2020

A FRAMEWORK FOR HETEROLOGOUS BIOSYNTHESIS OF NATURAL PRODUCTS IN MAMMALIAN CELLS VIA POLYMER-MEDIATED TRANSFECTIONS

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Digital Object Identifier: https://doi.org/10.13023/etd.2020.274

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TRANSFECTIONS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Engineering
at the University of Kentucky

By
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Lexington, Kentucky
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2020

ABSTRACT OF DISSERTATION

A FRAMEWORK FOR HETEROLOGOUS BIOSYNTHESIS OF NATURAL
PRODUCTS IN MAMMALIAN CELLS VIA POLYMER-MEDIATED
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With the promise to treat a multi-faceted list of serious inherited and acquired diseases, such as cancer, neurodegenerative and infectious diseases, and inherited genetic indications, gene therapy has continued to push the boundaries of traditional medicine since its earliest implementation. While much progress has been made, clinical success has largely remained elusive. Immunogenicity, difficulty producing commercially relevant quantities, and having a limited genetic payload still limits the ability of viruses to act as directed delivery agents for genetic material. As such, researchers have turned to cationic synthetic materials as a means of delivering nucleic acids, which can circumvent the immune response but suffer decreased delivery efficiency relative to viral vectors. To advance non-viral vectors towards clinical relevance, special consideration of the various barriers associated with nucleic acid delivery must be applied to the design of the synthetic agents. How does the material interact with the cell surface, are the vectors stable in circulation, can the structure escape the endocytic environment of cells, and will the structure release the genetic payload? All of these questions, and many others, need to be explored and implemented in a set of design criteria that optimizes the ability of a certain vector to consistently induce adequate expression for the desired application.

In chapter 3, we apply the aforementioned considerations to polyethylenimine (PEI), the "gold standard" material for polymeric gene delivery vectors (polyplexes). Through simple modifications of PEI via succinylation, we generated zwitterion-like polymers (zPEI) that increased transfection efficiency by up to 50 fold over that of traditional PEI vectors when in the presence of serum proteins. These vectors also show remarkable resilience when lyophilized and stored for long periods, maintaining transfection efficacies similar to freshly prepared polyplexes over the course of 8 weeks. Since vector development is quick, efficient, consistent, and can be stored for long periods of time, zPEI could serve as a platform for testing applications of non-viral gene therapies or for explicit expression of specific proteins.

In chapter 4, we explore this notion by investigating applications where non-viral particles have shown success and elucidate situations where certain vectors may be preferred over others. Finally, in chapters 5 and 6 we deployed our own application of non-viral genetic engineering to induce production of the plant-derived biomolecule, curcumin, in a mammalian cell. This is the first time an entire plant enzyme cluster has been expressed in a mammalian cell, as well as the first time a plant biomolecule has been synthesized in a mammalian cell. We optimized the transient expression of the gene cluster and maximized the production of bisdemethoxycurcumin, demethoxycurcumin, and curcumin. Bioactivity of curcuminoids produced in cells were assessed via metabolic and migration assays. When cultured in the presence of curcuminoid-producing HEK293 cells, metabolic

activity of MDA-MB-231 and MCF7 cancer cells was reduced and cell migration was inhibited. We believe this work may represent the first step towards a drastic shift in how diseases are treated, focusing not on the external delivery of drugs, but instead engineering patients' bodies to produce their own supply of therapeutic compounds.

KEYWORDS: Genetic Engineering, Polymer Transfection, Gene Therapy, Cancer

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2/24/2020
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CHAPTER 1. ADVANCEMENT OF NON-VIRAL GENE DELIVERY STRATEGIES

1.1 Gene Therapy: Early Trials, Setbacks, and a Hopeful Future

The concept of gene therapy can be traced back nearly a century when scientists first observed transfer of viral genetic material between bacteria [1]. Fast forward to present day, gene therapy has evolved tremendously, seeking to treat acquired and inherited diseases such as cancer, neurodegenerative and infectious diseases, and genetic defects. Gene therapy in its simplest form is the delivery of a recombinant nucleic acid with the intent to regulate, repair, replace, add, or delete a genetic sequence. The therapeutic effect comes directly from the protein encoded within the nucleic acid, or as a product of gene regulation that is targeted by the genetic sequence. This present day interpretation of gene therapy was not realized until 1990 when researchers used a recombinant retroviral vector to treat patients with severe combined immunodeficiency (SCID) [2]. It was not until later in 2000 when the first clinical success of SCID treatment via recombinant viral gene therapy was reported. However, due to the nature of the viral vector used in the trial, the genetic sequence that was meant to treat the patient was inserted into a region of DNA that disrupted the normal cellular processes, leading to unregulated cell growth and division [3]. This case of insertional mutagenesis led to the patient developing a leukemia-like condition. In an equally tragic trial in 1999, a young man died four days after injection with a modified adenovirus [4]. The fallout from these trials slowed progress in gene therapy and possible severe immunogenic response and insertional mutagenesis remain pervasive even today. This fear sparked researchers to look for genetic carriers other than viruses, and eventually led to the development of synthetic, or non-viral, vectors.

Despite the initial tragedies and setbacks, research continued on viral vectors in pursuit of greatly increase safety. Perseverance led to the first regulated and approved gene therapy product in 2012 in the European Union. The United States would not see its first gene therapy product for another five years. In 2017 Kymriah (Novartis) and Yescarta (Kite Pharma), two chimeric antigen receptor (CAR) T-cell therapies, were approved by the FDA to treat acute lymphoblastic leukemia and non-Hodgkin lymphoma, respectively. These therapies are based on *ex-vivo* principles, where patients' cells are harvested and modified outside the body, before being reinjected, preventing the possibility of the virus infecting unintended targets. The pipeline for these products is continuing to expand, with 16 trials currently in Phase I or II [5]. *In-vivo* treatments are also gaining traction, with the first adeno-associated virus product, Luxturna (Spark Therapeutics), entering the market for treatment of retinal disease [6]. With a growing list of remarkable candidates, FDA released a set of guidelines in January 2020 to streamline development, indicating a shift in policy and, more importantly, hope for a field budding with potential.

1.2   Gene Delivery Vectors

Genetic material in the form of DNA or RNA can regulate gene expression to produce a therapeutic response. In order to achieve this, the genetic material must reach its intended destination, the inside of a cell. What may seem like a simple concept quickly becomes complicated when considering the many biological systems contained with the human body. These barriers will be expanded upon later, but in brief, the genetic material must be ferried to the target cell, and only the target cell, while avoiding circulatory proteins and enzymes that will either remove the material or degrade it to a point of non-functionality. Once at the cell, the material must interact with the membrane in such a way

that facilitates endocytosis. Once inside the cell, the material must escape the endocytic vesicle, avoid lysosomal degradation, and release into the cytosol. For siRNA, which regulates gene expression from the cytosol, the nucleic acid has reached the final destination. For DNA, the vectors must go one step further and localize to the cell nucleus for transcription. Taking into consideration all of these difficulties, it is easy to envision why DNA and RNA alone are not well suited for gene therapy. Thus, there is need for a carrier that can protect the genetic material and deliver it to the cell while retaining transcription potential. In general, three strategies have emerged throughout the history of gene therapy; viral, non-viral, and hybrid-vectors. The remaining portion of this chapter will briefly explore each of these carriers.

1.2.1 Viral Vectors

Since the implementation of viral vectors as vehicles for genetic material three decades ago, the gamut of viral vectors has expanded immensely [7]. Vectors include both RNA and DNA viruses that contain either single-stranded or double-stranded genomes. The main viruses utilized today include adeno-associated viruses (AAV), adenoviruses, retroviruses, lentiviruses, alphaviruses, herpes simplex viruses, rhabdoviruses, flaviviruses, measles viruses, poxviruses, coxsackieviruses, and Newcastle disease viruses. Of these, four greatly standout in both clinical trials and number of publications. Adenoviruses, AAV, retroviruses, and lentiviruses make up over 50% of clinical trials conducted to date [8].

1.2.1.1 Adenoviruses

Adenoviruses are non-enveloped viruses with double-stranded DNA genome approximately 30-35 kb in length encoding about 50 viral polypeptides [9]. Adenoviruses are non-integrating viruses, meaning once the virus has infected the cell, the DNA is transferred into the nucleus and transiently transcribed for days to weeks, after which expression will diminish [10]. This characteristic is attractive for *in-vivo* applications where direct gene transfer is desired without permanent modification of the genome. However, adenoviruses are very common, often persisting in the tonsils of humans. It is estimated that nearly 90% of adults have antibodies against the most common serotypes of adenoviruses, specifically serotype C5 which is a foundation of adenoviral vectors [10, 11]. Due to pre-established antibodies and diminishing expression, many adenovectors need to be re-administered. As adenovectors distribute through the blood, interaction with the reticuloendothelial system promotes production of multiple proinflammatory cytokines such as IL-6, IL-8, and TNF-α, potentially leading to toxicity [12, 13]. First generation adenovectors also inadvertently targeted macrophages and dendritic cells that activated as an initial immune response to administration, leading to upregulation of antigens and certain chemokines which could result in severe immunogenic shock [14]. Later generations of adenovectors have had significant portions of the viral genome deleted in an attempt to reduce cytopathic effects [15]. These later generations are characterized by greatly reduced immunogenicity, and high infection rate in both quiescent and non-dividing cells, making adenovectors candidates for applications where short term expression is desired [16, 17].

1.2.1.2 Adeno-Associated Viruses

Initially thought to be an impurity or byproduct of adenoviruses, this relatively simple and small family of parvovirus was aptly named adeno-associated virus (AAV) [18]. AAV are small (18-25 nm), non-enveloped, single stranded DNA viruses that infect humans with relatively little immune response and integrate specifically in chromosome 19 [19]. AAV naturally only encodes for 3 genes, *rep*, *aap*, and *cap*, flanked by inverted terminal repeats (ITR) which are involved in the viral replication and integration process [20]. The viral genes can be replaced with therapeutic proteins of interest, essentially creating a protein-based nanoparticle engineered to target and traverse the cell membrane that is ultimately only limited by the relatively small size of the genetic payload. The flanking ITR regions allow the transgenes to form episomes allowing for transcription weeks after the initial transfections, making AAV ideal for slowly dividing cells where the episomal DNA will not be diluted by division, such as in the central nervous system [21, 22]. There is a slight chance (0.1-1.0%) that recombinant AAV can integrate in chromosome 19 similar to the wild type at locus AAVS1 [23]. This locus is not involved in any major cellular processes, eliminating the potential of random insertional mutagenesis. Interestingly, this has recently been taken advantage of serving as a popular target for CRISPR/Cas9 integration sites in mammalian cells [24].

