNA nanospheres to protect their DNA cargo from DNA nucleases and to

interact with cells remained uncharacterized.

2.3.1 DNA integrity

The integrity of the plasmid DNA integrity is necessary for efficient gene delivery, specifically maintenance of the supercoiled conformation; thus integrity measurements were previously made to ensure that encapsulation of the DNA within the zein spheres did not damage the DNA structure (Regier 2011). Agarose gel electrophoresis revealed that DNA was not damaged by encapsulation under the conditions used in this study (Figure 2-1A). DNA integrity was also maintained in spheres during seven days of incubation in PBS for the release studies (Figure 2-1B and 1C). While the stability of the DNA in and released from zein particles is critical for the success of gene delivery, it also suggests 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, Bengali et al. 2006).

DNA is also susceptible to *in vivo* biological clearance through degradation by cellular defense enzymes, known as endonucleases (Agarwal and Mallapragada 2008). These endonucleases are enzymes that cleave and damage DNA resulting in a nonfunctional plasmid. An essential determinant of transfection efficiency of these spheres is their ability to protect encapsulated DNA against endonucleases and therefore they were evaluated with a DNase I assay. Zein nanospheres showed protection of encapsulated DNA from DNase I degradation, with DNA concentrated at the top of the gel and unable to migrate from the loading well, due to its strong interaction with zein (Figure 2-1D).

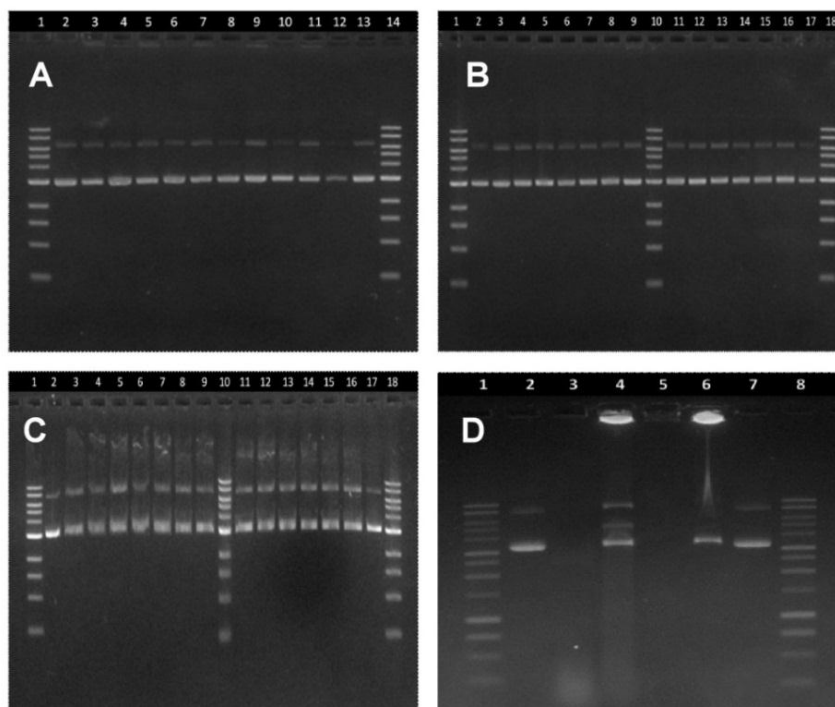


Figure 2-1: Agarose gel electrophoresis images of extracted samples for spheres made at various zein to DNA ratios (A): lane 1, ladder; lane 2, stock DNA; lane 3, 20:1 spheres; lane 4, 20:1 supernatant; lane 5, 40:1 spheres; lane 6, 40:1 supernatant; lane 7, 80:1 spheres; lane 8, 80:1 supernatant; lane 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. (D) Agarose gel electrophoresis images of DNA in DNase I degradation assay: lane 1, ladder; lane 2, stock DNA; lane 3, Naked DNA + DNase I; lane 4, 80:1 spheres + DNase I; lane 5, blank spheres (zein with no DNA); lane 6 80:1 spheres; lane 7, stock DNA; lane 8, ladder.

2.3.2 Cell studies

Cell studies were conducted to determine biocompatibility, cellular association and internalization of the zein/DNA particles. Biocompatibility of the zein/DNA spheres with HEK 293T and Caco-2 cell lines was measured using a WST-1 assay. For HEK 293T cells, absorbance values at a wavelength of 430 nm after 24 hours showed statistically significant differences between cells that were dosed with zein/DNA particles (2 µg) and the control (cell only) condition, but at 48 and 72 hours, all zein/DNA conditions resulted in absorbance values that were not statistically significantly different from the control condition, indicating no cytotoxicity (Figure 2-2A). For Caco-2 cells, absorbance values at a wavelength of 430 nm at all times points (24, 48 and 72 hours) showed no statistically significant differences between cells that were dosed with zein/DNA particles and the control (cell only) condition, except for two concentrations of zein/DNA nanoparticles (2µg and 1µg of DNA at 48 and 72 hours, respectively), where absorbance values were significantly increased ($p < 0.05$) over the control condition, suggesting an increase in cell proliferation, similar to previous studies that have shown that the degradation products of zein can enhance cell proliferation (Sun, Dong et al. 2005). For both cell types a lack of a dose response was observed, in that the addition of more spheres did not result in increased cytotoxicity as measured by the WST-1 assay, indicating that even at high doses, the zein/DNA spheres were biocompatible. In addition, morphology appeared normal for both cell types incubated with zein/DNA nanospheres as seen in Figure 2-3. These results demonstrate that the spheres in this current study possess biocompatibility with cells.

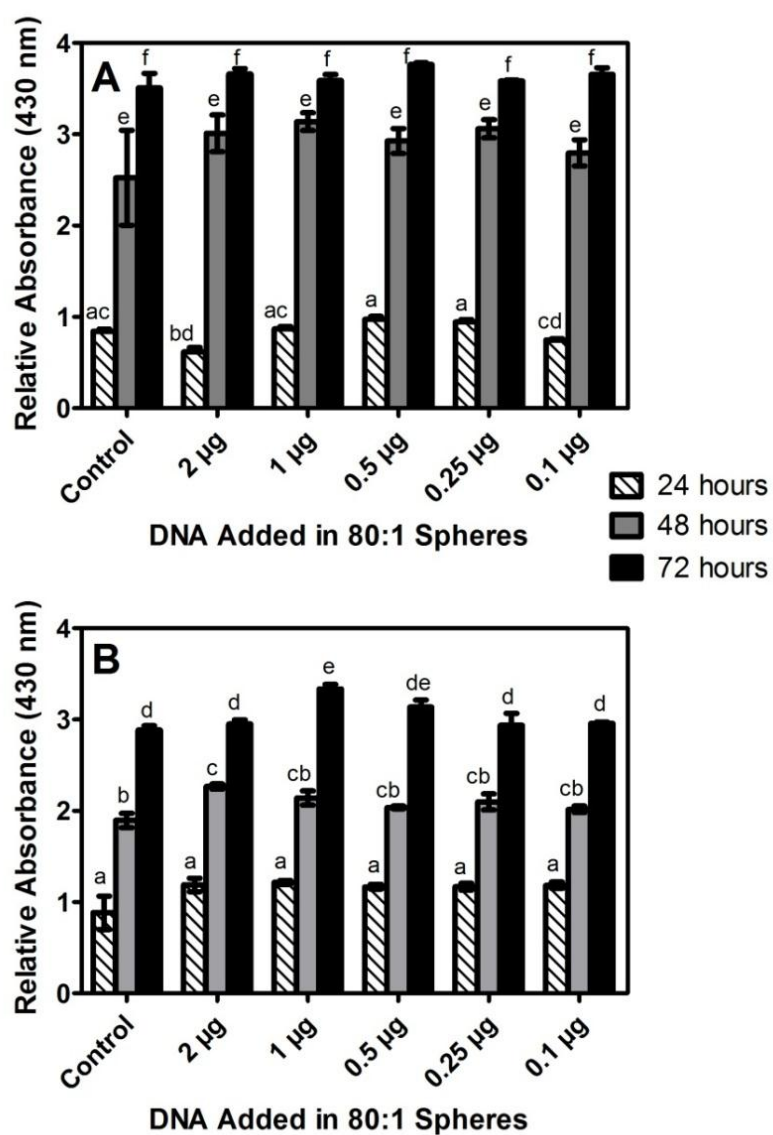


Figure 2-2: Cytotoxicity of zein-DNA nanospheres quantified by WST assay for (A) HEK 293T and (B) Caco-2 cells as a function of time and DNA dose. Control condition represents cells without the addition of particles. Data points labeled with the same letter are not significantly different ($p < 0.05$). Data reported as mean \pm standard error of the mean, with $n=3$.

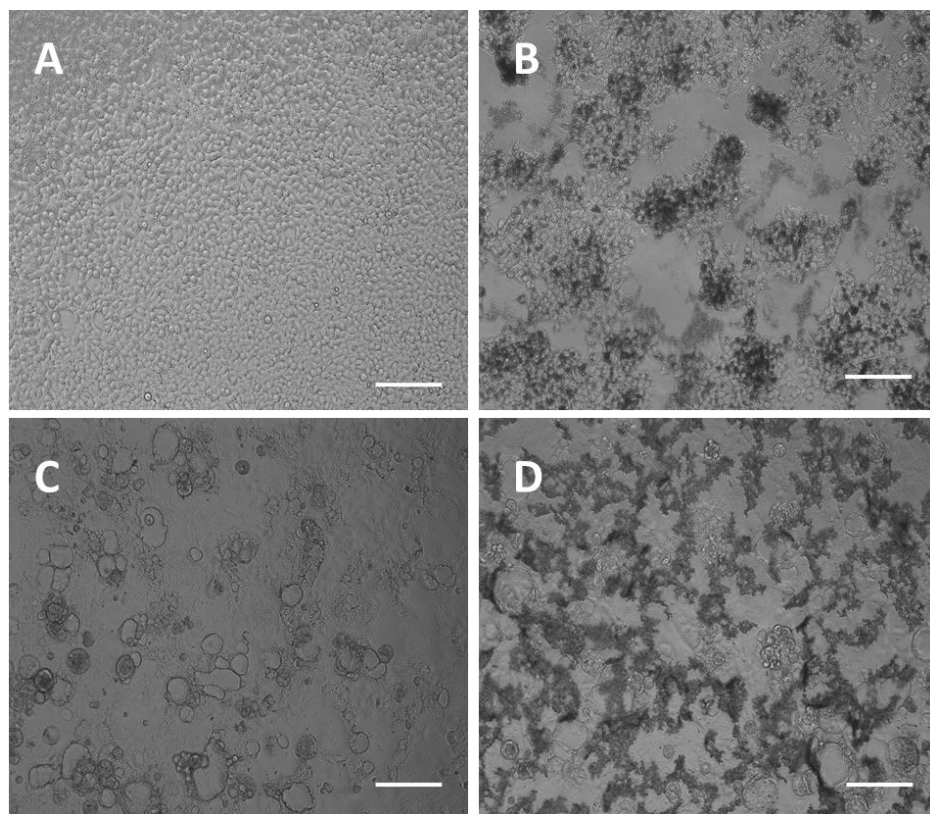


Figure 2-3: Cell images (A) HEK 293T control (B) HEK 293T with 0.5 μg zein/DNA spheres (C) Caco-2 control (D) Caco-2 with 0.5 μg zein/DNA spheres. All images taken at 100X. Scale bar represents 100 μm .

To further assess internalization of the zein nanospheres within the Caco-2 cells, cellular membranes were stained with Vybrant DiO and then confocal imaging was used to visualize both the membrane and sphere locations (Figure 2-4). Sphere internalization was observed in over half of the cells in any particular image (Figure 2-4A). Further analysis of individual cells that were shown to be associated with spheres, through examination of individual z planes within the plane of the cell, confirmed internalization of the spheres. For instance in Figure 2-4B, two zein particles in the XZ plane are clearly internalized, which is further confirmed in the YZ plane. Punctate green staining within cells is indicative of endosomes. While transfection was not observed within the time

course of these internalization studies due to slow release profile of DNA from the spheres, these results suggest that DNA/zein nanospheres can enter cells and thus be used to deliver DNA when properly optimized.