1.2.1.3 Retroviruses

Retroviruses are enveloped RNA viruses that transfect dividing cells. Retroviruses carry virally encoded elements that reverse transcribe the RNA payload into dsDNA that is transported to the cell nucleus and stably integrated into the host genome by means of a

virally encoded integrase [9]. Integration into the genome means permanent alteration and production of the encoded protein, creating the potential for long-term therapeutic effects. The proteins contained within the viral envelope naturally target specific receptors on the cell surface. Needless to say, this has been exploited to create many variants of viral envelopes that target different cells [25]. Retroviruses suffer from severe immune responses similar to that of adenoviruses, but have a lower transfection efficiency and can only infect proliferating cells [11]. However, these characteristics make the virus ideal for *ex-vivo* modification where stable integration is desired in a simple *in-vitro* environment before re-implantations such as CAR T-cell modification [26]. Although retroviruses have a preferred integration site, those loci tend to be in actively transcribed regions close to origins of transcriptional units, leading to a high chance of insertional mutagenesis [23].

1.2.1.4 Lentiviruses

Lentiviruses are a specific group of retroviruses that have more preferable integration sites away from transcriptional origins that reduce the prevalence of oncogenesis, with the added benefit of being able to transduce non-dividing cells [27]. Lentiviruses particularly exemplify how far viral engineering has come. Generally, lentiviruses are classified by generations, with each generation having differences in packaging and final viral composition. First generation lentiviruses contained all regulatory and accessory genes. With the identification of genes responsible for gene transfer in HIV, second and third generation lentiviruses omitted the accessory genes *vif*, *vpr*, *vpu*, and *nef*, increasing the safety of the viruses while retaining efficiency [28]. Development of a lentiviral vector without a viral integrase creates a situation similar to

AAV where the genetic payload will remain in the nucleus as an episome instead of being integrated into the genome. Lentiviral vectors having a larger capacity for genetic payload in comparison to AAV have garnered a significant amount of attention in recent years, evidenced by the fact that Kymriah, the first gene therapy drug approved in the US, employs a lentiviral vector [29].

1.2.2 Non-Viral Vectors

There is no doubt that viral vectors have improved tremendously since the first implementation in the 1990s. Yet, viral vectors have not been able to escape the concern for safety and strict regulatory practices enacted in response to the initial trials. Consequently, research efforts have diverged into two fields; continued improvement and development of viral vectors and development of synthetic, or non-viral, vectors. Synthetic vectors generally comprise DNA or RNA complexed with a cationic biomaterial, such as a lipid, dendrimer, peptide, or polymer. Non-viral complexes have the potential to address many of the issues that face viral vectors including reduced immunogenicity, larger capacity for genetic payloads, and more facile manufacturing [30-32]. However, unlike their viral analogues that have inherent components designed by evolution to infect and deliver genetic material to cells, non-viral vectors are relatively simple and suffer from reduced transfection efficiency [33]. The cationic nature of synthetic carriers may encourage favorable interactions with the cell membrane, but those same interactions will occur with circulatory proteins *in vivo* leading to rapid systemic clearance or toxicity [34]. Condensation of genetic material in the complex will protect the integrity of the material during transport, preventing serum nucleases from accessing the nucleic acids, but that

same steric hindrance can prevent transcription machinery from interacting with the payload [35]. These are just two examples that highlight the complexity facing the design of synthetic vectors, as most situations must require a compromise to maintain transfection efficacy while minimizing toxicity. These concepts will be explored further in chapter 2, but first we must explore the advances made in recent years to the design of synthetic vectors and the different materials that comprise the carriers.

1.2.2.1 Naked DNA and RNA

The simplest form of non-viral gene therapy is using solely DNA or RNA, without any accompanying carriers. From the brief mention of problems associated with more complex delivery methods, it is apparent that naked nucleic acids have a near impossible task of reaching the nucleus of a cell intact. The human body is bombarded with foreign genetic material daily and has developed a sophisticated system of DNase and RNase throughout the body to promptly remove any potential threats, including any exogenously delivered nucleic acids. Even so, there are some specialized delivery methods that have been shown to successfully induce expression. First is simply injecting the nucleic acid at the target site. Chloramphenicol acetyltransferase, luciferase, and P-galactosidase encoding DNA and RNA were injected into mouse skeletal tissue *in vivo*, and all three proteins were found to be expressed at levels comparable to *in vitro* experiments [36]. Another method is to introduce the nucleic acids into cells via electric pulse, or electroporation. A low voltage field employing <1 ms pulses can be employed to both permeabilize the cell membrane and move the DNA/RNA into the cell [37]. Although

these methods have shown some degree of success *in vivo*, clinical relevance is ultimately limited.

1.2.2.2 Lipoplexes

Liposome-mediated gene transfer is one the most widely used strategies for non-viral gene delivery. First observed in the 1980s, cationic lipids can condense DNA into a partially complexed structure with an ordered substructure [38]. The cationic structure interacts with the negatively charged cell membrane, where it is subsequently endocytosed. After internalization, the lipoplex destabilizes the endocytic vesicle triggering a flip-flop of the lipids contained in the endosomal membrane which causes the membrane to laterally diffuse into the complex, displacing the genetic material [39]. Cationic lipids consist of a cationic headgroup, a hydrophobic tail or anchor, and a linker that conjoins the two. Toxicity and low transfection efficacy plagued early lipofection studies, attributable to the cationic nature of lipoplexes. An intravenous injection of liposome-DNA complex in mice showed as little as 2% of the initial lipoplexes remain in circulation after 10 min, with the majority localizing in the liver or lungs [40, 41]. Many efforts to reduce induced toxicity center around modifying the positively charged head group, replacing the ammonium group with alternative moieties. Floch et al., demonstrated that replacing ammonium with groups that would form larger complexes, with lower charge densities such as arsonium and phosphonium, resulted in lower toxicological profiles while efficacy was maintained [42, 43]. Another strategy is to modify the headgroup with polymers or dendrimers that will decrease the cationic charge density, or allow the lipoplex to remain neutral at physiological pH, which would increase the ability of the lipoplex to stay in circulation

[44, 45]. Modifying the hydrophobic tails to include longer chains or chains that alternate in size has also shown some promise in increasing the transfection efficacy [46, 47].

1.2.2.3 Polypeptide Vectors

One of the more recent developments in non-viral gene therapy is the use of polypeptides as gene carriers. These peptides, dubbed 'cell penetrating peptides' (CPPs), consist of short (<20 amino acids) cationic peptides that can traverse the cellular membrane unconstrained by endocytosis [48]. The basis of CPPs is their ability to initiate movement across the cell membrane, with cargo, attributable to their specific peptide motif, or protein transduction domain (PTDs). This was first observed when the HIV protein transactivator of transcription was observed to enter cells and translocate to the nucleus [49]. It was not until three years later that Joliot et al. solidified the idea of PTDs by demonstrating *Drosophila antennapedia* homeodomain could be internalized by cells [50]. Since then many CPPs have been designed, primarily around specific PTDs. CPPs offer the advantages of having no apparent immunogenic or inflammatory response and can transduce a wide array of cells [51]. Although, there does not seem to be a limit to the size of the cargo, higher molecular weight cargo can promote endocytosis and membrane disruption, negating the CPPs benefit [52]. Since CPPs can transduce many different types of cells, special consideration must be taken to ensure the cargo is delivered to the intended cell. This has led to the recent development of pH-sensitive, thermally responsive, and ligand-conjugated CPPs with a much higher cell specificity [53].

1.2.2.4 Polymeric Vectors

In the mid-1990s, several 'off the shelf', or commercially available, cationic polymers were found to condense nucleic acids into nanostructures that could traverse the cell membrane and deliver a genetic payload to the nucleus [54-57]. The discovery of these abilities was rather fortuitous, since many of these polymers were not developed for biological applications. Polyethylenimine (PEI), widely considered the 'gold standard' of polymeric vectors, was originally developed as a chelator for the mining industry. An abundance of polymers based on these original polymers have been developed over the past two decades, attempting to imitate the desired properties or improve upon the design (Figure 1.1).
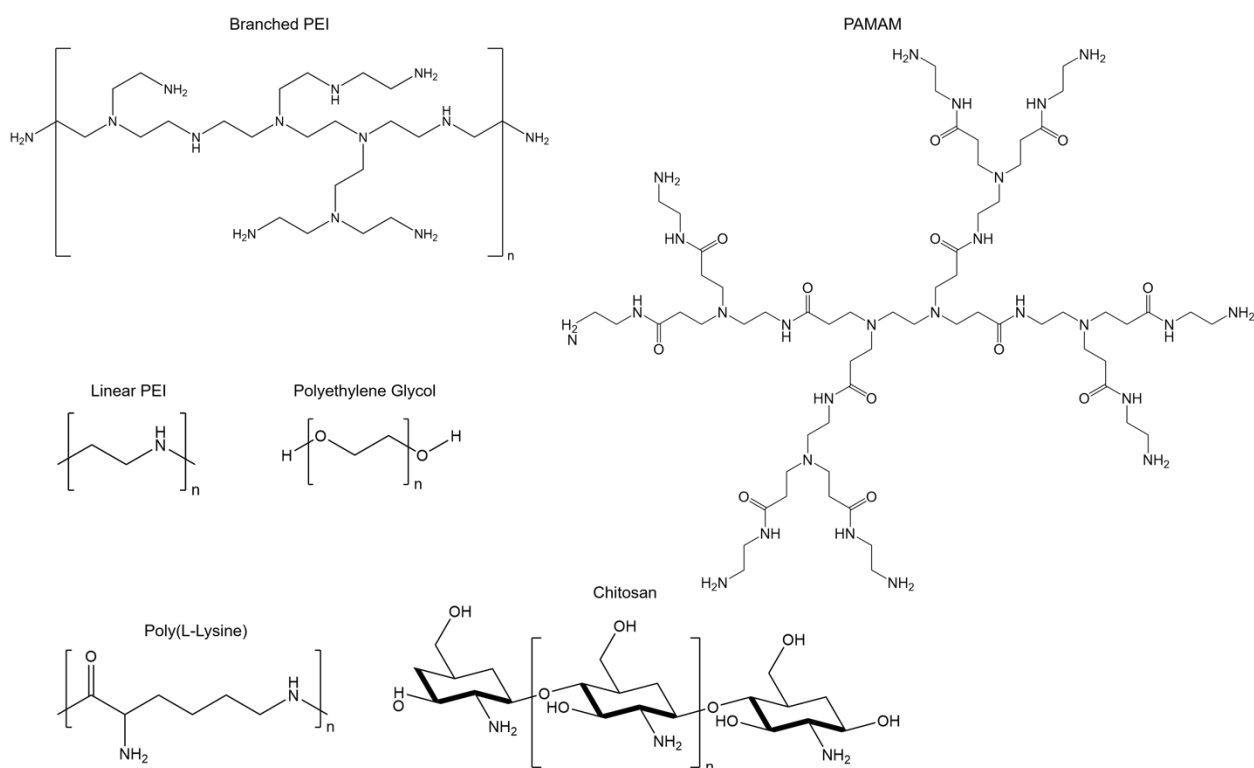
Figure 1.1: Examples of commonly employed polymers for polymeric gene carriers.