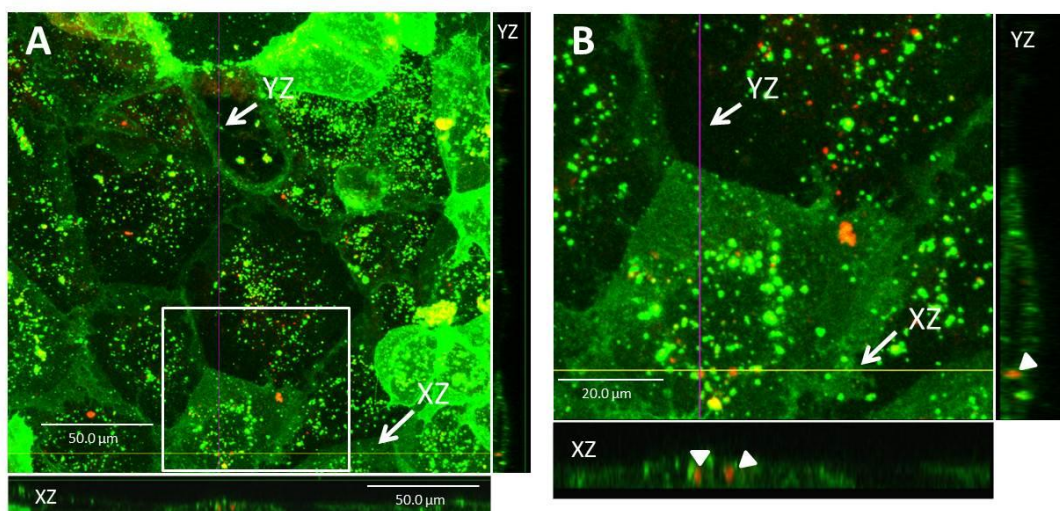


Figure 2-4: Analysis of sphere internalization within Caco-2 cells, with DiO membrane staining, using merged fluorescence confocal images of entire z-stack with orthogonal views, XZ and YZ (A) and a digital magnification of the area outlined by a white square in A (B). Punctate green staining within cells are indicative of endosomes. Filled arrows indicate internalized, autofluorescent nanospheres. Scale bars in A and B represent 50 and 20 μm, respectively.

2.4 Conclusions

In this work, zein, a natural protein, was further investigated as a possible material for the formation of spheres encapsulating DNA for gene delivery. Zein is a protein that is insoluble under physiological conditions, which can allow for sustained release of encapsulated compounds and enable zein to act more like a hydrophobic polymer (e.g. PLGA). Zein is biocompatible and has degradation products that can enhance cell proliferation (Sun, Dong et al. 2005). Furthermore, part of the N-terminal region of γ -zein has been shown to interact with cell membranes and has served as a peptide

carrier for drugs across cell membranes (Fernandez-Carneado, Kogan et al. 2004). Zein is commonly 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 also shown great potential in the field of drug delivery (Mathiowitz, Bernstein et al. 1993; Sun, Dong et al. 2005; Gong, Wang et al. 2006), but previous studies have focused on small molecule, hydrophobic drugs. However, properties of zein make it well-suited for the development of nonviral gene delivery systems. Here, zein nanospheres encapsulating DNA, formed and optimized from previous work (Regier 2011), demonstrated the ability to protect cargo DNA from endonucleases, and when delivered to cells showed acceptable biocompatibility, with high levels of cell association and internalization (Regier, Taylor et al. 2012). Future work will include modifications to improve cellular uptake and transfection, as well as design components to engineer these particles for particle DNA delivery applications, including oral gene delivery, intramuscular injection, and in the fabrication of tissue engineering scaffolds.

CHAPTER 3: Zein Films for Substrate-Mediated Gene Delivery

3.1 Background

Gene delivery, the introduction of exogenous DNA into cells with subsequent expression, is applicable to the fields of gene therapy (Niidome and Huang 2002), DNA vaccination (Donnelly, Ulmer et al. 1997) functional genomics and diagnostics (Pannier, Ariazi et al. 2007), tissue engineering (Shea, Smiley et al. 1999), and biomedical devices (Zilberman, Kraitzer et al. 2010). Nonviral gene delivery is often explored due to its simplicity of manufacturing, flexibility in transgene capacity, and lower immunogenicity over its counterpart of viral delivery (Mintzer and Simanek 2009). Typically, formation of nonviral vectors is accomplished through complexation of negatively charged DNA with cationic polymers or lipids and then is delivered as a bolus to cells (Segura and Shea 2001). However, this bolus addition of complexes has limitations in mass transport to cells or can be hindered by deactivation processes such as degradation, aggregation or clearance of DNA complexes from cells or tissues (Segura, Volk et al. 2003), resulting in uneven distribution of complexes and lower transfection levels. The need for a controlled and efficient gene delivery system is a crucial component to many applications and thus, alternative delivery strategies have been investigated for increasing location and levels of transfection efficiency. Substrate-mediated gene delivery (SMD), also termed reverse transfection (Ziauddin and Sabatini 2001), or solid-phase delivery (Bielinska, Yen et al. 2000), describes a delivery system where complexes are immobilized to a surface that supports cell adhesion; cells are then seeded on top of the immobilized DNA complexes (Segura, Volk et al. 2003; Bengali, Pannier et al. 2005; Bengali and Shea 2005; Pannier,

Anderson et al. 2005). The immobilization of complexes allows for concentrated amounts of DNA in the local environment of cells (Shea, Smiley et al. 1999; Luo and Saltzman 2000) and limited aggregation of complexes, and has been shown to enhance gene delivery (Bengali, Pannier et al. 2005; Bengali, Rea et al. 2009; Segura, Volk et al. 2003; Pannier, Anderson et al. 2005). SMD can also be used as a patterning strategy to spatially control delivery on a surface, in applications such as transfected cell arrays (Pannier, Anderson et al. 2005) and tissue engineering scaffolds (Jang, Bengali et al. 2006). This alternative approach to gene delivery has been examined widely, using polyplexes (Bengali, Pannier et al. 2005; Bengali, Rea et al. 2009), lipoplexes (Segura, Volk et al. 2003; Pannier, Anderson et al. 2005; Bengali, Rea et al. 2009), and viral vectors (Levy, Song et al. 2001; Shin, Salvay et al. 2010). Vectors can be immobilized through non-specific interactions (hydrophobic, electrostatic and van der Waals) (Bielinska, Yen et al. 2000; Kneuer, Sameti et al. 2000; Manuel, Zheng et al. 2001; Zhang, Chua et al. 2004; Bengali, Pannier et al. 2005) or specific interactions (antigen-antibody or biotin-avidin) (Levy, Song et al. 2001; Segura and Shea 2002) to the substrate.

Previous work has demonstrated the potential of SMD with different substrates including polystyrene (Bengali, Pannier et al. 2005), metal stents (Fishbein, Alferiev et al. 2006) and self-assembled monolayers (SAMs) of alkanethiols on gold (Pannier, Anderson et al. 2005), as well as surface-coatings including fetal bovine serum (FBS)-coated polystyrene (Bengali, Pannier et al. 2005) and PLGA (Jang and Shea 2003; Jang, Bengali et al. 2006), collagen- coated polyurethane (Stachelek, Song et al. 2004) and PLGA (Shin, Salvay et al. 2010), and fibronectin- coated polystyrene (Bengali, Rea et al. 2007) and PLGA (Shin,

Salvay et al. 2010). While SMD has demonstrated increased gene transfer compared to the traditional bolus technique in several previous studies (Segura, Volk et al. 2003; Bengali, Pannier et al. 2005; Bengali, Rea et al. 2009), SMD has only been used with a few different surfaces and model biomaterial substrates; other surface materials, in particular surface coatings consisting of natural biomaterials, need to be explored for their ability to translate or improve SMD to applications including biomedical devices (e.g. implants or stents), tissue engineering scaffolds, and transfected cell arrays.

Zein is the prolamin or storage protein from corn and has many physical and biological properties that make it a promising candidate material for substrate-mediated gene delivery, in particular its ability to naturally form films. Zein is amphiphilic (Wang, Wang et al. 2004) with more than 50% of its 225 amino acid residues (Geraghty, Peifer et al. 1981) being hydrophobic (Cabra, Arregun et al. 2005), including high percentages of leucine, proline, and alanine, in addition, zein has a high glutamine content (Pomes 1971; Padua Graciela and Wang 2009), contributing polar, protonable side chains. With its amphiphilic character (Wang, Wang et al. 2004), zein is most commonly dissolved in aqueous ethanol. Zein films can be formed by a simple technique of evaporation induced self-assembly (EISA), that relies on the different evaporation rates of zein's solvent (i.e., ethanol and water), to form a film upon drying (Wang and Padua 2012; Dong, Padua et al. 2013). The resulting zein films are tough, glossy, hydrophobic, and resistant to microbial attack, while maintaining flexibility and compressibility (Shukla and Cheryan 2001). Due to these excellent film properties, zein films have been widely used in the food industry as coatings and packaging materials for fruits, vegetables, and nuts (Shukla

and Cheryan 2001). In addition, zein is biocompatible, degrades slowly in physiological conditions, and its degradation products have been shown to enhance cell proliferation (Sun, Dong et al. 2005), thus zein films have been used in the pharmaceutical industry, as coatings for medical tablets for controlled release systems (Winters and Deardorff 1958). Due to all of the aforementioned properties, zein films have also been investigated as a scaffold to support cell adhesion for biomedical implants (Gong, Wang et al. 2006; Wang, Gong et al. 2007), such as a drug- eluting film for cardiovascular devices (Wang, Lin et al. 2005), and as a particulate for DNA delivery (Regier, Taylor et al. 2012). With such versatility, the objective of this chapter was to investigate zein films as a surface coating for substrate-mediated gene delivery, examining specific parameters of the films for their influence on transfection.

3.2 Materials and Methods

3.2.1 Plasmid preparation

All experiments used pEGFP-Luc (Clontech, Mountain View, CA), a plasmid encoding for both the green fluorescent protein, GFP, and the firefly luciferase protein, LUC, under the direction of a CMV promoter. The plasmid was purified from bacteria culture using Qiagen (Valencia, CA) reagents and stored in Tris-EDTA (TE) buffer solution (10 mM Tris, 1mM EDTA, pH 7.4) at -20 °C. Only plasmids with purity of 1.8 or better measured by 260/280 ratio (Nanodrop 2000 Spectrophotometer, Thermo Scientific, Waltham, MA) were used.

3.2.2 Film formation

Zein films were formed using an evaporation-induced self-assembly (EISA) technique. Films were formed on 8 mm round cover glass slides (Electron Microscopy Sciences, Hatfield, PA). The glass slides were placed on a stage inside a well of a 24-well plate. Several fabrication conditions were varied. For studies investigating zein concentration, zein (Freeman Industries LLC, Tuckahoe, New York) was first dissolved in 55% ethanol forming a 0.2%, 0.4% or 0.8% w/v zein solution. Subsequently, the zein solution pH was adjusted using 1M sodium hydroxide to obtain a pH value of 9. For studies investigating the effect of pH, zein (Freeman Industries LLC, Tuckahoe, New York) was first dissolved in 55% ethanol forming a 0.4% w/v zein solution. Subsequently, the zein solution pH was adjusted using 1M hydrochloric acid or 1M sodium hydroxide to obtain pH values of 2, 4, 7, 9 or 12. For each condition tested, concentration or pH, 60 μ L of the zein solution was pipetted onto the glass slide resting on a stage in a well of a 24-well plate. The lid of the well plate was placed on top, leaving a small gap for air movement for evaporation overnight. Films were used the next day.

3.2.3 Film surface characterization

Scanning electron microscopy (SEM, S-3000N Hitachi, Japan) was used to image the entire film surface (from center to edge) for subsequent analysis of film morphology and uniformity. For SEM imaging films were formed on glass slides as described above and dried in an oven for 1 hr at 120° C. The glass slides with zein films were placed on carbon tape (Electron Microscopy Sciences) and mounted for sputter coating. Films were then sputter coated with chromium and imaged at 20 kV and varying magnifications. A

minimum of two micrographs for each concentration and pH were analyzed using ImageJ (NIH, Bethesda, MD) to determine average sphere diameter. A macroscopic image was also taken with a Galaxy SIII phone camera, to observe difference in appearance of films with regards to concentration and pH.

3.2.4 Contact angle measurements

Static water contact angle (WCA) measurements were carried out using a Ramé-Hart Instruments Goniometer. An image was taken after 10 μ L of ddH₂O was pipetted onto the surface of pH 2 and pH 4 zein films. Angle measurements were analyzed using Ramé-Hart Imaging Software.

3.2.5 Degradation

Degradation studies were carried out for a predetermined time in 1X phosphate buffered saline (PBS) at a temperature of 37° C to mimic the physiological environment of the human body. Zein films were placed in a 48-well plate with 300 μ L of PBS. After specified times, the PBS was removed and zein films were dried in an oven at 120° C for 1 hr. SEM, as described above, was used to observe and analyze degradation of the zein films.

3.2.6 Complex formation

DNA complexes were formed using either a cationic polymer, polyethyleneimine (PEI) (Sigma-Aldrich, St Louis, MO, USA), or cationic lipid, Lipofectamine 2000 (LF2000; Invitrogen). For polyplexes, branched 25 kDa PEI was dialyzed with 10,000 MWCO

Slide-A-Lyzer against ddH₂O, lyophilized, dissolved in TE buffer at 1 mg/ml and stored at -20° C. PEI/DNA polyplexes were formed at a nitrogen to phosphate (N/P) ratio of 25 in 1X Tris buffered saline (TBS) solution by dropwise addition of PEI solution to plasmid DNA (pDNA) solution, the PEI/DNA mixture was briefly vortexed for 10 s, and incubated for 15 min at room temperature. LF2000/DNA lipoplexes were formed following manufacturer's instructions. Briefly, DNA complexes were formed at a DNA:lipid ratio of 1:2 (µg of DNA to µl of LF2000) in serum-free, Opti-MEM media (Invitrogen) by adding transfection reagent diluted in media to DNA in media, mixing by gentle pipetting, and incubating for 20 min. Once complexes were formed, 60 µL (final volume of complexes with 2µg of DNA) was added to each of the zein films for loading, release and transfection studies. All complexation conditions were selected based on optimization of transfection.