Polylysine (PLL) was one of the first polymers researched as a gene carrier [58]. As with many of these polymers, polylysine contains an amine group that is positively charged at physiological pH, allowing for electrostatically driven condensation interactions with the negatively charged phosphate backbone of DNA. Additionally, the primary amine group provides for easy conjugation to targeting ligands, allowing for cell targeting and specificity of the polyplex [59-61]. One important discovery that came from polylysine was the importance of endosomal escape. Polylysine-DNA complexes are prone to lysosomal degradation due to the relatively poor buffering capacity of polylysine [62]. When formulated with a lysosomotropic agent such as chloroquine, which raises the pH of acidic vessels inhibiting enzymatic degradation, transfection efficiency increases [58, 63]. Although polylysine is not widely used today, it played an important role in early polymer-mediated gene transfer and laid much of the groundwork for polymeric gene carriers that are used today.

Polyethylenimine (PEI) built upon the discoveries from polylysine. A polymer with strong cationic nature and high buffering capacity, PEI has shown strong performance in mediating transgene expression without the need an accompanying endosomolytic agent [54]. The high transfection capability of PEI is thought to come from the ability of the polymer to buffer the influx of protons while compartmentalized in endolysosomes. Osmotic swelling resulting from the chloride influx destabilizes the endosome and facilitates escape, the underlying phenomenon known as the proton-sponge effect [64]. The repeating amine structure allows for easy modification with sugars, targeting ligands, and antibodies, much like polylysine [65-68]. The high nitrogen density of PEI also leads to favorable cell membrane interactions, but has the potential to be disruptive to the cell

membrane leading to significant cytotoxicity [69, 70]. Unfortunately, increasing the molecular weight, and thus increasing the cationic charge density and potential for cytotoxicity, increases transgene expression in PEI-DNA complexes [71]. Researchers have attempted to address these issues by decreasing the cationic charge density through simple modifications such as acetylation and succinylation, which were found to increase transfection efficiency while decreasing cytotoxicity [35, 72].

Polyamidoamines (PAMAM) are spheroidal dendrimers, a highly ordered macromolecule that cascades out into a starburst-like pattern. PAMAM contains both internal tertiary amines and surface primary amines. Depending on the number of iterative Michael additions, or generations, the number of dendritic branches and subsequent surface amines can be controlled [73]. The unique molecular architecture of PAMAM allows for very specific and diverse alterations of the surface amine profile including modifications with lipids, fluorine moieties, folate, saccharides, peptides, proteins, and photosensitizers [74, 75]. Transfections with PAMAM have shown benefit from lysosomotropic agents like chloroquine, but the internal tertiary amines provide a large enough buffering capability ($pK_a \sim 6.0$) that endosomal escape via proton-sponge mechanism should be comparable to PEI [76]. Cytotoxicity of PAMAM is more favorable than that of high molecular weight PEI and polylysine since the PAMAM-cell membrane interactions are less disruptive and do not cause lysis [77]. The lower cytotoxicity makes PAMAM an attractive candidate for *in-vivo* applications, with success already being shown with *in-vivo* expression in the eye, heart, and lungs of animal models [78].

Chitosan has long been employed as a drug delivery system due to its biodegradability and low toxicity [79-81]. Chitosan, much like the previously mentioned

polymers, has ample amines that allow for modification such as conjugation to folate or transferrin, or addition of polyethylene glycol (PEG) chains [82, 83]. The amines of chitosan are neutral at physiological pH, and thus the polymer is poorly soluble in water. By acetylating the amine network to varying degrees, however, it is possible to control properties like solubility, charge density, and hydrophobicity [84]. The programmable nature of chitosan makes it an attractive carrier for 'hard-to-transfect' regions where particles may need to traverse a mucosal membrane, where the lipophilic tendencies of chitosan can be exploited.

1.2.3 Hybrid Vectors

One final strategy to consider for a gene carrier is a hybrid vector that employs principles from both viral and non-viral systems. Hybrid vectors are reverse-engineered, virus-like particles that have a functional virus core that can still transport, transcribe, and integrate the genetic payload, but the viral envelope has either been replaced or coated by a cationic material [85, 86]. This strategy attempts to maintain the inherent efficacy of viruses, such as stable expression, while increasing the cell-specific targeting and bypassing the immune response, which ultimately increases safety. For example, adenoviruses have been encapsulated with both lipids and polymers and then had functional ligands conjugated to the surface of the synthetic coating. This allowed for successful retargeting and escape from antibodies [87, 88]. In other situations, the cationic material can replace the function of the viral envelope. When non-infectious Moloney murine leukemia and human immunodeficiency virus-like particles were encapsulated by

PEI or PLL, the virus was able to enter the cell through polymer-mediated transfection and

complete the normal infection cycle after being released into the cytosol [86, 89].

CHAPTER 2. NON-VIRAL GENE DELIVERY STRATEGIES

Though non-viral gene delivery has shown great promise *in-vitro* and *ex-vivo*, many potential applications require successful delivery and transfection inside the body. For this to happen, a gene carrier must successfully evade arrest, capture, and clearance by serum and immune constituents of the circulatory system, localize to the target area, facilitate endocytosis, escape intracellular vesicles, and finally release the genetic material (Figure 2.1). One may first consider optimizing each step of the pathway and consolidating the findings into a super carrier at the end. However, many of the optimization steps tend to be contradictory. For example, if the goal is to evade serum proteins, one could neutralize the surface charge of the gene carrier to shield the particles from non-specific binding interactions. However these interactions largely constitute particle-cell membrane association. Thus, endocytosis of neutral particles would suffer. Conversely, an increase in particle-cell membrane interaction by increasing the cationic charge density of the carrier could increase endocytosis, but may also increase cytotoxicity or inhibit release of the genetic payload inside the cell. Many of the barriers associated with non-viral gene delivery will require compromises to find an optimal formulation that maximizes circulation lifetime and stability, transfection, and expression.
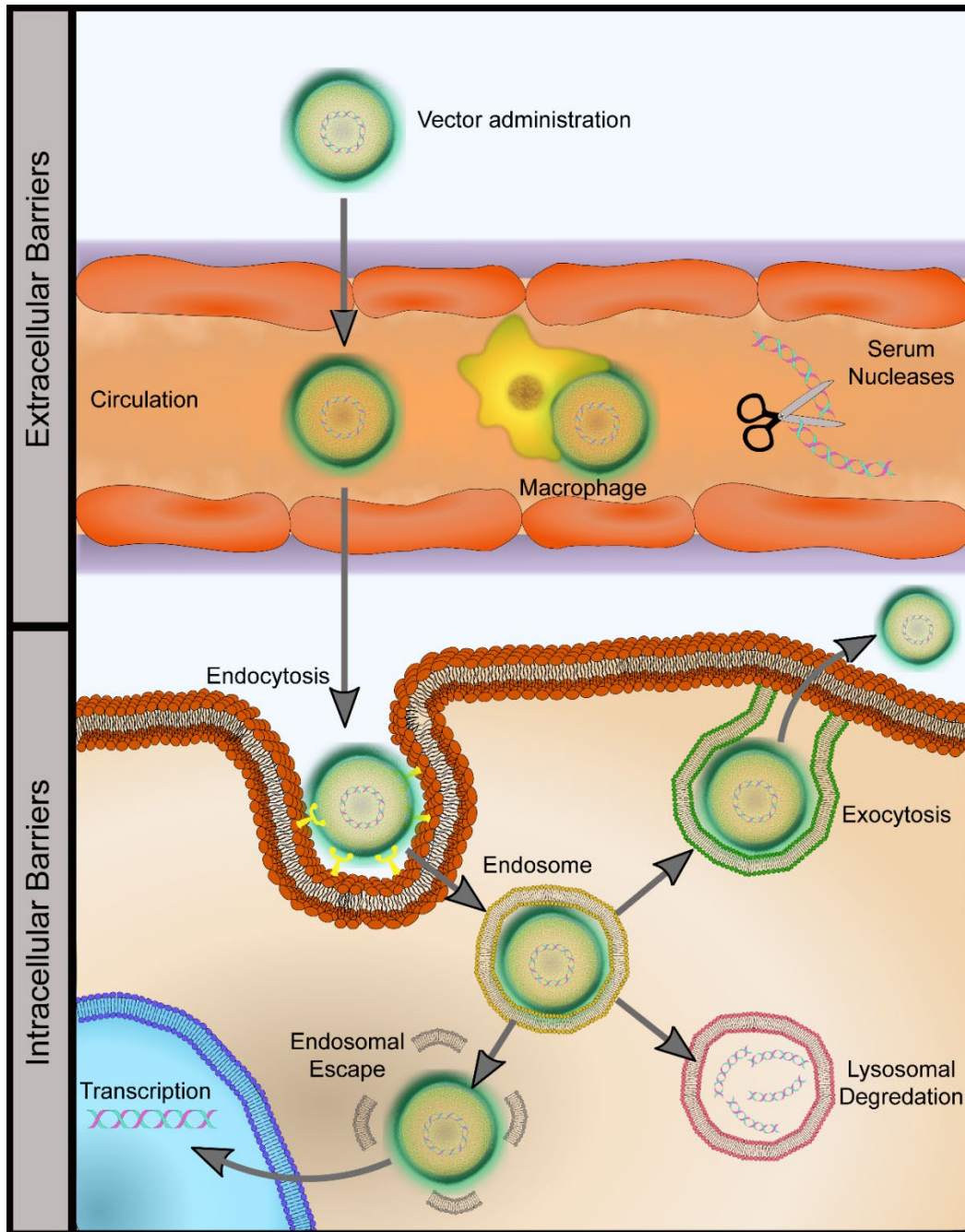
Figure 2.1: Barriers to non-viral gene delivery. Once vectors enter circulation the arduous path to the cell begins. Vectors must evade serum proteins and immune components to avoid clearance and degradation. Vectors must then extravasate or diffuse through the vasculature wall to reach the cell surface. From here the vectors can be endocytosed into cells after binding to the cell membrane through either non-specific electrostatic interactions or receptor-mediated interactions. Some endocytic pathways can lead to lysosomal or exocytic vesicles that will either degrade the vector or lead to cell ejection, respectively. Vectors must escape endosomal compartments and transport through the cytosol and localize within the nucleus for transcription

2.1 Barriers to Non-Viral Gene Delivery

2.1.1 Circulatory and Extracellular Stability

One of the first obstacles genetic material must navigate when entering into circulation is avoidance of host nucleases [90]. To protect the integrity of the DNA or RNA, most vectors consist of a cationic shielding material. Although this solves one problem, it introduces several others. First, at physiological pH and ionic strength, cationic complexes tend to aggregate [91]. Large aggregates can encourage phagocytosis from immune cells and also prevent extravasation [92, 93]. The cationic charge of the carrier can also promote non-specific electrostatic interactions between the complex and negatively charged serum proteins leading to rapid renal clearance [59]. The positively charged particles can also bind to the membrane of circulating erythrocytes leading to accumulation in the lungs, and bind to plasma proteins initiating a coagulation cascade, both of which can lead to serious toxicity [94].

If non-viral vectors can successfully mitigate the threats posed by the circulatory system, the next issue is localization to the desired tissue. As mentioned before, cationic complexes tend to be unstable in physiological conditions leading to aggregation. If these aggregates grow to sizes larger than 500 nm, diffusion through gaps of the vascular wall will be prevented [32, 59, 92].

2.1.2 Cell Membrane Interactions and Internalization

Once a vector has reached its intended destination, it must then enter the cell. To do this a vector must bind to the surface of the cell in one of two ways. The first method

again involves the cationic nature of most gene carriers. Scattered throughout the cell membrane are transmembrane proteins, namely proteoglycans, which perform a wide range of functions for the cell [95]. Proteoglycans are highly anionic, consisting of a core protein covalently linked to sulfated glycosaminoglycans. The anionic nature of these proteins makes for an excellent ionic attractor to cationic vectors. Polymer- and lipid-mediated transfections performed on cells that are under proteoglycan inhibitors or on cells that have been modified to be devoid of proteoglycans result in expression levels decreased >50-fold [96]. Complexes that are neutrally or negatively charged can undergo a similar phenomenon, but are restricted to scarcer cationic regions and likely cluster to the few cationic receptors [97, 98].

The second method involves receptor-mediated interactions driven by cellular recognition of a ligand. Cell membranes have receptors for a multitude of processes, with some cell types having a greater concentration of certain receptors, increasing their affinity to specific molecules. This has been exploited for decades in order to increase the cell-specific targeting of vectors [99]. Common ligands include sugars, antibodies, cholesterol, integrin, folate, and transferrin [59-61, 67, 83, 100-107].

Once a vector has attached to the surface of the membrane it is then engulfed by the cell into a vesicle that detaches from the membrane inside the cell, a process known as endocytosis. Due to recent work, it is becoming ever more important to consider the endocytic pathway that results from surface interactions [108, 109]. Generally speaking, there are three prominent categories of endocytosis to consider: clathrin-mediated endocytosis, caveolin-mediated endocytosis, and macropinocytosis [110]. In clathrin-mediated endocytosis the vector is first compartmentalized into a clathrin-coated vesicle

(CCV) formed from the plasma membrane [111]. From here it is understood that the contents of CCVs are acidified and exposed to degradative enzymes such as proteases and nucleases as the CCVs fuse with endosomes and lysosomes. Therefore, the clathrin pathway is largely considered detrimental to gene delivery due to the propensity for late endosome/lysosome fusion. Interspersed throughout the lipid membrane of cells are ordered domains of lipids termed 'lipid rafts' [112], within which the majority of clathrin-independent endocytosis occurs. Caveolin-mediated endocytosis is one such pathway where cargo is invaginated via flask-shaped plasma membrane domains containing caveolin-1 [113]. Caveosomes, unlike CCVs, are presumed to avoid lysosomal targeting, providing an advantageous route to circumvent complex degradation [114]. Macropinocytosis is categorized by the large sweeping uptake of fluids, in which nanoparticles may be suspended [115]. Ruffles are formed by actin on the surface of the plasma membrane that are extended out and then swept in forming macropinosomes [116]. After internalization the intracellular route can vary depending on the type of cell. In macrophages, macropinosomes are acidified and then fused into lysosomes [117]. In other cells the macropinosomes avoid interaction with other endocytic compartments, and thus, avoid acidification and trafficking into lysosomes. However, the vesicle can be trafficked back to the cell surface and the cargo exocytosed [116]. Since both macropinocytosis and caveolin-mediated endocytosis avoid low pH environments and are not subjected to degradative enzymes, it is easy to see why these pathways are starting to be preferentially targeted in vector design.

2.1.3 Endosomal escape

No matter which specific pathway a vector traverses, at some point the vector must facilitate endosomal escape. Each of the three main categories of non-viral gene carriers—lipids, polymers, and CPPs—demonstrate three distinct methodologies for escape [118]. As briefly mentioned previously, cationic lipid carriers are widely believed to escape via a 'flip-flop' mechanism. Endosomes have a lipid bilayer primarily comprising anionic lipids facing the cytosol and zwitterionic lipids facing the compartment interior. Electrostatic interactions between the cationic carrier lipids and the anionic endosomal lipids cause the endosome to laterally diffuse into the lipoplex [119]. This change in morphology causes the genetic material to be displaced from the lipoplex into the cytosol.

CPPs also utilize a morphology change to escape endosomes. When the endosome starts to mature and become acidified, some CPPs will undergo a conformational change into a helical structure [120]. The helical structure can then aggregate with other carrier peptides and integrate into the endosomal membrane in a similar manner to lipid 'flip-flop'. The aggregate acts as a small pore, or channel, that could allow nucleic acids to exit the endosome [121].

Many polymeric carriers inherently have large buffering capacities due to a large number of protonatable amine groups at physiological pH [118]. As endosomes mature, protons are actively pumped into the vesicle compartment to lower pH and activate hydrolytic enzymes. Some polymers will buffer the incoming protons and prevent acidification, resulting in a continuous influx of protons [64]. Counter ions, primarily chloride, also enter the vesicles to maintain charge neutrality, causing an osmotic induced swelling leading to membrane permeation or lysis, effectively releasing the vector.

2.1.4 Complex Dissociation and Nuclear Transport

After escaping the endosome, the only steps that remain are nucleic acid unpacking to allow transcription machinery to access the genetic material and transport into the nucleus. These are arguably some of the least understood aspects of polymeric gene delivery. Where lipoplexes and CCPs simultaneously release the genetic material and escape the endosome, polyplexes have been observed to remain, to some degree, complexed even after entering the nucleus [122, 123]. There are many proposed mechanisms for polyplex dissociation. After acidification, buffering, and escaping the endosome, the cationic charge density of the polymer will be reduced, lessening the electrostatic interaction between the carrier and genetic material. This could allow transcription machinery to access the once sterically hindered nucleic acid. The genetic cargo could also be displaced from the polymer by a competing anion, such as glycosaminoglycans, cytosolic RNA, or chromosomal DNA, or by polymerases found within the nucleus [124-126].

Though it is not known which mechanism dominates, the genetic cargo must enter the nucleus for successful expression to occur. If the nucleic acid is displaced before the vector is localized in the nucleus, the genetic cargo can traverse the nuclear membrane through nuclear pore complexes [32]. These complexes only allow diffusion of small molecules (<11 nm), thus nucleic acids still complexed to polymers would not be able to navigate through this pathway. Instead, non-dissociated polyplexes can associate with the nuclear envelope and enter the nucleus as the envelope is disassembled during cell division [127, 128].

2.2   Design of Polymeric Gene Carriers

Considering the relatively simple nature of non-viral vectors in comparison to viral carriers, it is easy to see why understanding the aforementioned barriers is crucial to developing a set of criteria for designing non-viral vectors. As the barriers become more defined, changes can be made to the synthetic materials that comprise non-viral vectors to better navigate the extra- and intra-cellular environment. For the remainder of the chapter we will discuss some of the lessons learned throughout the history of polymeric vectors and how they have shaped the modern design of polymers for gene delivery.

2.2.1   Size of Polyplexes

Size can very often be overlooked when designing polymers for gene delivery. However, size is probably one the most well understood and crucial properties for successful *in vivo* applications. As mentioned earlier, vectors must extravasate through the vascular wall. The channels within the wall are only large enough to allow diffusion of structures less than 500 nm in size [129]. If vectors cannot overcome this first barrier, they will stay in circulation until ultimately being cleared. One might then think it is intuitive that the smaller the particle, the better, since smaller particles could diffuse through the channels faster, have higher colloidal stability in solution, and lead to more facile endocytosis due to the lower energy requirement [130]. This is not the case for many polymeric vectors. PEI-pDNA polyplexes greater than 100 nm exhibit increased transfection efficiency in comparison to smaller counterparts [130]. Multiple explanations have been proposed to account for this observation. First, smaller particles do in fact exhibit a higher solution stability compared to large particles, which may sediment onto the cell

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surface facilitating higher interactions. This same effect can be achieved in smaller particles if the complexes are centrifuged onto the cells [131]. Another explanation is that size can dictate endocytic fate. For polymers that depend on buffering the endosome and escaping via the proton-sponge phenomenon, large complexes resulting from higher polymer weight have increased buffering capacity. This is evidenced by the fact that large complexes received little to no benefit from transfections with lysosomotropic agents, while smaller complexes saw significant increases in efficiency [130]. Furthermore, vector size can control the route of internalization. CCVs measure approximately 200 nm in diameter. Thus, particles that enter via this route must adhere to that constraint [132]. Larger particles instead enter via clathrin-independent pathways, avoiding the harsh acidification and trafficking to lysosomes.

As alluded to earlier, many design parameters have a point of compromise, and the spectrum of small to large complexes is no different. Large complexes, formed from excess polymer:DNA charge ratios, result in highly cationic surface potentials. As discussed earlier, cationic polyplexes readily aggregate with circulatory constituents such as serum proteins and erythrocytes leading to clearance or toxicity, respectively. Smaller and more neutral polyplexes avoid this fate by having decreased electrostatic and non-specific binding interactions. Large polyplexes also suffer from decreased cytosolic mobility and must be actively transported by the microtubular and microfibril networks [133, 134]. Thus, the compromise for polyplex size is maintaining favorable endocytic trafficking and cellular interactions while optimizing cytotoxicity and cytosolic mobility.