3.2.7 Quantification of DNA complex loading

For loading studies, DNA complexes were adsorbed to zein films formed at different pH conditions, as described above. At predetermined time points, complexes were removed and collected from zein films. Films were then washed with either TBS or Opti-MEM and this wash solution was also collected. In order to quantify the amount of DNA adsorbed to the surface of zein films, the amount of DNA that did not adsorb to the films was determined and subtracted from the total amount of DNA added to the well. For LF2000/DNA complexes, removed complexes were dissociated with a 2 mg/mL final concentration of heparin (Sigma-Aldrich, St Louis, MO). Solutions were briefly vortexed and incubated at room temperature for 30 min. After incubation, solutions were then

diluted appropriately in 1X TNE buffer (10 mM Tris; 0.2 M NaCl; 1 mM EDTA; pH 7.4), mixed with one volume Hoechst dye solution (200 ng/mL) and fluorescence was measured by a fluorometer (Modulus Luminometer/Fluorometer, Turner Biosystems, Sunnyvale, CA) after 5 min. incubation at room temperature. A Hoechst standard curve was produced by graphing raw fluorescence versus DNA concentration for various dilutions of the stock DNA solution. For PEI/DNA complexes, removed complexes were diluted appropriately in 1X TNE buffer (10 mM Tris; 0.2 M NaCl; 1 mM EDTA; pH 7.4), mixed with one volume Hoechst dye solution (200 ng/mL) and fluorescence was measured by a fluorometer (Modulus Luminometer/Fluorometer, Turner Biosystems, Sunnyvale, CA) after 5 min. incubation at room temperature. A Hoechst standard curve was produced by graphing raw fluorescence versus PEI/DNA complex concentration for various dilutions of the stock DNA solution. For either complex type the standard curve was then used to quantify the amount of DNA removed from the films, using fluorescence measurements that were first normalized to the fluorescence of blank complex solutions (no DNA added). Equation 1 was used to determine the amount of DNA adsorbed on the zein films.

% DNA Adsorbed

$$= \frac{\text{Amount of DNA added} - \text{Amount of DNA removed}}{\text{Amount of DNA added}} \times 100\% \quad (1)$$

3.2.8 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 1 mM EDTA. Cells were seeded into 48-well plates at a density of 30,000-33,000 cells per well for transfection and cell viability studies.

3.2.9 Transfection

Transfection studies were performed on zein films formed at different zein concentrations and also different pH conditions containing a final DNA amount of 0.4 µg per well. After complex formation and adsorption (2 hrs), HEK 293T cells were seeded onto zein films as described above. Fluorescence microscopy was conducted at 48 hours after seeding cells to confirm the successful expression of the EGFP protein using a Leica DMI 3000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Transfection levels were analyzed following 48 hours of culture by quantification of luciferase activity using the Luciferase Assay System (Promega), for which cells were lysed and assayed for enzymatic activity using a luminometer (Turner Designs, Sunnyvale, CA), and luciferase activity (RLUs) was normalized to area (1cm²). LF2000/DNA transfection experiments were performed in triplicate on duplicate days (n=6), except for pH 2 and pH 4 films (n=5). PEI/DNA transfection experiments were performed in triplicate (n=3), for all zein films at different pH conditions.

3.2.10 Cell viability

Cell viability was assessed using a Water Soluble Tetrazolium (WST-1) salts cell proliferation assay kit (Roche, Indianapolis, IN). For the WST-1 assay, films containing a final DNA amount of 0.4 μg of DNA were formed. Cells were seeded in 48-well plates on the zein films as described above and after 24, 48 and 72 hours of culture, cells were imaged to observe cell morphology (Leica DMI 3000B, Bannockburn, IL) and the WST-1 assay was conducted according to the manufacturer's protocol. Briefly, cells were washed with 1X PBS, transferred to a new well and incubated at 37°C with the WST-1 solution (10 vol.% WST-1 reagent in phenol-free DMEM, 250 μl /well). After incubation for 3 hours, absorbance values of WST-1 solution (150 μl from each well) were measured on an Epoch Microplate spectrophotometer (BioTek, Winooski, VT) at a wavelength of 430 nm and normalized to area (1 cm^2). Assays were performed in triplicate.

3.3 Statistics

All experiments were performed in triplicate, unless otherwise stated. Comparative analyses were completed using a one- or two-way ANOVA followed by Tukey's multiple comparison test or Bonferroni posttest, respectively 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 are reported for all data.

3.4 Results

3.4.1 Optimization of Zein Concentration for Film Formation

Zein films were formed by EISA and investigated for their ability to support SMD. First, the concentration of zein solution used to form the films was varied, to optimize film morphology and coverage. From SEM images and analysis (Figure 3-3A-F), zein films formed from all concentrations were comprised of spheres, as previously reported (Dong, Padua et al. 2013). Different zein concentrations impacted the uniformity of the film and sphere size. Films formed with the lowest concentration of zein, 0.2%, were comprised of 0.99 ± 0.07 nm spheres and did not produce a uniform film over the underlying glass substrate, as seen in Figure 3-1A and 3-1B and shown by others (Dong, Padua et al. 2013). The highest zein concentration tested, 0.8%, formed a microsphere film comprised of the largest spheres (Figure 3-1E), 1.8 ± 0.11 nm, however the sphere sizes varied dramatically around the edge of the film, ranging from 1.5 ± 0.14 nm to 5.6 ± 0.99 nm (Figure 3-1F). The most uniform film was formed from a 0.4% zein solution, with homogenous spheres of 0.99 ± 0.08 nm, and full film coverage of the underlying surface from center to edge (Figure 3-1C and 3-1D). Differences in zein films formed at various concentrations can also be observed macroscopically (Figure 3-3G), which confirms SEM results that show films formed from 0.4% zein solutions have the most uniform appearance.

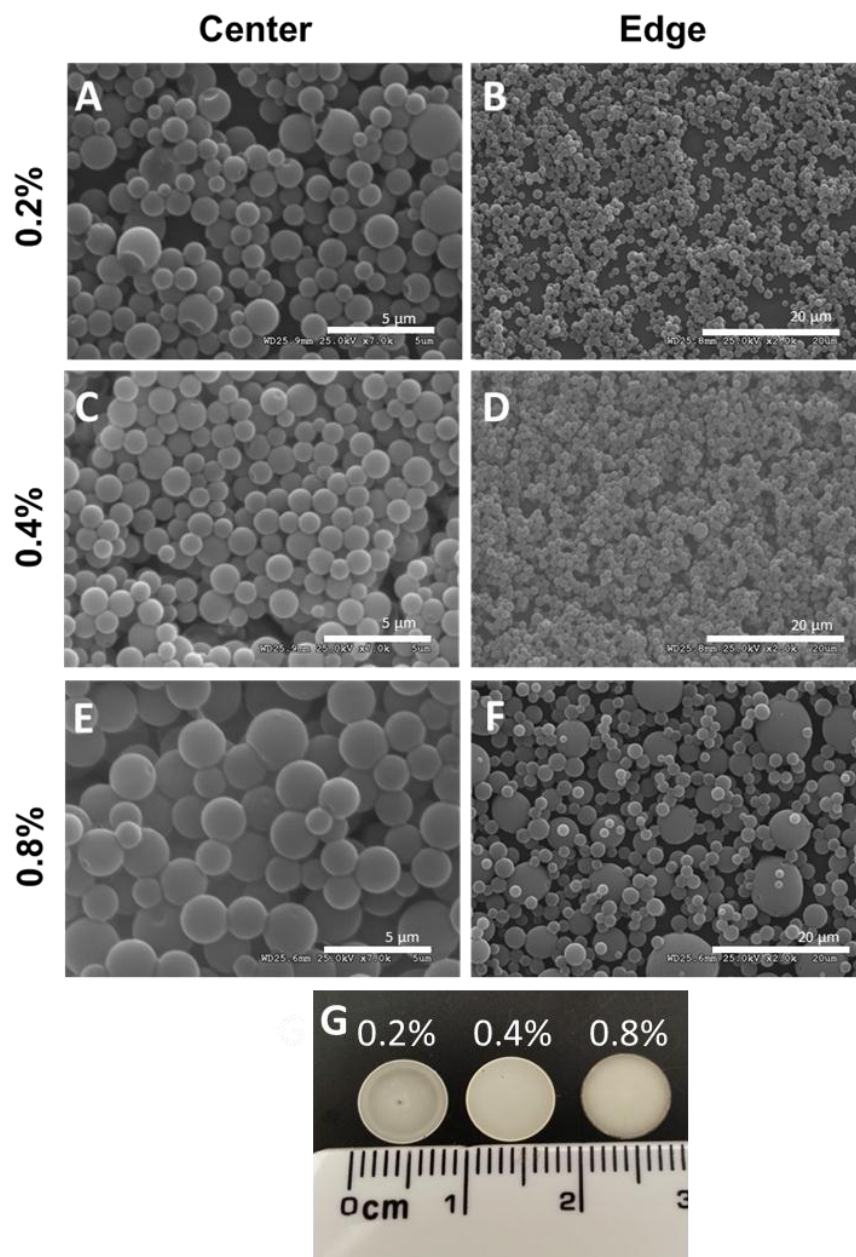


Figure 3-1. SEM of zein films comprised of spheres, (A, B) 0.2% (0.99 ± 0.07 nm spheres with incomplete surface coverage), (C, D) 0.4% (0.99 ± 0.08 nm spheres with full surface coverage), (E, F) 0.8% (1.8 ± 0.11 nm spheres in center with 1.5 ± 0.14 nm to 5.6 ± 0.99 nm spheres around edge, with uneven surface coverage); and (G) Macroscopic image of different concentrations of zein films.

In addition to optimizing zein solution concentration for film uniformity, the concentration was also optimized for SMD transfection in HEK 293T cells. By varying the amount of added DNA, the DNA:lipid ratio for lipoplexes (data not shown), the optimal conditions for high transfection and low cytotoxicity were found to be at a DNA:lipid ratio of 1:2 for polyplexes, and 2 μ g of DNA concentration. Thus, complexes formed with 2 μ g DNA and LF2000 at a 1:2 DNA:lipid ratio were added to these films for adsorption over two hours. While all surfaces supported transfection, films formed from 0.2% and 0.4% zein solutions exhibited significantly higher transfection levels ($p < 0.05$, $p < 0.01$, respectively), than transfection on the films formed from 0.8% zein concentration, determined through luciferase activity (Figure 3-2A) and fluorescent cell images (Figure 3-2B-D) of EGFP expression. Thus, films formed using 0.4% (w/v) zein solution were used throughout the remainder of this chapter due their uniformity in surface coverage and high transfection levels.

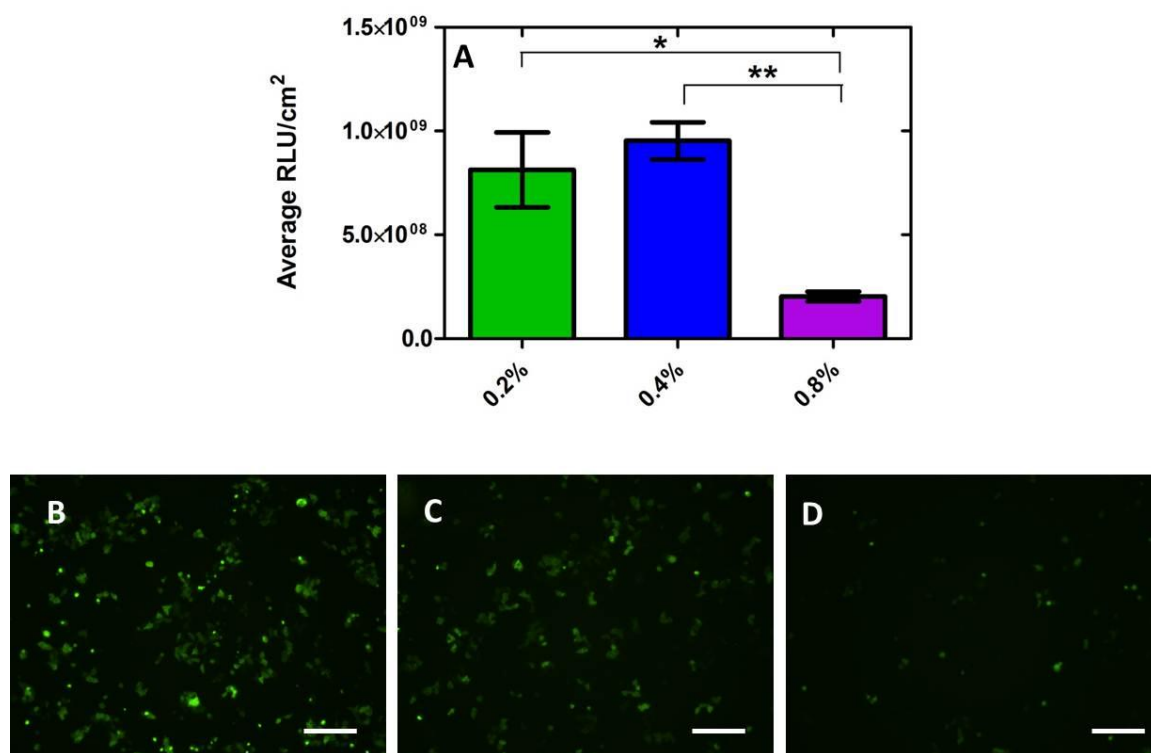


Figure 3-2. SMD Transfection levels of different concentrations of zein films, reported as relative light units (RLU) normalized to area (A), and SMD transfection images of HEK 293T cells with LF/DNA complexes on (B) 0.2%, (C) 0.4%, and (D) 0.8% zein films.