2.2.2   Preparation

The way polyplexes are prepared can affect the size and polydispersity. Traditionally, polyplexes are prepared by conventional bulk mixing methods where a polymer solution is added to a solution of plasmid DNA and mixed. This preparation can lead to a very high polydispersity index, with significant populations of aggregates which can be cytotoxic [135]. This effect can be exacerbated when preparing vectors with greater complexity than typical binary polyplexes [136]. Researchers have developed two robust methods of preparing complexes that can give a greater degree of control on particle preparation and even streamline some aspects of gene therapy, opening doorways to industry [137]. The first method employs a microfluidic device to hydrodynamically focus two laminar streams of polymer and DNA solution into a single channel [135, 138, 139]. From here the two solutions will mix via diffusion. Complexes prepared with microfluidic devices result in much narrower PDIs and can benefit from increased transfection efficiency. Less common than microfluidic preparation is electrohydrodynamic spraying (EHDS). In EHDS materials are positioned in annular needles set up with pDNA occupying the inner needle and polymer occupying the outer needles. As pressure is applied and flow commences, a positive voltage is applied to the inner needle causing a Taylor cone to be formed at the tip. Drops of polymer and DNA solution disperse as a mist from the needle tip and mix together [140]. This method has some of the same advantages as microfluidics, with increased control over particle size and enhanced transfection efficiency, but with the added benefit of being able to apply polyplex aerosol directly to the cells. For polymers that result in large aggregates, using a more robust preparation method may increase transfection efficiency without further modification.

2.2.3 Charge and Functionalization

Two of the more investigated aspects of polymeric gene carriers are the resulting charge and zeta potential, as well as specific chemistries that have been functionalized to better address targeting, cell association, and trafficking. The charge of a polymer and the density of cationic groups have received intense focus due to the numerous facets of polymeric gene delivery that depend on electrostatics. First and foremost, the electrostatic condensation of genetic material with polymers greatly depends on the charge density. When polymers of equivalent molecular weights are used to condense DNA, the polymers with a higher cationic charge per mass led to the formation of smaller complexes [141, 142]. Surface charge of the resulting complexes has a tremendous effect on the remaining lifecycle of the vector. Due to the numerous anionic moieties in circulation, highly cationic particles have a high propensity of being cleared from circulation quickly. In *in vitro* environments, the cationic nature facilitates strong electrostatic association to the cell membrane, enabling endocytosis. However, these strong interactions can cause severe membrane disruption leading to cell lysis. Furthermore, the strong electrostatic pairing of the polymer to the DNA backbone could hinder unpackaging and ultimately prevent expression. Thus, there exists a balance between maintaining favorable cell membrane interactions and optimal complex size, while decreasing adverse cytotoxicity and clearance.

In attempts to address some of these issues, the specific chemistry of polymeric carriers have been modified to decrease cationic charge in circulation. A particularly common method employed for polymers such as PEI and PAMAM is conjugating polyethylene glycol (PEG) chains to the various amine groups within the polymers [143].

This method effectively neutralizes an amine group, reducing the cationic charge density and decreasing interactions with circulatory constituents [144, 145]. This can increase the stability of polyplexes in circulation, but transfection efficiency tends to suffer due to the low cell membrane interactions. Many alternatives to PEG have been explored, with some producing more promising results by maintaining or even increasing transfection efficiency, while decreasing protein interactions [35, 72, 146]. A third route to reducing cationic charge density is by complexing the binary polyplex with an anionic material to create a ternary polyplex. These complexes create a modified surface chemistry of both anionic and cationic material, resulting in polyplexes that have decreased protein interaction with increased transfection performance [147]. However, ternary particles suffer from poor polydispersity profiles and can be difficult to manufacture with consistent properties without the aid of a microfluidic device [148].

2.2.4   Concluding Remarks

With mounting interest in gene therapy, the list of novel non-viral genetic carriers is continuously expanding. However, as laid out in the previous chapters, many barriers to non-viral gene therapy are still not fully understood. With the influx of specialty polymers and difficult chemistries in literature, it can be difficult to cypher through the data to discern meaningful design criteria. While some insight has been gained on a few high performing polymers, the field of polymeric gene carriers is lacking clinical relevance. Therefore it is crucial we focus on understanding how our modifications affect the gene delivery mechanism and interactions of the vector in the body.

CHAPTER 3. SUCCINYLATED POLYETHYLENIMINE DERIVATIVES GREATLY ENHANCE POLYPLEX SERUM STABILITY AND GENE DELIVERY IN VITRO

Preface

Parts of the following chapter are adapted from previously published work. All experiments, excluding lyophilization of succinylated polyethylenimine particles, were included in Warriner, L. W., Duke III, J. R., Pack, D. W., & DeRouchey, J. E. (2018). Succinylated Polyethylenimine Derivatives Greatly Enhance Polyplex Serum Stability and Gene Delivery In Vitro. *Biomacromolecules*, 19(11), 4348-4357. This work was performed in collaboration with the group of Dr. DeRouchey at the University of Kentucky Department of Chemistry. Polymer reaction schemes, DNA binding assays, and dextran sulfate displacement assays were designed and performed by Joseph R. Duke III and Dr. Jason DeRouchey. Succinylation of polyethylenimine and pH titrations were performed by Joseph R. Duke III and the author. Toxicity, elemental analysis, NMR, dynamic light scattering, zeta-potential, protein interaction, transfection, and lyophilization experiments were designed and performed by Dr. Daniel W. Pack and the author.

3.1   Introduction

To date, recombinant viruses account for roughly 70% of clinical trials due to their inherent gene delivery activity [32], yet viruses present important safety concerns, such as immunogenicity [149] and oncogenicity [150], while also being difficult to produce in commercially relevant quantities [151, 152] and having a limited genetic payload [153]. Non-viral, or synthetic, vectors utilize polymers, dendrimers, lipids, or peptides as the

delivery vehicle for genetic material. Synthetic vectors have several advantages over viral vectors, including reduced immunogenicity [154], larger capacity for their genetic payloads [30], and facile manufacturing [94], but delivery efficiency relative to viral vectors is significantly lower [32]. Polyethylenimine (PEI), often considered the benchmark for polymeric, non-viral gene delivery vectors, serves as an excellent model for investigating gene delivery mechanisms [31]. The highly cationic polymer readily condenses DNA through electrostatic interactions to provide a relatively effective gene delivery vehicle [31, 32, 94, 154, 155]. The repeating amine structure of PEI results in a large positive charge density, which is conducive to condensing DNA and leads to favorable electrostatic interactions with the negatively charged cell membrane [70]. In addition, the substantial quantity of secondary and tertiary amines in branched PEI also results in a large buffering capacity, thought to facilitate escape from endocytic vesicles via the 'proton-sponge' mechanism [64]. However, PEI was originally developed nearly 50 years ago as a chelator for use in the water purification and mining industries. As such, the properties of PEI should not be expected to be optimal for gene delivery [64, 155]. The excess positive charge typical of PEI/DNA polyplexes induces nonspecific binding to negatively charged serum proteins, which leads to polyplex aggregation and, ultimately, clearance from the intended treatment area by the reticuloendothelial system [59, 156]. The high charge density can also contribute to cytotoxicity, causing changes in cellular morphology, damage to the cell membrane, decreased metabolism, and lysis [157]. Finally, the strong electrostatic interactions between PEI and DNA can hinder vector unpackaging, which is required for transcription [158, 159].

Acetylation of primary and secondary amines on PEI resulted in polymers exhibiting dramatically enhanced gene delivery activity, demonstrating the relative importance of buffering capacity and polymer/DNA binding strength in PEI-mediated gene delivery [35, 146]. Generally, acetylation lowered the protonation constant of PEI, which resulted in weaker polymer/DNA interactions and significantly lower buffering capacities. The acetylated PEI/DNA polyplexes were shown to dissociate more readily within cells, presumably enhancing access of cellular transcription machinery to the plasmid. In addition, acetylation of up to 43% of primary amines enhanced transgene expression, while further modification decreased expression, suggesting there exists an optimal balance between the ease of polymer/DNA dissociation, which increased with the degree of acetylation, and buffering capacity providing endosome escape, which decreased with acetylation. Despite the significant increases in gene delivery efficiency realized by acetylated PEI, transfections in the presence of serum resulted in gene delivery activity lower than that of unmodified PEI in the absence of serum, suggesting that the acetylated polymers still suffered from serum instability, likely due to aggregation with anionic proteins.

Protein adsorption and biofouling are important in fields other than gene and drug delivery. Medical implants and biosensors in particular require high resistance to nonspecific protein binding and cell adhesion to avoid leukocyte activation, tissue fibrosis, thrombosis, and infection [160]. Attention has recently turned to polyampholytes, or polymers that have both positively and negatively charged moieties that provide an electrostatically induced hydration that is relatively strong and stable [161]. The tightly bound water molecules form both a physical and energetic barrier that inhibits protein

adhesion to biomaterials, which is the first step in the coagulation cascade that can lead to rejection of biomaterials [160, 161]. This phenomenon is an attractive design criterion, as the hydration barrier could potentially increase the longevity and stability of synthetic vectors while in circulation.

Addition of negatively charged groups to polycationic gene delivery materials is one approach to generating such polyzwitterions. For example, charge-shifting polymers, which under physiological conditions convert neutral esters to anionic carboxylates, have been shown to modify electrostatic interactions between the polymer and the DNA and enhance gene delivery efficiency of linear PEI [162, 163]. Little work to date, however, has initially formed polyplexes using mixed-charged polymers to condense DNA. Recently, we have shown that succinylated PAMAM (zPAMAM) dendrimers with up to 40% modification of the primary amines were not only able to condense DNA but also induced a phase transition in the DNA packing with increasing degrees of succinylation [164]. Similar succinylation of PEI on 9 and 19% of amines was shown by Wagner and coworkers to increase the effectiveness of PEI as a vector for siRNA-mediated gene silencing [165]. Upon modification of 9% of PEI amines with succinic anhydride, gene expression was reduced to nearly 10% of the relative control, a significant improvement over unmodified PEI, which generally acts as a poor siRNA carrier in comparison to its ability as a plasmid DNA carrier. Though this previous study explored succinylated PEI as a siRNA carrier, the ability of succinylated PEI in plasmid DNA delivery had not been reported until our recent work [72]. Furthermore, potentially important characteristics of succinylated PEI such as polymer-DNA interactions, cellular internalization, non-specific

protein binding interactions, and the relative ease of unpackaging had yet to be investigated.