Data reported as mean \pm standard error of the mean, with $n=3$.

(* $p < 0.05$, ** $p < 0.01$).

3.4.2 Morphology of Zein Films formed at Different pH conditions

Given that zein is amphiphilic and its surface charge varies with pH (Deo, Jockusch et al. 2003; Wang, Wang et al. 2004), the effect of the pH of zein solution on the morphology of the resulting zein films was investigated. Films formed from zein solutions at pH 2 formed a smooth, flat film with minor cracks (Figure 3-3A). Films formed from zein solutions at higher pH levels (4-12), were comprised of spheres (Figure 3-3 B-E), as seen above with the different concentrations of zein (Figure 3-1A). Average sizes of the spheres that constituted the films ranged from $1.07 \pm 0.5 \mu\text{m}$ (Figure 3-3B), $1.56 \pm 0.4 \mu\text{m}$ (Figure 3-3C), $0.66 \pm 0.2 \mu\text{m}$ (Figure 3-3 D), to $0.59 \pm 0.2 \mu\text{m}$ (Figure 3-3 E) for pH

4, 7, 9 and 12 zein films, respectively, and are shown in Figure 3-3G. Differences in zein films formed from solutions at different pH conditions were also observed macroscopically (Figure 3-3F), ranging from a translucent appearance (pH 2) to more opaque films at higher pH levels.

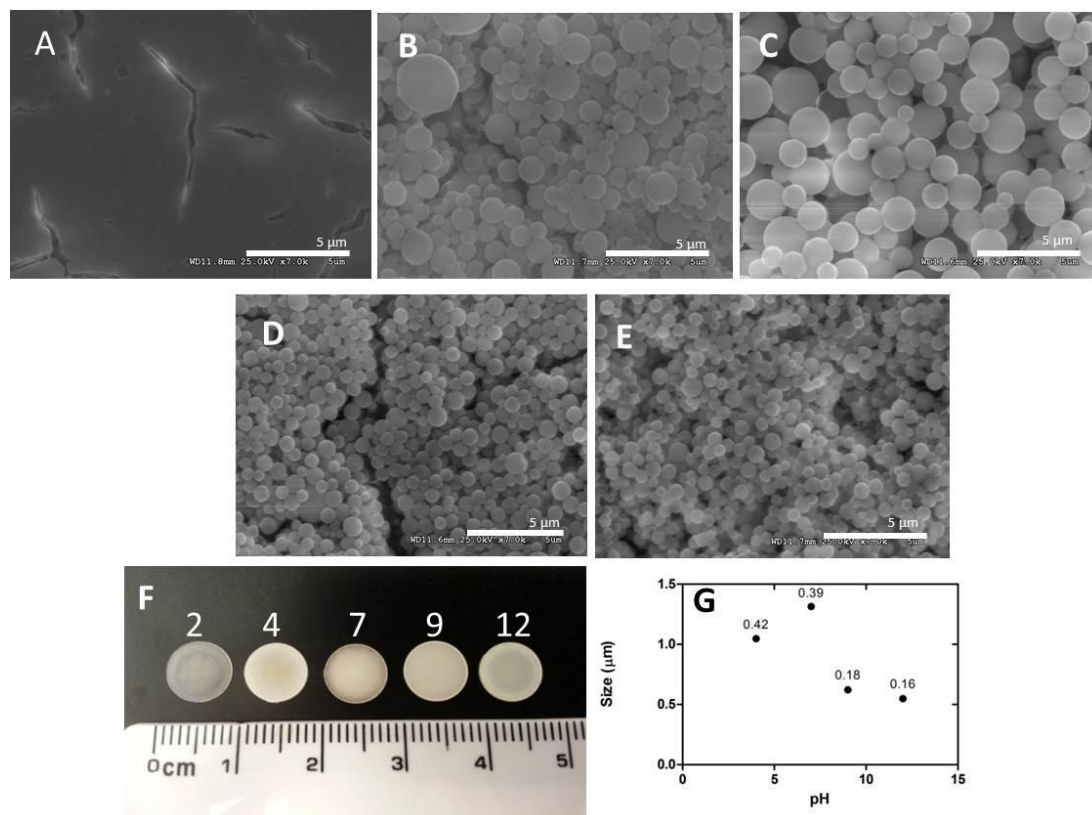


Figure 3-3. SEM images (7k magnification, scale bar 5 μm), of zein films formed at solution at pH 2 (A), (B) pH 4, (C) pH 7, (D) pH 9, (E) pH 12; and (F) Macroscopic images of zein films formed from different pH conditions and (G) average diameter of spheres from each pH level measured from SEM images, reported as mean ± standard error of the mean.

3.4.3 Contact Angle

The WCA of the pH 2 and pH 4 zein films were measured due to the morphological difference between the two films and revealed a WCA of 21.7° for pH 2 (Figure 3-4A) and 9.8° for pH 4 (Figure 3-4B).

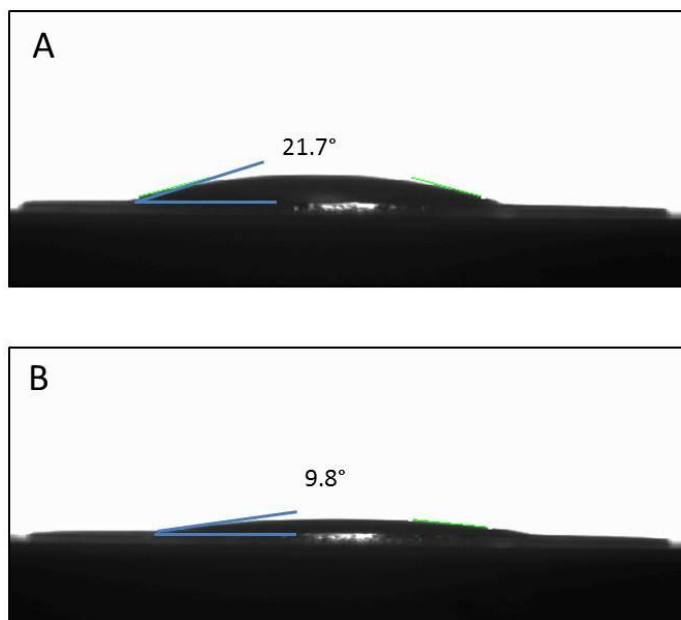


Figure 3-4. The water contact angle of (A) pH 2, and (B) pH 4 zein films.

3.4.4 Degradation

Zein films formed from solutions at different pH conditions were incubated in PBS over a determined period of time and degradation of the films was evaluated through SEM (Figure 3-5). After 72 hours of incubation in PBS, pH 4, 7 and 12 films displayed larger degradation pore sizes of approximately 1.8 μm , compared to films formed from solutions at pH 2 and 9, which maintained a smoother film surface with smaller degradation pores. The addition of PBS to zein films formed from solutions of pH 12 resulted in partial removal of the film from the glass slide; therefore this condition was not further evaluated.

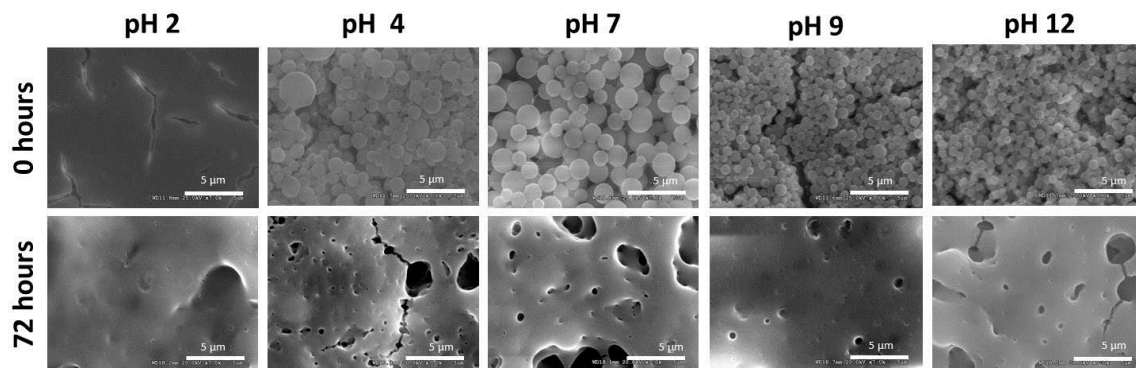


Figure 3-5. SEM images (7k magnification, scale bar 5 μm) of zein film degradation in PBS after 72 hours.

3.4.5 Quantification of Surface Adsorbed DNA complexes

As varying the pH of the zein solution used to form the film could result in a change in surface charge (confirmed by WCA), the effect of pH on the loading of non-specifically adsorbed complexes on the films was analyzed. Both LF/DNA and PEI/DNA complexes were adsorbed on zein films for specified time points and then complexes that did not adsorb were collected. The amount of DNA immobilized to the surface was quantified by measuring the DNA removed and collected. Quantifying the DNA in the lipoplexes was performed by dissociating the complexes with heparin and then using Hoescht, a fluorescent dye that binds DNA, to measure the fluorescence of the sample. For PEI/DNA complexes, a standard curve of known concentration of complexes was used to measure the amount of complexes removed using Hoescht. The amount of DNA immobilized to the surface increased with time for both type of complexes (Figure 3-6), but varied per substrate. For lipoplexes, the rate of absorption was initially slow, as there was only a 4% increase in mass over two hours (Figure 3-6A). After 4 hours of lipoplex adsorption, zein films at pH 4 had a significant increase in absorption of complexes compared to zein film at pH 9 ($p < 0.01$). At the end of the 8 hour adsorption period,

statistically significant differences of DNA absorption between pH 4 and pH 7 ($p<0.01$), pH 4 and pH 9 ($p<0.001$), and pH 7 and pH 9 ($p<0.01$) with a maximum DNA absorption of 51 %, 44 %, and 41% for pH 4, 7 and 9 films, respectively, were observed. This maximum absorption decreased linearly with increasing pH ($r^2=0.99$). However, after two hours of incubation, all zein films showed DNA loading of approximately 20% of the total amount added to the surface (Figure 3-6A), indicating a total of 0.4 μg of DNA, which was used for further experiments in this chapter. Like lipoplexes, PEI/DNA absorption mass and rate was significantly different between zein film conditions. After two hours, DNA absorption on pH 2 and pH 9 zein films was significantly lower compared to both pH 4 and pH 7 zein films (Figure 3-6B). At later time points, zein films formed at pH 2 had significantly reduced DNA absorption as compared to the other zein films at pH 4 ($p<0.01$), pH 7 ($p<0.05$) and pH 9 ($p<0.01$) . However, 100% of polyplexes were adsorbed on all zein films by 24 hours. PEI/DNA complexes absorbed faster than LF/DNA complexes in that 46-56% of polyplexes were absorbed on zein films after two hours, while 21% of DNA from lipoplexes were absorbed on zein films. The two hour adsorption of PEI/DNA polyplexes resulted in a range of 0.92 μg to 1.12 μg of total adsorbed DNA, and was chosen for further experiments in this chapter.

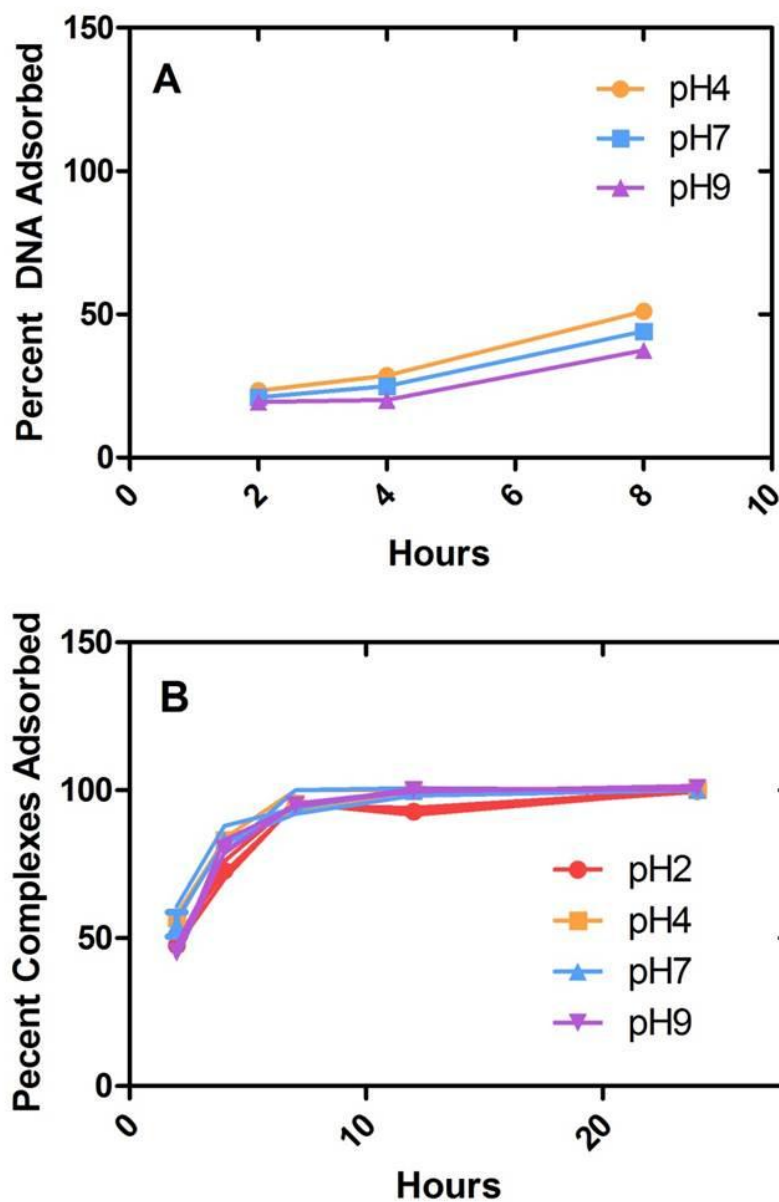


Figure 3-6. Absorption of (A) LF/DNA and (B) PEI/DNA complexes on zein films formed at different pH conditions. Data reported as mean \pm standard error of the mean, with $n=3$.

3.4.6 Substrate-Mediated Transfection on Zein Films

To examine the influence of different zein film fabrication parameters on SMD transfection, LF/DNA or PEI/DNA complexes previously optimized for high transfection and low cytotoxicity (data not shown), were adsorbed onto zein films (prepared from solutions at different pH conditions) for two hours, HEK 293T cells were seeded and transfection profiles were acquired 48 hours following seeding. Transfection profiles were measured using both fluorescence microscopy to image EGFP expression, and the luciferase assay, where average relative light units (RLU) of luciferase luminescence for each sample were normalized to area. Although the amount of DNA adsorbed to the surface of zein films (at two hours) was independent of pH for lipoplexes, but dependent on pH for polyplexes, transfection levels in HEK293T cells for both types of complexes displayed a dependent trend with pH (Figures 3-7A-D and 3-8A).

Transfection mediated by LF/DNA complexes adsorbed to the zein films formed from different pH solutions generally increased with increasing pH of the films (Figures 3-7A-D, Figure 3-8A). Cells seeded on pH 9 films achieved the highest transfection levels in terms of average RLU/cm² for lipoplex transfection, which was nearly 2.5-fold greater than transfection on pH 4 zein films (Figure 3-8A). Transfection levels mediated by PEI/DNA complexes adsorbed on the zein films were significantly lower than lipoplex transfection, however a similar trend was observed with varying pH of the zein films (Figure 3-8B). Gene expression levels were statistically different between pH 2 and pH 9 films ($p < 0.05$), with nearly 2.8-fold enhancement of PEI-mediated transfection on the pH

9 zein films as compared to pH 2 films (Figure 3-8B). These results demonstrate that zein films can support substrate-mediated transfection, in a pH-dependent manner.

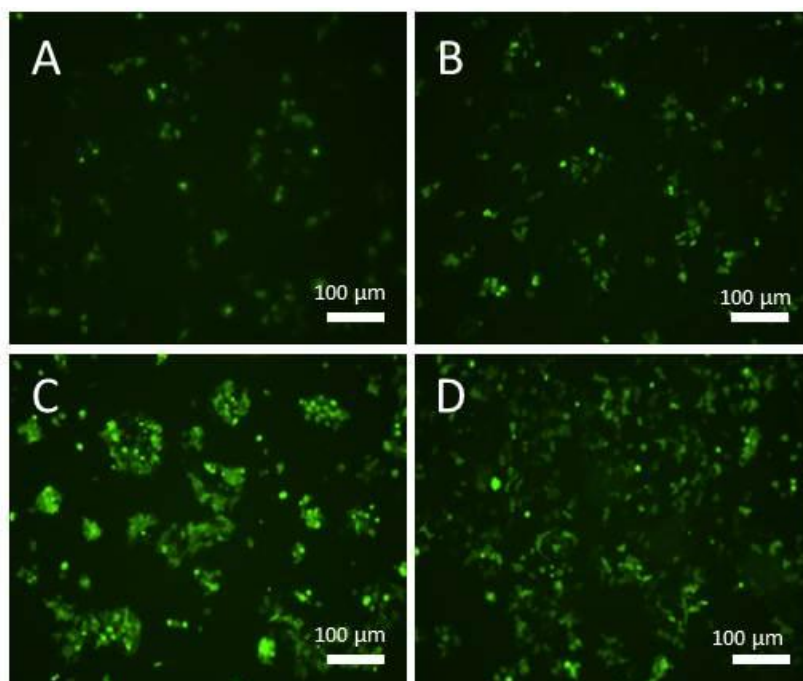


Figure 3-7. SMD transfection images of HEK 293T cells with LF/DNA complexes on (A) pH 2, (B) pH 4, (C) pH 7 and (D) pH 9 zein films. Scale bars represent 100 μm .

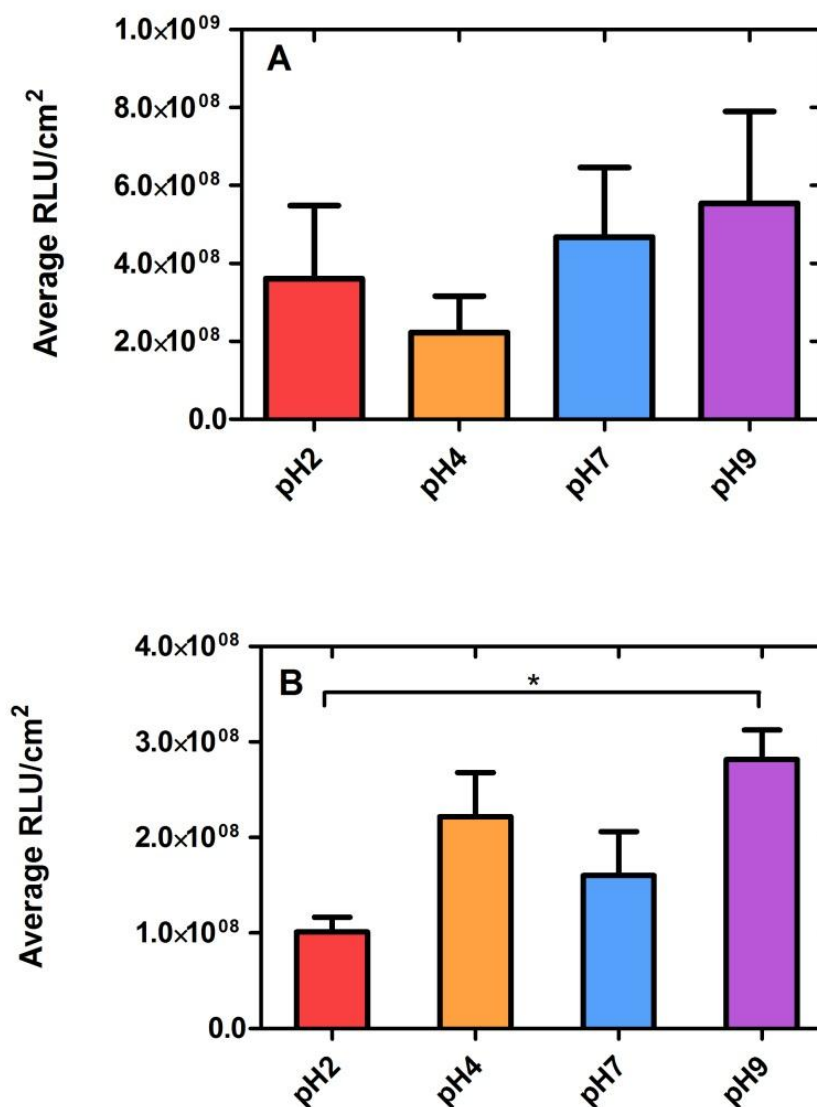


Figure 3-8. Normalized SMD transfection profile for HEK 293T cells plated on zein films formed from different pH solutions, mediated by (A) LF/DNA complexes (n=5 or 6) and (B) PEI/DNA complexes (n=3). Data reported as mean \pm standard error of the mean.

3.4.7 Cell Viability

The viability of HEK 293T cells seeded onto the zein films loaded with LF DNA complexes was measured using a WST-1 assay over 72 hours (Figure 3-9). Polystyrene (PS) with adsorbed complexes was used as a control. Cells on zein films displayed a

slower proliferation rate compared to those on the control PS surface from 24 to 48 hours. However, from 48-72 hours, pH 7 and 9 zein films had similar or higher proliferation rates than PS.

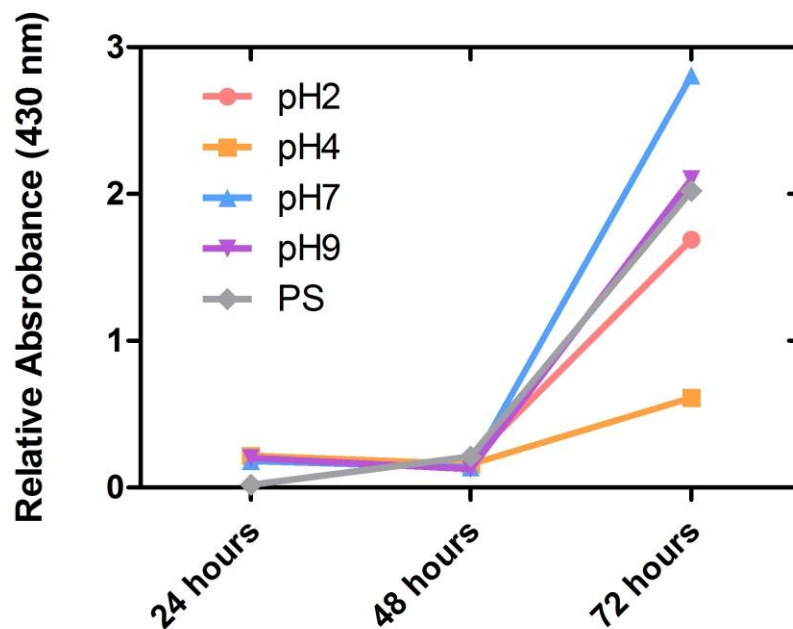


Figure 3-9. Viability of cells on zein films with adsorbed LF/DNA complexes quantified by WST-1 assay for HEK 293T cells as a function of time. The slope of the lines connecting each set of consecutive time points is indicative of the rates of cell proliferation. Data reported as mean \pm standard error of the mean, with $n=3$, except for pH 2, where no data was collected at 24 hours.

3.5 Discussion

Substrate-mediated delivery (SMD) is an alternative approach to traditional bolus gene delivery, and describes the immobilization of DNA to a cell-adhesive substrate prior to cell seeding (Bengali, Pannier et al. 2005; Bengali and Shea 2005; Pannier, Anderson et al. 2005; Jang, Bengali et al. 2006; Bengali, Rea et al. 2007). This mechanism not only allows for an increase in concentration of DNA within the direct microenvironment of

cells (Luo and Saltzman 2000), but also limits aggregation of complexes and has been shown to enhance nonviral gene delivery, compared with bolus delivery, with the potential for spatially controlling transfection through patterned delivery (Pannier, Anderson et al. 2005; Houchin-Ray, Whittlesey et al. 2007). However, limitations of SMD have included the limited understanding of interactions between the properties of both vector and substrate for optimal transfection profiles, as well as ill-defined surface coatings that may be difficult to translate to *in vivo* biomedical devices and scaffolds. Thus, there is still a need for investigating new surface coatings to further develop an efficient and controlled DNA delivery system. Here, zein films were explored for their ability to support SMD.