We generated zwitterion-like polymers by reacting PEI with varying amounts of succinic anhydride, resulting in modification of 9-55% of the primary and secondary amines (named zPEI X, where X represents the percentage of modification). In vitro gene delivery efficiency was assessed via transfections in the absence and presence of serum and compared to the efficiency of unmodified PEI. While succinylation of PEI showed slightly improved transfection efficiencies out of serum, the most surprising aspect of the zPEIs were the highly effective in-serum transfection efficiencies, showing enhanced gene delivery surpassing that of unmodified PEI/DNA out of serum. Possible mechanisms for this enhanced gene delivery activity were investigated by characterizing the interactions of the polymers with DNA and serum proteins, polymer cytotoxicity, and cellular internalization.

3.2   Methods and Materials

3.2.1   Cells and Plasmids.

HeLa human cervical cancer and MDA-MB-231 human breast cancer cell lines were purchased from the American Type Culture Collection. The MC3T3-E1 mouse fibroblast cell line was received as a gift from Dr. David Puleo (University of Kentucky, Lexington, KY). Cell lines were cultured at 37 °C, 5% CO2, and in the presence of 10% fetal bovine serum per ATCC recommendations. The pGL3 luciferase expression vector was purchased from Elim BioPharm (Hayward, CA).

3.2.2 Succinylation of Polyethylenimine.

Succinic anhydride and branched 25-kDa polyethylenimine (PEI) were purchased from Sigma-Aldrich (St. Louis, MO). Half a gram of PEI was placed in a 7 mL scintillation vial and dissolved using 3 mL of sodium bicarbonate buffer (pH 9). Succinic anhydride was added in amounts necessary to achieve the desired degree of modification. Scintillation vials were sealed and the contents were allowed to react at 60 °C for 4 h under constant agitation. The reaction product was transferred to a 2K MWCO Slide-A-Lyzer dialysis cassette and dialyzed against double distilled water for 48 h. Water was replaced every 8 h. The dialysis product was frozen overnight and then lyophilized for 24 h. The dried product was placed in a -80 °C freezer for storage. The degree of modification was analyzed by elemental analysis performed by Midwest Microlab (Indianapolis, IN). The O, C, H, and N contents were used in a system of equations to determine the mole percent of succinyl and PEI monomers present in each sample. The modification was expressed as a ratio of modified amines (mole of succinyl) to the total number of amines (mole of PEI monomer). 1H NMR was used to track the relative area of the succinate methylene peaks (δ 2.3-2.5 ppm) in comparison to the peaks of unreacted amines associated with the PEI backbone (δ 2.6-3.3 ppm), as a qualitative check for the presence of succinyl groups on the modified polymers.

3.2.3 pH Titrations.

Approximately 2 mg of both the unmodified and modified polymers were dissolved in 5 mL of double distilled water. The pH of each sample was adjusted to approximately 11.5 with 1 M NaOH. Samples were then titrated using 5 µL aliquots of 1 M HCl. The solution

pH was measured after each addition of HCl with a pH meter (Accumet AB15, Hudson, MA).

3.2.4 DNA Binding Assay.

Agarose gel electrophoresis was utilized to assess DNA binding efficiency of modified polycations. A 0.8% (w/v) agarose gel was prepared by dissolving agarose in a 0.2 μm PES-filtered TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA). Polyplexes containing 200 ng pUC19 plasmid DNA were formed in 10 mM Tris (pH 7.5) by addition of the appropriate amount of each polymer to achieve the desired polymer:DNA weight ratio and diluted to a total volume of 12 μL. Polyplexes were incubated at room temperature for 45 min. After incubation, 2.4 μL of 6x loading dye was added to each sample, mixed, and 10 μL of each solution was then loaded onto the gel. Electrophoresis was performed at 100 V for approximately 120 min and DNA was visualized with ethidium bromide. A BioRad ChemiDoc MP Imaging System was used to image each gel using Image Lab software.

3.2.5 Dextran Sulfate Displacement.

To determine the stability of DNA/zPEI complexes, samples of 200 ng pUC19 were complexed with a fixed polycation:DNA weight ratio at a total volume of 5 μL in 10 mM Tris buffer at pH 7.5. The amount of polycation added corresponded to the observed optimum transfection ratios for each polymer. Samples were well-mixed and incubated for 45 min at room temperature to ensure polyplex formation. Dextran sulfate was then added

at various weight ratios relative to the DNA and the final solution volume was brought to 12 μL per sample. Samples were mixed and incubated for 30 min prior to adding loading buffer and running on a 0.8% agarose gel as described above. DNA was visualized with ethidium bromide and a BioRad ChemiDoc MP Imaging System was used to image each gel.

3.2.6 Dynamic Light

Polymer/DNA complexes were formed in 0.1x phosphate buffered saline (PBS) at the optimum transfection weight ratio for each of the modified and unmodified polymers. After the 30 min incubation period, the polyplexes were diluted to 1 μg of DNA/mL using 0.1x PBS. The solution was then immediately read using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). DLS measurements were performed prior to ζ-potential measurements. Each measurement was taken in triplicate.

3.2.7 Cytotoxicity.

Toxicity of both the modified and unmodified polymers was quantified using the CellTiter-Blue cytotoxicity assay (Promega, Madison, WI). Each model cell line was grown in 96-well plates at 2×10^4 cells/well for 24 h at 37 °C and 5% CO_2 in serum-supplemented media. After 24 h, growth medium was replaced with serum-free media. Polymer was then added to achieve concentrations from 0-50 μg/mL and allowed to incubate for 4 h. After the incubation period, the polymer containing medium was replaced with serum-supplemented growth medium. The cells were allowed to grow undisturbed for 20 h, after which 20 μL of CellTiter-Blue reagent was added to each well and allowed

to incubate for 4 h at 37 °C and 5% CO2. Then, cells were immediately excited with a 560-nm laser and the fluorescence was read at 590 nm using a Synergy 2 plate-reader (BioTek, Winooski, VT). Fluorescence was normalized to cells containing no polymer.

3.2.8 Polyplex Transfection.

HeLa and MDA-MB-231 cell lines were cultured in EMEM supplemented with 10% FBS, while MC3T3-E1 cells were cultured in alpha MEM with 10% FBS according to ATCC recommendations. Cell lines were seeded in 24-well plates at 7.5 x 104 cells/well 24 h prior to transfection. Polymer/DNA complexes were formed by diluting 20 μL of 0.1 μg/μL DNA solution with 80 μL of PBS in a 1.5 mL microcentrifuge tube. Polymer solutions (100 μL) at various concentrations were added dropwise to the DNA solution under constant agitation to achieve the desired polymer:DNA weight ratio. Particles were allowed to incubate at room temperature for 30 min. Immediately before transfection, 200 μL polyplex solution was deposited into 2.8 mL of fresh serum-free or serum-present medium, depending on the specific study. Regular growth medium was aspirated from cells and replaced with 750 μL of polyplex/growth medium solution (0.5 μg DNA/well). After 4 h, the transfection medium was replaced with serum-supplemented growth medium. Transfection efficiency was quantified via luciferase expression 24 h post initial transfection. A Promega luciferase assay kit (Madison, WI) was used to measure protein activity in relative light units (RLU) using a Synergy 2 plate-reader (BioTek, Winooski, VT). Results were normalized to total cell protein using a bicinchoninic acid (BCA) assay from G-Biosciences (St. Louis, MO).

3.2.9   Protein Interaction Study.

The affinity for nonspecific protein binding interactions was assessed for both free polymer and polyplexes. Free polymer interaction was quantified by mixing 0.5 mL of bovine serum albumin (BSA) standard (2 mg/mL) with 0.5 mL of each polymer (1 mg/mL). Polyplexes were prepared using the aforementioned procedure at a concentration of 1.5 μg/mL of DNA with a final volume of 0.5 mL and were also mixed with 0.5 mL of BSA standard. Each mixture was incubated at 37 °C for 1 h. The mixtures were then centrifuged and samples of the resulting supernatants were collected. The protein concentrations of the samples were determined through the use of a BCA assay and a standard BSA calibration curve. The protein interaction value, A, was defined as:

$$A = 1 - \frac{C_s V_s}{C_i V_i}$$

Where Ci is the initial BSA concentration (2 mg/mL); Cs is the BSA concentration in the supernatant determined by BCA; Vi is the initial volume of the BSA solution (0.5 mL); Vs is the total volume of the BSA after adsorption measurement (1 mL).The interaction value A, as it has been described, is essentially a measure of how much protein has been removed from the initial solution via aggregation with polymer or polyplexes, and thus, ranges between 0 (no removal of protein) and 1 (complete removal of protein).

3.2.10 Flow Cytometry.

DNA complexes were formed with both the modified and unmodified polymers at their respective optimum weight ratios. The intercalating dye YOYO-1 was added according to the ratio 15 nl YOYO-1 per 1 μg of DNA. The complexes were then incubated

at 4 °C for approximately 30 min. Cells (HeLa, MDA-MB-231, or MC3T3-E1) were cultured as described above and seeded in 24-well plates at 7.5x104 cells/well 24 h prior to transfection. Immediately before transfection, 50 μL of polyplex solution was deposited into vials containing 700 μL of fresh, serum-free or serum-supplemented medium, depending on the specific study. The polyplex/growth medium solution was then added to each well (0.5 μg plasmid/well). Two hours post-transfection, the cells were rinsed twice with 0.001% SDS in PBS and PBS, respectively, to remove surface-bound complexes. Next, 250 μL of 0.05% trypsin in PBS was added to each well. The cells and trypsin were allowed to incubate for 5 min before 50 μL of FBS was added to each well. The cells were then collected and centrifuged at 6400 rpm for 5 min. The resulting supernatant was discarded and the cells were resuspended in 1 mL of fresh PBS. Samples were transferred into 15 mL polystyrene tubes and sealed. The polystyrene tubes were placed in a sealed container and then transported in a secondary container to an Attune flow cytometer, where FACS analysis was performed. Data was processed using FloJo software (FlowJo LLC., Ashland, OR).

3.2.11 Long Term Stability of Polyplexes.

To test the shelf stability of the polyplexes formed with succinylated PEI, DNA complexes were prepared, frozen, and lyophilized for up to 8 weeks. Polyplexes were formed using unmodified PEI, zPEI 9, zPEI 14, and zPEI 25. Trehalose was added to the DNA solution as a cryoprotectant at a ratio of 200:1 trehalose:DNA (w:w) to prevent aggregation during the drying process. Polyplexes were formed using the protocol described above for transfections and immediately placed in a -80 °C freezer. After 4 h,

the frozen complex solution was placed in a Labconco Freezone drying apparatus (Labconco, Kansas City, MO) and allowed to dry for 12 h. Dried polyplexes were stored in a -20 °C freezer until needed for transfection. To reconstitute the polyplexes, 200 uL of DNase and RNase free water was added to each dried sample and allowed to incubate for 10 min, after which transfection proceeded as described previously.