Zein, a natural protein from corn, is biodegradable, biocompatible, amphiphilic, resistant to microbial attack, promotes cell proliferation and can encapsulate plasmid DNA (Shukla and Cheryan 2001; Wang, Wang et al. 2004; Sun, Dong et al. 2005; Regier, Taylor et al. 2012). In addition, zein is known as an excellent film former and commonly used in pharmaceutical tableting and coatings, which makes it a promising material for biomedical applications (Fernandez-Carneado, Kogan et al. 2004). Due to the desirable properties of this natural biomaterial, zein films that can support SMD could be used in a multitude of biomedical applications such as coated- implants, tissue engineering, and transfected cells arrays (Shea, Smiley et al. 1999; Pannier, Ariazi et al. 2007; Zilberman, Kraitzer et al. 2010). In this chapter, zein films were formed after evaporation induced self-assembly (EISA). This mechanism involves the preferential evaporation of one of the solvents of a binary or tertiary solvent, causing a polarity change in the solution, which

drives the self-assembly of zein (Wang and Padua 2010; Wang and Padua 2012).

Different zein structures, such as spheres, hexagonal, sponge and lamellar phases have been formed after EISA (Wang, Yin et al. 2008; Wang and Padua 2010). The zein films formed in this chapter were all composed of spheres, except for films formed from a pH 2 zein solution. The sphere formation in the zein films can be attributed to zein being amphiphilic and having defined hydrophobic and hydrophilic (glutamine-rich) regions (Pomes 1971; Matsushima, Danno et al. 1997). Zein was dissolved in an aqueous ethanol solvent and subsequently, during EISA, the polarity of the solvent began to increase as the ethanol evaporated faster than the water. This increase in polarity forced the hydrophobic regions of zein to aggregate and reduced their exposure to the hydrophilic solvent, resulting in spheres. This mechanism results in a hydrophilic film surface, as confirmed by WCA, composed of spheres (Dong, Padua et al. 2013). Aggregation of increased number of zein molecules causes the direct relationship of the increased size of spheres with increased concentration of zein solution, as seen in Figure 3-1 and previously shown (Dong, Padua et al. 2013).

As stated above, the films formed at pH 9 for all zein concentrations tested were comprised of spheres and as the concentration of zein increased, the size of spheres increased (Figure 3-1). The size of spheres comprising the zein films correlates to surface roughness, with larger sphere sizes corresponding to increased surface roughness. Surface roughness is a good indicator of hydrophilicity (Sun, Feng et al. 2005). As surface roughness decreases, the hydrophilicity of the surface increases and thus hydrophilicity can be controlled through varying surface roughness (Sun, Feng et al. 2005; Dong, Padua

et al. 2013). Hydrophilic surfaces have previously been shown to support SMD better than hydrophobic surfaces due to the reversible interactions between the substrate and complexes (Bengali, Pannier et al. 2005; Pannier, Anderson et al. 2005). Here, zein films formed with 0.2% and 0.4% zein concentration achieved higher transfection than 0.8% zein films, as seen in figure 3-2. This increase in transfection could be the result of lower surface roughness (i.e. smaller sphere size) of 0.2% and 0.4% compared to that of 0.8% zein films (Blacklock, Vetter et al. 2010).

Next, the pH of the zein solution was investigated for its effect on the morphology of the zein films. Zein films formed at pH 2 were flat, smooth and featureless, which is similar to zein films casted in acetic acid (Shi, Kokini et al. 2009). Films formed at pH values of 4, 7, 9 and 12 showed an inverse relationship between pH of zein film and size of spheres. As the pH of the film increased, the size of spheres decreased. Zein films formed at pH 4 and 7 were comprised of larger spheres (greater surface roughness and hydrophobicity) than pH 9 and 12 zein films. The morphology of zein films can be explained by the mechanism of EISA. The isoelectric point of zein is 6.8 (Cabra, Arregun et al. 2005), indicating that when solution $\text{pH} < 6.8$, the glutamine-rich regions (hydrophilic) of zein are positively charged and negatively charged when solution $\text{pH} > 6.8$. When coating the glass slides, which contain mainly negatively charged silicate groups, the electrostatic interaction between zein molecules and glass slide will force the zein molecules to lie down flat to minimize the interaction energy between zein and glass, as observed in pH 2 solution resulted in a smooth surface seen in Figure 3-2A. For the other zein films formed at pH values of 4, 7, 9 and 12, the repulsive interactions

between the zein molecules and glass slide lead to the formation of a film surface being comprised of spheres (Figure 3-2B-E). The solvent used in zein solution preparation has previously been shown to affect the orientation of molecules on a substrate (Shi, Kokini et al. 2009). Zein films formed from acetic acid formed smoother surfaces as compared to those cast from 95% ethanol aqueous solutions, which were rough and composed of granules. This morphological difference was due to the conformational difference of the protein, due to how the zein molecules aggregate on the substrate (Shi, Kokini et al. 2009).

Degradation of zein films in PBS is shown in Figure 3-5. Zein films, pH 2 and pH 9, appear to have the slowest degradation, which could be attributed to their lower surface roughness compared to the other zein films. This lower surface roughness may help maintain the structure of the film longer than the other zein films, especially pH 4 and pH 7. The larger sphere size in pH 4 and pH 7 zein films could have resulted in the increased pore sizes from degradation upon zein sphere aggregation in PBS, as previously reported (Regier, Taylor et al. 2012). Despite these slight differences in film degradation, the zein films demonstrated a slow degradation profile in PBS, which mimics physiological conditions and can be a good indicator for release of DNA, or an encapsulated drug. Also, zein degradation products have been shown to enhance cell proliferation (Sun, Dong et al. 2005), which is another advantageous factor for zein as a natural polymer choice for SMD compared to commonly used PLGA, where degradation products are acidic and can induce cell death (Jang and Shea 2003). Degradation of zein films in PBS confirms the slow breakdown of zein by hydrolysis which is due to zein's hydrophobic nature (Hurtado-Lopez and Murdan 2006). This slow degradation could allow for a

sustained release of complexes over a long period of time that is critical for tissue engineering applications.

The amount of DNA immobilized to the surface increased with time for all zein films, but varied with zein films formed at different pH conditions. The maximum adsorption of DNA from lipoplexes measured from the zein films decreased with increasing pH (increasing hydrophilicity). Substrate hydrophobicity has been previously shown to influence the local DNA concentration adsorbed to the surface of the substrate. This trend of decreased adsorption on hydrophilic surfaces supports other's results of greater immobilization of complexes on hydrophobic substrates compared to more hydrophilic surfaces (Bengali, Pannier et al. 2005; Pannier, Anderson et al. 2005). Even though zein is hydrophobic, the formed zein film surface appeared to be hydrophilic through surface roughness by EISA in Figure 3-3 and contact angle measurements as shown in Figure 3-4, again due to the forced exposure of the hydrophilic residues through EISA. For polyplexes, the adsorption rate for pH 2 zein films (a more hydrophobic surface) was significantly lower than other zein films (hydrophilic), which contradicts the above statement and our LF/DNA adsorption trend of increased adsorption on hydrophobic surfaces. However, Pannier et al demonstrated decreased absorption on hydrophobic surfaces when the hydrophilic surface contained 100% charged functional groups (Pannier, Anderson et al. 2005). In contrast, pH 7 zein films showed a significant increased adsorption of PEI/DNA complexes compared to pH 9 zein films, which again agrees with increased adsorption on hydrophobic surfaces. Our results coincide with others and suggest that non-specific DNA complex adsorption to zein films might be

mediated by both charge-charge interactions and hydrophobic interactions (Pannier, Anderson et al. 2005).

Here, zein films loaded with DNA complexes were shown to mediate successful gene transfer. Transfection mediated by LF/DNA complexes adsorbed to zein films increased on films formed from solutions of higher pH, which corresponds to surfaces of increasing hydrophilicity and decreasing surface roughness, confirming other's work with both SMD and fabrication of zein films, separately (Bengali, Pannier et al. 2005; Pannier, Anderson et al. 2005; Dong, Padua et al. 2013). The same trend was seen with PEI/DNA complexes but at much lower transfection levels, which could be due to an almost 2-fold increase of PEI/DNA complexes adsorbed to the zein films compared to LF/DNA complexes resulting in increased toxicity and reduced transfection. However, with PEI/DNA complexes, transfection was statistically lower on pH 2 zein films than at pH 9. This difference demonstrates the influence of both hydrophilicity and complex conformation on transfection since adsorption of complexes was not significantly different between pH 2 and pH 9 zein films. Pannier et al showed increased transfection on hydrophilic surfaces as compared to a more hydrophobic surface and suggested the results were possibly due to the reversible interactions between the substrate and complex (Pannier, Anderson et al. 2005), where surfaces with greater hydrophobicity may induce irreversible binding of complexes due to conformational changes upon binding to the hydrophobic surface (Stuart, Fleer et al. 1991). Adsorption of complexes on hydrophobic surfaces may alter the complex conformation, resulting in decreased transfection.

Cell viability is another factor influencing transfection. The biocompatibility of the zein films was demonstrated with a WST-1 assay and revealed excellent cell viability compared to the PS control, except with pH 4 zein films. This lower cell viability on pH 4 zein films could be due to the increased adsorption of LF/DNA complexes compared to other zein films (Figure 3-6A), which could lead to increased toxicity and reduced transfection (Figure 3-7A). Conversely, the proliferation rate (slope between two time points) of zein films at pH 7 and pH 9 was higher than tissue culture PS at 72 hours, demonstrating that these films are increasing cell proliferation. This increase in proliferation can be connected with the degradation of zein at 72 hours shown in Figure 3-5 as zein's degradation products have been shown to enhance cell proliferation (Sun, Dong et al. 2005).

These studies demonstrate the potential for using zein films as coatings to support SMD. Further investigation of both the properties of zein films, such as surface charge, and the properties of the DNA complexes (conformation and release) are needed in order to better understand the mechanism and design of SMD systems on zein films. Future improvements could entail embedding of complexes within the film, which would occur before EISA. Future studies will also examine another method to control release of DNA by forming DNA-loaded zein spheres, as shown by our previous work (Regier, Taylor et al. 2012), and then casting them into a film for SMD. However, the above results reveal the potential for zein films as a surface-coating material for biomedical devices including drug/DNA-eluting stents, wound dressings, tissue engineering scaffolds and cell arrays (Shea, Smiley et al. 1999; Zilberman, Kraitzer et al. 2010).

3.6 Conclusions

The ability of zein films to support substrate-mediated gene delivery was demonstrated in this chapter. The influence of pH of the zein solution on surface roughness and hydrophilicity was investigated for its effect on transfection. Zein films formed at a higher pH, exhibited lower surface roughness and higher hydrophilicity, showing increased transfection levels, as well as prolonged stability against degradation and robust biocompatibility. This study provides proof of concept that zein films with adsorbed complexes can result in high levels of transfection and suggests that zein films have applications in the fields of gene therapy, tissue engineering, and transfected cells arrays.

CHAPTER 4: Conclusions and Future Directions

4.1 Introduction

This thesis describes further analysis of DNA-loaded zein nanospheres. Zein/DNA nanospheres, formed through a simple coacervation process, demonstrated the ability to protect DNA against nucleases, displayed robust biocompatibility, and were able to be internalized by cells. In addition, zein films were developed for substrate-mediated gene delivery. Zein films were formed by evaporation- induced self-assembly and were comprised of particles that increased in size with zein concentration, up to 1.8 ± 0.11 nm. Films formed at different pHs (2-12) resulted in morphological differences, from a smooth surface at pH 2 to films composed of spheres that decreased in size as the pH of the zein solution used to form the film increased. Zein films degraded minimally in PBS, and were able to adsorb DNA complexes, with 0.4 μ g and 1.12 μ g of total DNA on film surface for lipid/DNA and polymer/DNA complexes, respectively, which was sometimes independent of pH. However, transfection levels increased on films with increasing pH and highest gene expression was achieved on pH 9 films with lipid/DNA complexes. This increase in transfection on pH 9 films could be attributed to the decreased surface roughness and increased hydrophilicity of the film. The results presented in this thesis demonstrate both the zein spheres and films are novel and show great potential to the field of gene delivery. However for both zein nanospheres and zein films, future improvements are needed to improve and create a controlled and efficient DNA delivery system for applications in oral gene delivery, tissue engineering and biomedical devices.

4.2 Future Directions for Zein Nanospheres

Particulate delivery for DNA is promising due to the ability to protect DNA and provide for controlled, localized, and sustained release resulting in transfection (Agarwal and Mallapragada 2008). Zein, a natural protein, has many unique biological and physical properties such as innate biocompatibility, slow degradation by hydrolysis, and degradation products that enhance cell proliferation (Sun, Dong et al. 2005; Hurtado-Lopez and Murdan 2006) that makes it a good candidate for particulate gene delivery. Zein nanoparticles can encapsulate DNA using a simple coacervation technique (Regier, Taylor et al. 2012). These DNA-loaded zein particles ranged in size from 157.8 ± 3.9 nm to 396.8 ± 16.1 nm, depending on the ratio of zein to DNA (20:1 to 250:1) and all particles were negatively charged, which was expected due to the nature of zein (Cabra, Arregun et al. 2005) and DNA at physiological pH. DNA encapsulation efficiency was as high as $65.3 \pm 1.9\%$ with a maximum loading of 6.1 ± 0.2 mg DNA/g zein. The cumulative release of DNA from these nanoparticles in PBS was $17.8 \pm 0.2\%$ over seven days and encapsulation could protect DNA from DNaseI, which is critical for efficient gene delivery, specifically for maintenance of the supercoiled conformation. The DNA-loaded zein particles also displayed excellent biocompatibility and cellular internalization.

As a next step for examining zein particles for oral delivery applications, preliminary studies were performed to investigate the DNA-loaded zein particles in gastrointestinal-simulated conditions. The DNA-loaded particles released only 20% of their payload in the first 30 minutes of incubation in simulated gastric fluid (SGF) (Figure 4-1) and DNA remaining in these pepsin-treated spheres remained intact (Figure 4-2), demonstrating the

ability of zein to protect DNA in the stomach, which is a critical for oral delivery applications. However, in simulated intestinal fluid (SIF), containing pancreatin, release was nearly instantaneous (Figure 4-1), and the DNA would likely be degraded from intestinal enzymes, justifying the need for a secondary carrier to delay intestinal release for the application of these particulates for oral gene delivery. Therefore, several changes are recommended to this system for future use of these nanoparticles in oral gene delivery applications, specifically forming particulates with a zein coating around a core of DNA complexed with a material more suitable to the intestinal environment.

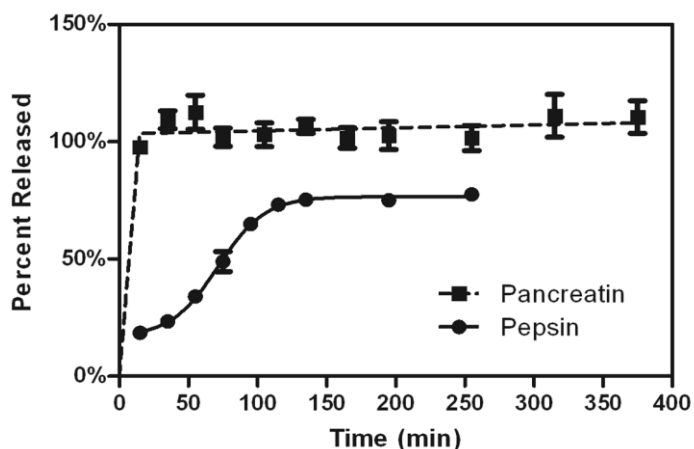


Figure 4-1. Enzyme-mediated release of DNA from zein nanoparticles (Regier 2011).

As demonstrated in literature and this thesis, zein possesses film-forming abilities, and has been commonly used in pharmaceutical tableting and coatings (Lawton 2002), since it has been shown to be especially well-suited for oral delivery applications (DiBiase and Morrel 1997; Parris, Cooke et al. 2005; Gong, Sun et al. 2011). In addition, as demonstrated above, zein particles can protect payload from the low pH and enzymes of the stomach, including DNA, as demonstrated from preliminary data (Figure 4-2).

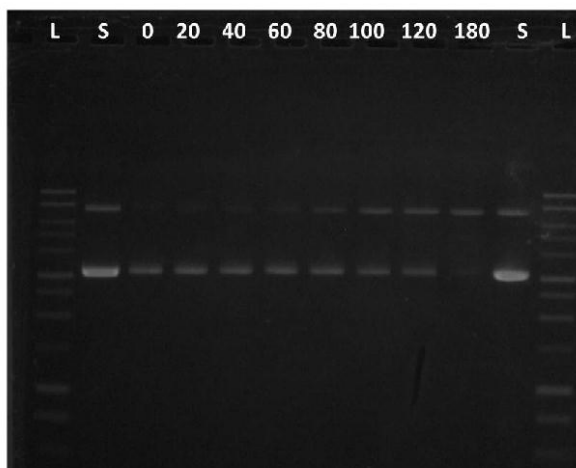


Figure 4-2. Integrity of released DNA from zein nanoparticles (in simulated gastric). Lanes “L” markers; lanes “S” stock DNA; lanes 0 -180 minutes in fluids.

However, zein is rapidly degraded in the intestine (as data shows above, Figure 4-1), making it an ideal material to serve as a coating for DNA-loaded nanoparticles rather than the DNA carrier alone. Therefore, in this proposed system, the zein-coated DNA nanoparticle would pass through the stomach, protected by the zein coating, which would degrade upon reaching the intestine, and then the inner DNA nanoparticle would be able to target cells and achieve transfection in the intestine. From surveying the literature, chitosan nanoparticles could provide for protection of DNA in the intestine, once the zein coating degrades.

Chitosan (CS), the N-deacetylation form of chitin, has not only inherent biocompatibility and biodegradability, but also unique mucoadhesive properties that allow for increased residence time at the cell surface of the intestines (Lai, Wang et al. 2009). Therefore, CS has been used to form nano- and microspheres loaded with DNA for oral delivery to treat

hypoparathyroidism (Chou, Huang et al. 2009), hemophilia (Dhadwar, Kiernan et al. 2010), and food allergies (Roy, Mao et al. 1999; Bengali, Rea et al. 2009). However, the efficacy of CS-mediated oral gene delivery is stunted due to the high solubility of CS in acidic conditions of the stomach, leading to fast release of cargos before arrival to the intestine (Luo, Zhang et al. 2010), and thus low transfection efficiency. Proper protections to CS/DNA nanoparticles are necessary to achieve effective gene transfection of intestinal cells.

Hence, the future direction for this project should investigate novel zein-coated, DNA-loaded chitosan nanoparticles with optimal properties for oral gene delivery. The zein coating will provide adequate protection to the CS/DNA nanoparticles until they reach the intestine, where zein will be instantly degraded. The exposed CS/DNA nanoparticles will have prolonged residence time in the intestine, due to the mucoadhesive nature of chitosan (Lai, Wang et al. 2009), leading to increased ability for transfection of intestinal cells. The significant advantage of this design is that the zein-coated CS/DNA nanoparticles can appropriately fulfill the requirements from different environments along the GI tract and allow for protection and targeted release of the DNA. Particles from zein/chitosan composites have been developed for oral delivery of selenite (Luo, Zhang et al. 2010) and α -tocopherol (Luo, Zhang et al. 2011), and the use of the two biomaterials resulted in prolonged release of encapsulated cargo versus particles from a single material. However, there have been no published reports on zein/chitosan nanoparticles for DNA delivery, making this exciting future work.

In preliminary studies, CS/DNA nanoparticles were prepared using a simple and mild method, ionic gelation, which is the complexation between oppositely charged macromolecules. During ionic gelation, low molecular weight (Mw) chitosan was dissolved in aqueous acidic solution to obtain the cation of chitosan, and DNA was dissolved in a polyanion, sodium Tripolyphosphate (TPP) solution. The two oppositely charged solutions were mixed and chitosan underwent ionic gelation and precipitated to form CS/DNA nanoparticles (Mitra and Dey 2011). This method demonstrated a linear increase of nanoparticle size with increasing CS concentration (1-2.5 % (w/v)) as seen in Figure 4-3, and loading efficiency similar to that reported in the literature for CS/DNA nanoparticles for gene delivery (Ozbas-Turan and Akbuga 2011). Also, the zeta potential was positive for all concentrations of CS/DNA spheres. This positive zeta potential is an important feature for gene delivery systems due to the increased interactions with negatively charged cellular membranes to enhance internalization of the nanoparticles and result in greater transfection (Harush-Frenkel, Debotton et al. 2007).

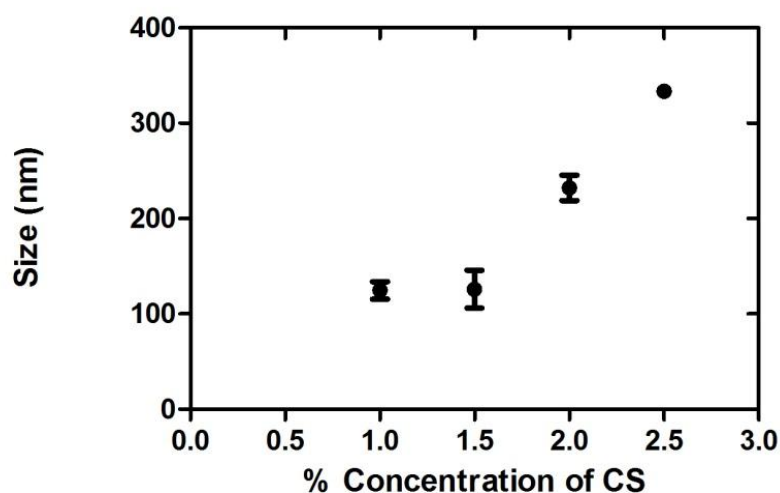


Figure 4-3. Size of CS/DNA nanoparticles at different concentrations of

Next, the release of DNA from these spheres was tested in SIF (Figure 4-4). In contrast to the instant burst release of DNA from zein spheres (Figure 4-1), DNA-loaded chitosan spheres demonstrated a slow and minimal release of DNA in SIF over 8 days (Figure 4-4). This minimal release of DNA is important because it allows for more DNA to be internalized with the CS nanosphere, strengthening the use of chitosan as a DNA carrier for oral gene delivery.

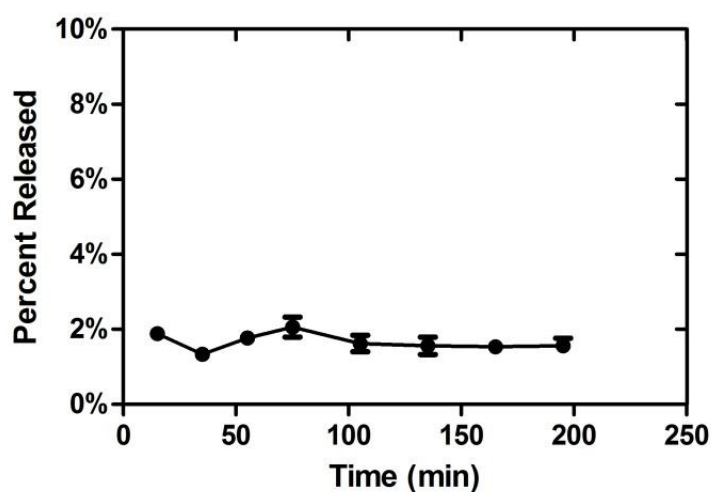


Figure 4-4. Release of DNA from CS/DNA nanoparticles in SIF.

Preliminary studies were also conducted to investigate the ability to coat CS/DNA nanoparticles with zein (Figure 4-5), showing that the size of coated nanoparticles increased with increasing zein/CS ratio. These results indicate that zein is a promising coating material for CS/DNA particles for oral gene delivery.

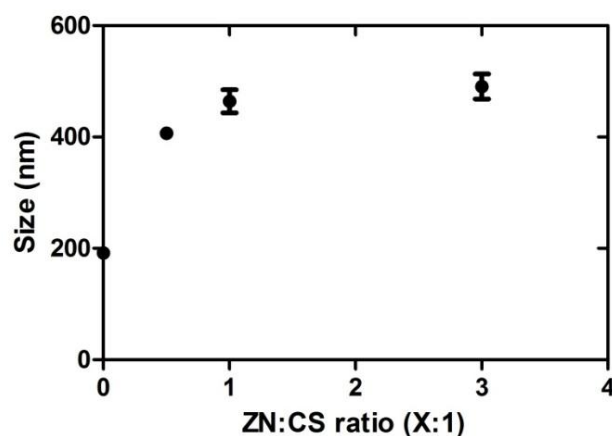


Figure 4-5. Size of CS/DNA and zein-coated CS/DNA nanoparticles at various zein/CS ratios.

Also, CS/DNA nanoparticles, prepared as described above, were added to cells and demonstrated excellent biocompatibility measured through a standard WST-1 assay (Figure 4-6A) and microscopic observation of cell morphology (Figure 4-6B). These results indicate that the zein-coated CS/DNA nanoparticles exhibit the required biocompatibility to be used for oral gene delivery

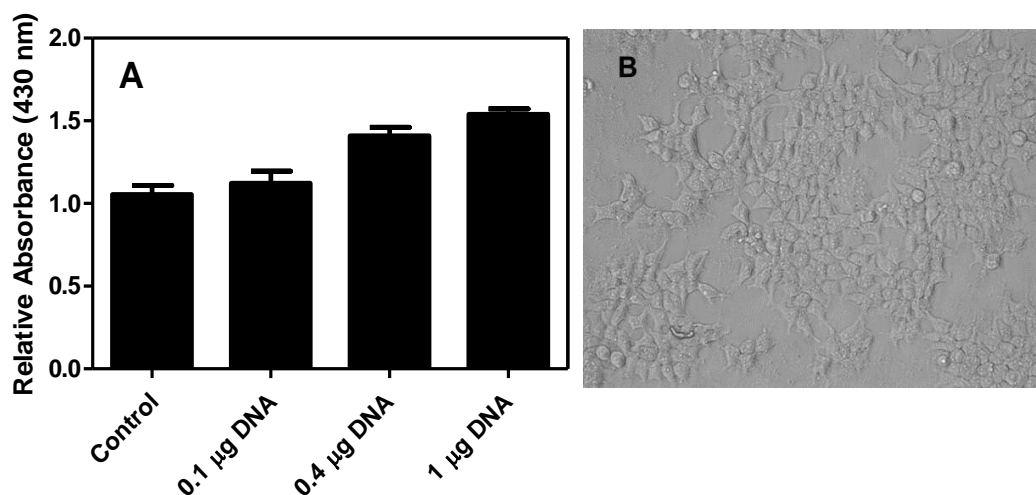


Figure 4-6. (A) Cytotoxicity of the zein-coated CS/DNA nanoparticles quantified by WST-1 assay for HEK 293T cells as a function of DNA amount in nanoparticles. (B) Cell morphology in presence of nanoparticles.