3.3   Results

3.3.1   Succinylation of Polyethylenimine.

In this study, zwitterion-like PEI derivatives exhibiting both reduction of positive charge density and introduction of negatively charged carboxyl moieties were generated through reaction of varying amounts of succinic anhydride with PEI (Figure 3.1). Succinic anhydride was added at 0.10, 0.25, 0.50, 0.75, and 1.00 moles anhydride per mole of nitrogen in PEI. ^{1}H NMR was used to track the relative area of the succinate methylene peaks (δ 2.3-2.5 ppm) in comparison to the peaks of unreacted amines associated with the PEI backbone (δ 2.6-3.3 ppm), as a qualitative check that the chemistry was proceeding as intended (Figure 3.2). The succinate methylene peaks to the right of the PEI backbone continued to grow as theoretical percent of modification increased, suggesting each PEI derivative achieved larger degrees of modification as intended. The extent of modification was determined by elemental analysis (Figure 3.2). Modifications of 8.5% (zPEI 9), 13.8% (zPEI 14), 25.2% (zPEI 25), 54.9% (zPEI 55), and 46.3% (zPEI 46*) were achieved for the aforementioned anhydride/nitrogen ratios, respectively. (The synthesis of zPEI 46* contained a higher anhydride/nitrogen ratio than zPEI 55 and, thus, was expected to yield a higher degree of succinylation. However, the large amount of succinic acid present in

this reaction may have resulted in intermolecular crosslinking of PEI, as evidenced by the

formation of a readily observable gel following the reaction, leading to higher degrees of

succinylation than suggested by the elemental analysis. Thus, we have named this

derivative with an asterisk to indicate this distinction.)
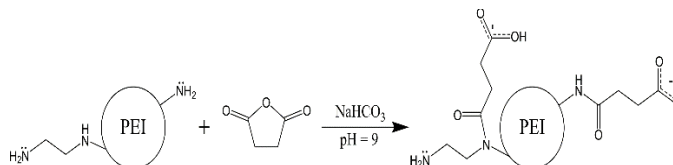
Figure 3.1: Generation of zwitterion-like PEI derivatives via succinylation. Primary amines and secondary amines react with succinic anhydride to generate secondary amides and tertiary amides, respectively. The polymers are named using the convention zPEI X, where X is the percentage of amines reacted with succinic anhydride, as determined by 1H NMR spectroscopy.
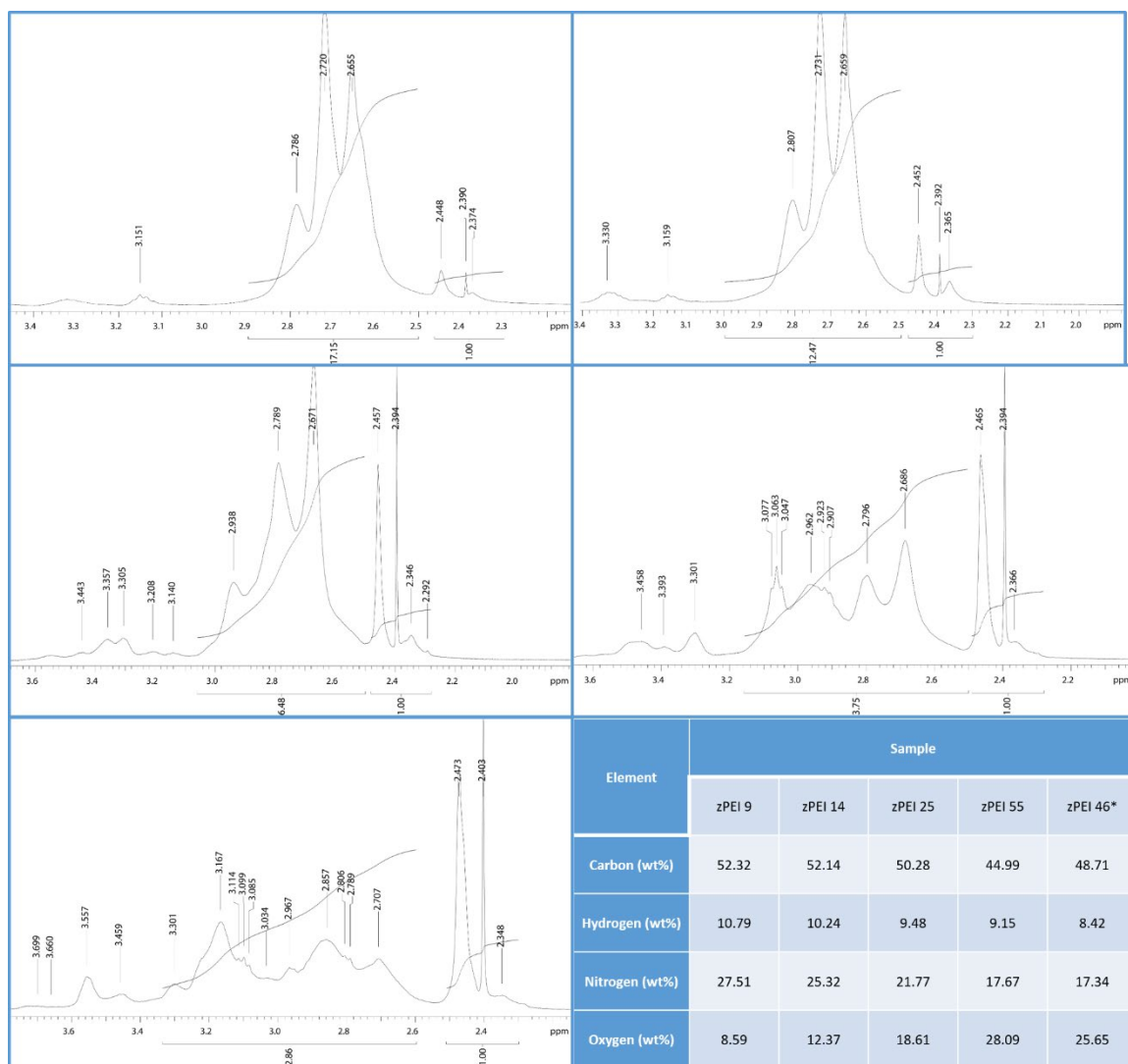
Figure 3.2: NMR spectra of (A) zPEI 9, (B) zPEI 14, (C) zPEI 25, (D) zPEI 55, and (E) zPEI 46* and the corresponding elemental analysis for each polymer.

Element	Sample				
	zPEI 9	zPEI 14	zPEI 25	zPEI 55	zPEI 46*
Carbon (wt%)	52.32	52.14	50.28	44.99	48.71
Hydrogen (wt%)	10.79	10.24	9.48	9.15	8.42
Nitrogen (wt%)	27.51	25.32	21.77	17.67	17.34
Oxygen (wt%)	8.59	12.37	18.61	28.09	25.65

3.3.2 pH Titrations.

The ability for a polyplex to escape endosomal and lysosomal vesicles can greatly affect the overall performance of a polymer to act as a gene carrier. PEI, having a large buffering capacity due to the large density of unprotonated secondary and tertiary amines, is often believed to escape vesicles through osmotic swelling via the proton sponge mechanism [54]. Conversion of primary and secondary amines to secondary and tertiary

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amides through addition of succinate is expected to decrease the pKa of the polymers and, thus, decrease the buffering capacity of zPEIs compared to unmodified PEI. We therefore titrated solutions of unmodified PEI and zPEIs from pH 11.5 to pH ~2. Buffering capacity was quantified here as the reciprocal slope of the titration plots over the pH range of 4 to 10 (with units of μL of 1 M HCl/pH unit). PEI, known to be a strong buffer, exhibited a slope of 15.8 μL/pH unit. In general, the buffering capacity of the zPEIs was lower compared to unmodified PEI, and the buffering capacity decreased as degree of modification increased (Figure 3.3). Buffering capacities of 13.3, 11.7, 9.2, 9.2, 6.7, and 1.7 μL /pH unit for zPEI 9, zPEI 14, zPEI 25, zPEI 55, zPEI 46*, and water, respectively.
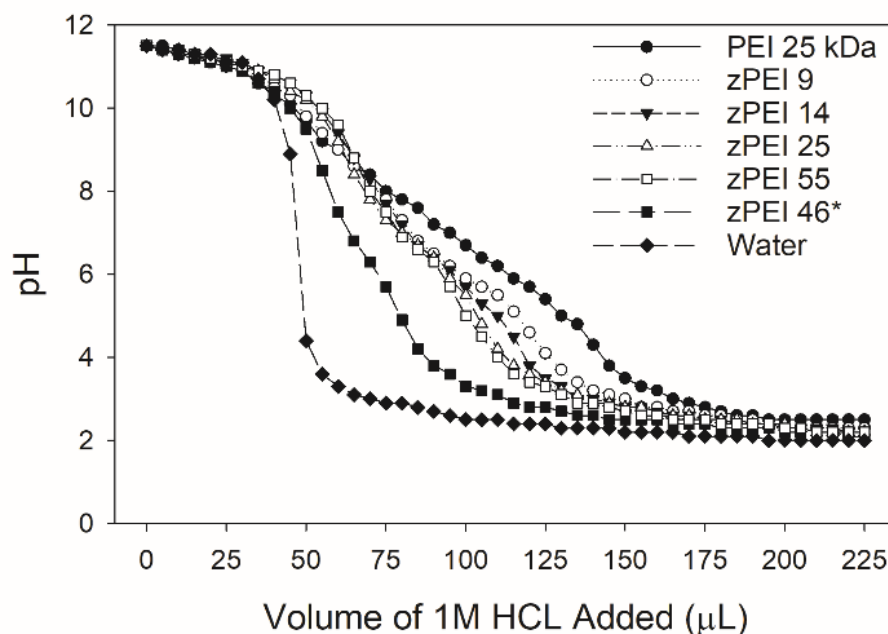
Figure 3.3: Titration of aqueous PEI and zPEI solutions (0.4 mg/mL) with 1 M HCl from pH 11.5 to pH ~2. Solutions were adjusted to pH 11.5 with 1 M NaOH, and 5 μL aliquots of 1 M HCl were added sequentially. The pH was measured after each subsequent addition.