All of the preliminary data above suggests the great potential of zein-coated DNA-loaded chitosan nanoparticles for oral gene delivery applications. However, there is still more work needed to optimize the zein-coated CS/DNA nanoparticles in order to create a controlled and efficient delivery system. Next steps on this project should include investigation of the ability of CS/DNA spheres to transfect cells effectively. Previous studies have shown that there are many factors affecting the size, charge, and stability of the CS/DNA spheres including Mw of chitosan, degree of deacetylation (DD), N/P ratio of amine CS to phosphate DNA and mass ratio of CS:TPP, which affects the affinity of CS to DNA, cellular uptake, dissociation of complex and therefore transfection levels (Koping-Hoggard, Tubulekas et al. 2001; Sato, Ishii et al. 2001; Romøren, Pedersen et al. 2003; Kiang, Wen et al. 2004; Liu, Sun et al. 2005; Strand, Danielsen et al. 2005; Lavertu, Méthot et al. 2006; Mao, Sun et al. 2010). Thus, optimization of transfection with CS/DNA nanoparticles should include investigating each of the above parameters. After the CS/DNA spheres are optimized for transfection and cytotoxicity, the concentration of zein needs to be explored for efficient coating of CS/DNA spheres to provide complete protection through the stomach environment. Then, zein-coated CS/DNA nanoparticles should be tested *in vitro* for transfection by delivering treated spheres (passed through SGF and SIF) to cells. Once the development of zein-coated CS/DNA nanoparticles are properly formed and optimized for efficient *in vitro* transfection, animal studies can then be carried out to examine the spheres efficacy *in vivo*, looking first at reporter gene expression in the intestine and then examining off target effects in other organs.

Gene delivery via the oral route for gene therapy and DNA vaccination has the potential to revolutionize the treatment and prevention of various diseases, thus making these outlined future directions of this project significant for human health. A novel and effective particulate system for oral DNA delivery could eliminate the need for administration by healthcare professionals and enhance convenience to the patient; the transient nature of nonviral gene delivery would require repeated administration for gene therapy applications to maintain a therapeutic effect and thus ease of administration as well as patient comfort and compliance are particularly important. The investigation of new materials and designs of gene carriers, which have properties required for efficient oral gene delivery, promises to fuel more breakthroughs and bring nonviral gene delivery via oral administration closer to clinical application.

4.3 Future Directions for Zein Films

In chapter 3, DNA lipoplexes and polyplexes were adsorbed to the surface of zein films for substrate-mediated delivery (SMD) for the first time. Transfection levels increased with increasing pH and highest gene expression was achieved on pH 9 films with LF/DNA complexes. However, these films need to be better fabricated to improve upon the DNA delivery efficiency in order to be applied as a coating on biomedical devices and thus the following improvements are recommended to the system.

Controlled and sustained release of plasmid from a tissue engineering scaffold or device coating is a crucial part of successful gene delivery by allowing for large numbers of cells to be transfected, but also providing localized delivery and circumventing the need for repeated injections. The zein films formed in Chapter 3 were not examined for release of

DNA from films and therefore a release profile of both lipid and polymer complexes needs to be investigated. If the release of DNA is found to be too fast from the surface, then DNA complexes could be embedded into the zein films to provide for a longer continuous release rate. This technique would allow the DNA complexes to be released as the film degraded and not simply through adsorption/desorption processes as described in Chapter 3. Previous works have embedded naked DNA and complexed DNA in polymer scaffolds. Shea et al, used a high pressure gas foaming procedure to incorporate DNA into a PLGA sponge, demonstrating sustained DNA release for a month and transfecting a large number of cells (Shea, Smiley et al. 1999). Another group incorporated a plasmid encoding for sperm-specific lactate dehydrogenase C₄ (LDH- C₄) into a poly(ethylene-co-vinyl acetate) (EVAc) matrix for vaginal DNA immunization through induced local mucosal immunity in mice. The DNA-loaded EVAc displayed continuous DNA release and long term production of LDH- C₄ in the vaginal tract as compared to naked DNA (Shen, Goldberg et al. 2003). Poly-caprolactone (PCL) films with complexed DNA embedded throughout the film showed enhanced transfection levels and suppressed burst release compared to embedded naked DNA (Ramgopal, Mondal et al. 2008). In order to embed DNA complexes into the zein film, complexes would be added to the zein solution prior to film formation by EISA. Problems such as complex aggregation and uneven distribution of complexes on the film surface may occur and would need to be further investigated through fluorescently labeling DNA and analyzing the dispersion of DNA complexes on the surface with confocal imaging.

Even though the release of DNA from the substrate is an important aspect to successful

gene transfer, the substrate properties are also important. Zein films are hydrophobic, glossy, greaseproof, and resistant to bacteria (Lawton 2002), but suffer from being brittle and susceptible to cracking and chipping as well as instability in water (Lawton 2002; Jiang, Reddy et al. 2010; Shi, Huang et al. 2010). These unwanted properties of zein films hinder its use in biomedical applications. However, mechanical and physical properties of zein films have been improved through the addition of a plasticizer or crosslinker for increased flexibility and increased tensile strength, Young's modulus and water stability, respectively (Parris and Coffin 1997; Xu, Karst et al. 2008; Reddy, Li et al. 2009; Jiang, Reddy et al. 2010; Shi, Huang et al. 2010).

Plasticizers are low molecular mass organic compounds which soften rigid polymers by reducing the glass transition temperature of polymers, reducing their crystallinity or melting point through separating individual polymer chains from one another (Hinrichsen 1996; Sperling 2005). A variety of plasticizers have been added to the fabrication process of zein films. Hydrophilic plasticizers, such as glycerol and polyethylene glycol, have increased the flexibility of zein films but with decreased strength and stiffness (Aydt 1989; Park, Bunn et al. 1994; Weller, Gennadios et al. 1998). Fatty acids, including palmitic acid, oleic acid, linoleic acid, and steric acid exceeded hydrophilic plasticizers by maintaining the strength of the films and improving the film flexibility (Lai and Padua 1997; Lai, Padua et al. 1997; Budi Santosa and Padua 1999). Another group of hydrophobic plasticizers are citrate esters, which are derived from citric acid. Citric esters are non-toxic and biodegradable. Zein films containing 10% tributyl citrate (TBC) demonstrated flexible and tough films (Shi, Yu et al. 2012). Incorporation of a

biocompatible and nontoxic plasticizer to the zein films formed in Chapter 3 could enhance flexibility for future applications of these films, including coating biomedical devices for gene delivery.

The addition of crosslinkers to zein films have included glyoxal, formaldehyde, glutaraldehyde, and citric acid, and butanetetracarboxylic acid (Yang, Wang et al. 1996; Woods and Selling 2007; Reddy, Li et al. 2009; Jiang, Reddy et al. 2010; Selling, Woods et al. 2012). Of all these crosslinkers that have been investigated, citric acid is the most promising due to its biocompatibility. Zein fibers crosslinked with citric acid have shown decreased rates of degradation, enhanced biocompatibility and improved water stability compared to uncrosslinked fibers (Jiang, Reddy et al. 2010). Thus, addition of a crosslinker, preferably, citric acid, should be investigated for its potential to improve the degradation rate and water stability of zein films, particularly films formed at pH 12 due to their instability upon addition of PBS.

Finally, future work is needed to examine the effect of storage time and humidity on zein film properties. Since zein is a biological material, it is affected by water and thus its properties are subject to change with humidity (Lawton 2002). Lai and Padua demonstrated increased water permeability, toughness, and elongation of zein films with high relative humidity (Lai and Padua 1998). However, they also showed the addition of oleic acid, a plasticizer, helped maintain these properties of zein films through increased humidity (Lai and Padua 1998). During the preliminary film studies reported in Chapter 3, there were changes in transfection levels with zein films of different ages. For instance,

with fresh (made the day before experiment) zein films, the transfection increased with increasing pH, but when zein films were aged (made weeks to months prior to use), the transfection levels decreased with increasing pH as seen in Figure 4-7. The incorporation of a plasticizer or crosslinker to not only improve mechanical and physical properties of zein films as mentioned above, but to decrease the film's water vapor permeability might help overcome these changes in film surface properties. Thus, there is a need to study the alteration in zein film properties over time to further understand the effect of surface properties of zein films on the transfection efficiency.

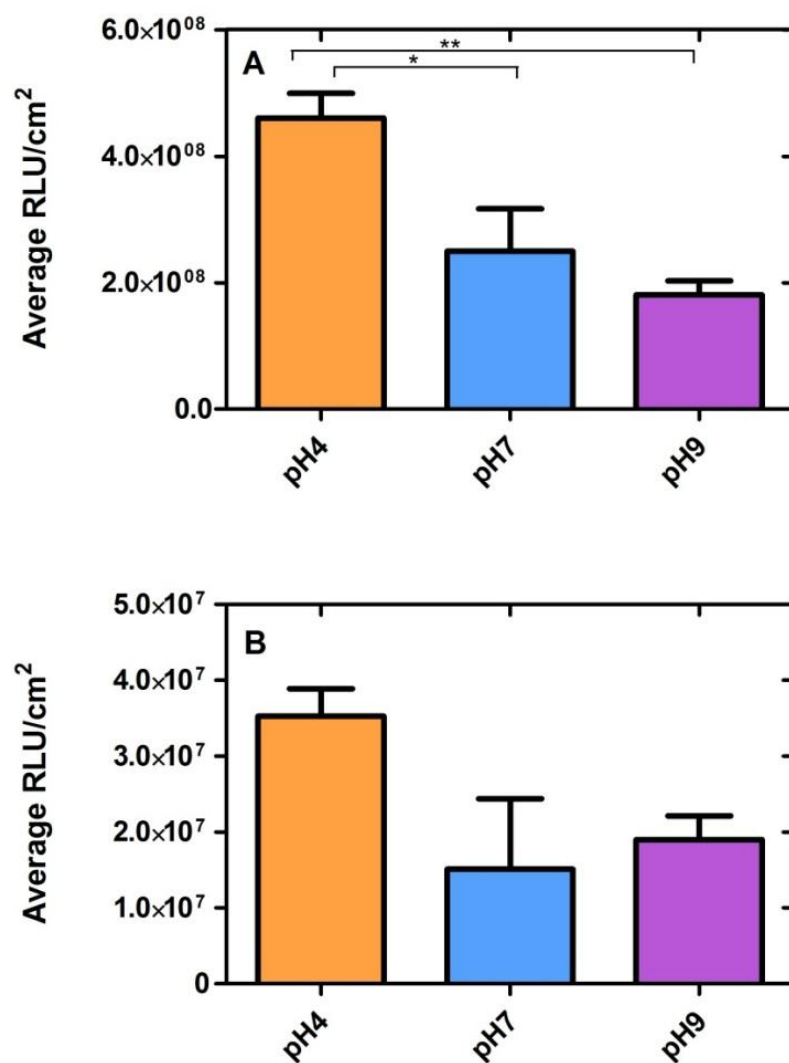


Figure 4-7. Normalized SMD transfection profile for (A) HEK 293T cells, n=6 and (B) NIH3T3 cells, n=3, plated on aged zein films formed from different pH solutions, mediated by LF/DNA complexes. Data reported as mean \pm standard error of the mean. (*p < 0.05, **p < 0.01).

4.4 Conclusions

In this thesis, a natural protein polymer, zein, was investigated for its ability to form DNA-loaded nanospheres and films for gene delivery. Once fabricated using a simple coacervation technique that resulted in sufficient DNA loading, zein/DNA nanospheres showed resistance to degradation against DNase I, cell biocompatibility and internalization, as well as protection of DNA in a gastric environment. However, it has been noted that zein might not be able to obtain sufficient oral gene delivery by itself and thus the addition of another polymer, such as CS, would allow for DNA nanoparticle stability in the intestine and these multilayer particles could revolutionize treatments and impact the field of oral gene delivery. Additionally, zein films used for SMD delivery of DNA were established for the first time. Zein films formed either a smooth, flat surface or that of particles depending on pH of zein solution, and demonstrated excellent cell biocompatibility and transfection with increasing pH of films. Future work still remains to better characterize these films for improved DNA delivery and improved mechanical properties, for applications in oral gene delivery, tissue engineering, and biomedical devices.

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