3.3.3 DNA Binding Assay.

Generally, cationic polymers condense DNA into nanostructures through entropically driven electrostatic interactions. PEI, having a high positive charge density, produces compact and tightly bound complexes with DNA and inhibits DNA migration in agarose gel electrophoresis. Polyplexes were formed at increasing polymer/DNA weight ratios using unmodified PEI and zPEIs to determine the point where charge-neutral polyplexes were formed, signified by retention of DNA in the loading wells (Figure 3.4). PEI achieved complete inhibition of DNA migration at 1 µg polymer/µg DNA, consistent with literature reports [35]. As the degree of succinylation increased, greater amounts of polymer were required to retard DNA mobility. Specifically, 2, 3, 3, 4, and 6 µg polymer/µg DNA were required to completely complex DNA with zPEI 9, zPEI 14, zPEI 25, zPEI 55 and zPEI 46*, respectively.
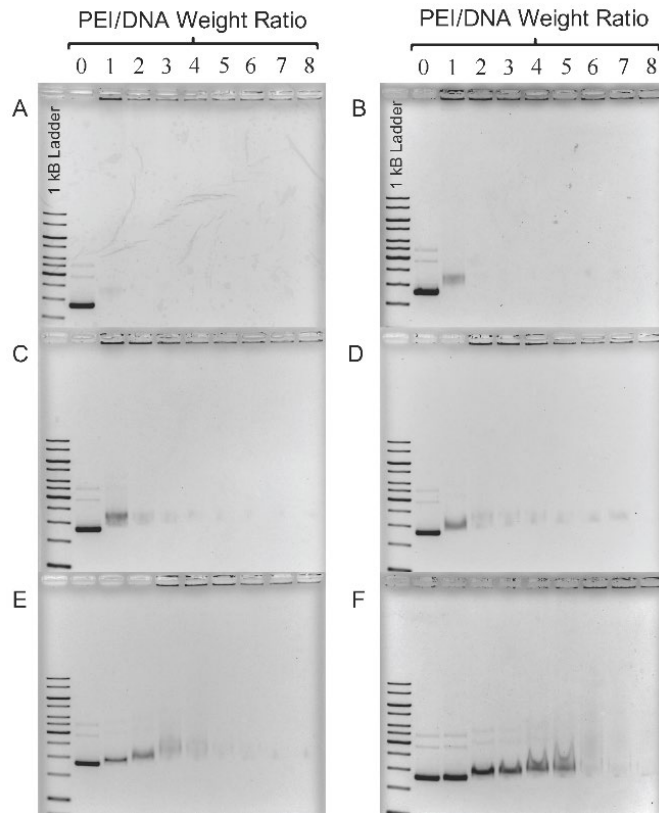
Figure 3.4: Agarose gel electrophoresis of polymer/pUC19 plasmid DNA complexes. (A) unmodified PEI, (B) zPEI 9, (C) zPEI 14, (D) zPEI 25, (E) zPEI 55, and (F) zPEI 46*. The number above each lane signifies the polymer to DNA weight ratio used to form each the corresponding polyplex.

3.3.4 Dextran Sulfate Displacement.

Once inside the cell, gene delivery polymers must dissociate from the DNA to allow transcription. Sulfated glycosaminoglycans are common anionic extracellular components that have been found to competitively displace DNA from cationic polymers in polyplexes [96]. The ability of the proteoglycan dextran sulfate (DS) to displace polycations from DNA has been used to quantify the relative strength of polymer-DNA interactions[166].

To assess the relative binding strengths of unmodified PEI and zPEIs with DNA, varying amounts of DS were incubated with polyplexes formed at polymer/DNA ratio of 6:1 (w:w), a weight ratio that resulted in complete complexation for all of the zPEIs,

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(Figure 3.5) and the polymer/DNA ratios that were found to be optimal for transfection (vide infra, Figure 3.6). At polymer/DNA ratio of 6:1 (w:w), decreasing amounts of DS were required to displace the polymer and restore DNA mobility as the degree of succinylation increased. Unmodified PEI required 10.4 μg DS/μg DNA to completely release DNA from the polyplexes, while 7.8, 5.8, 2.6, 2.6, and 0.9 μg DS/μg DNA were required for zPEI 9, zPEI 14, zPEI 25, zPEI 55 and zPEI 46*, respectively. Polyplexes formed at the optimum transfection weight ratios were disrupted by the addition of 2.2-6.8 μg DS/μg DNA with no clear correlation with the degree of succinylation. zPEI polyplexes formed at optimum transfection ratios contain 2-4 times more polymer than optimized unmodified PEI polyplexes, which may explain the increased amount of DS needed in order to restore DNA migration in zPEI polyplexes compared to unmodified PEI polyplex. Since more polymer is required to form zPEI complexes, more DS is needed to facilitate release.
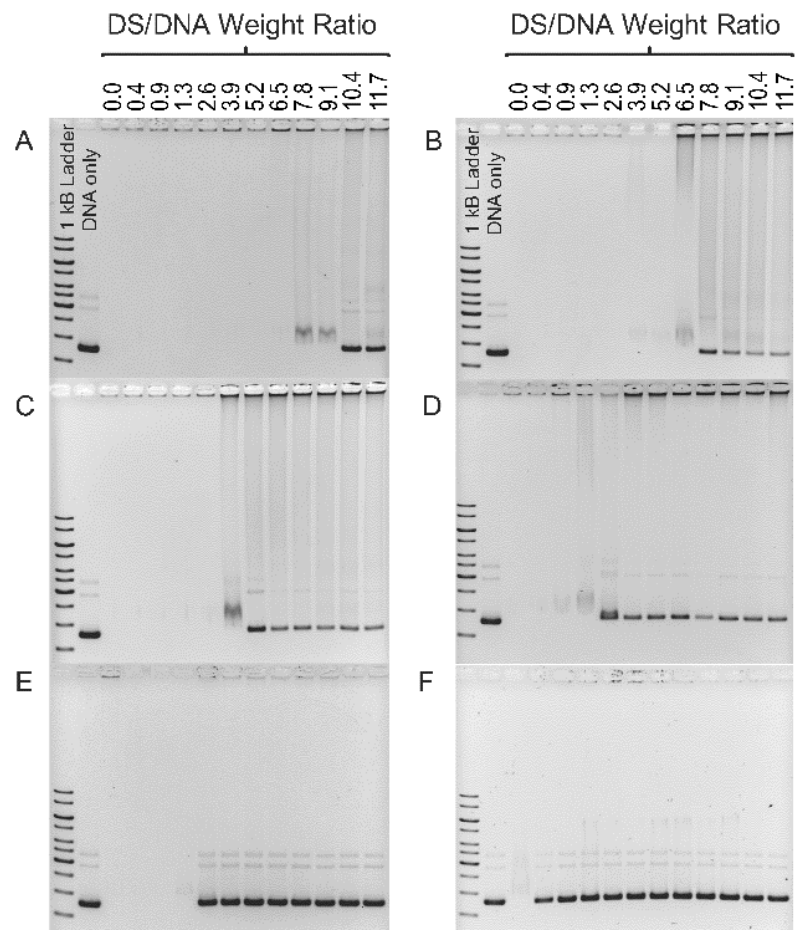
Figure 3.5: Stability of polyplexes in the presence of dextran sulfate (DS) by gel-shift assay. Shown are pUC19 plasmid DNA complexed with (A) unmodified PEI (B) zPEI 9 (C) zPEI 14 (D) zPEI 25 (E) zPEI 55 (F) zPEI 46*. All polyplexes were formed at a polymer/DNA ratio of 6:1 (w:w). DS:DNA weight ratio are as indicated.
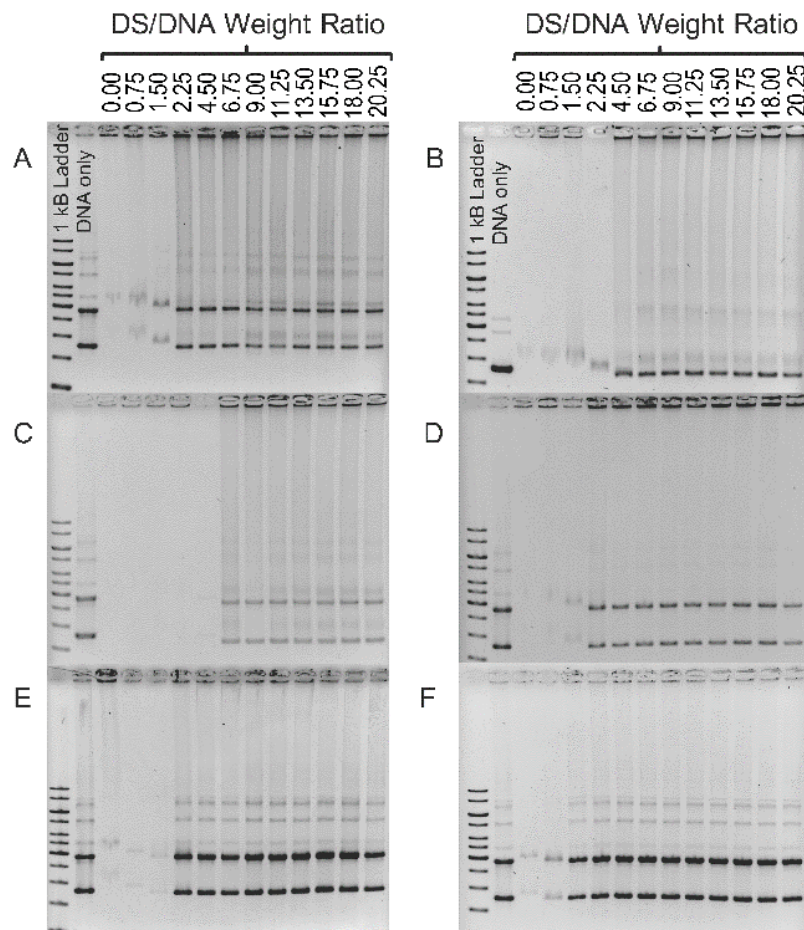
Figure 3.6: Stability of optimized polyplexes in the presence of dextran sulfate by gel-shift assay. Polyplexes were formed using pUC19 plasmid DNA at the optimal polymer/DNA weight ratio for mediating transgene expression in HeLa cells: (A) PEI 25 kDa, 1:1 (w:w); (B) zPEI, 9 2:1 (w:w); (C) zPEI, 14 4:1 (w:w); (D) zPEI, 25 4:1 (w:w); (E) zPEI, 55 4:1 (w:w); (F) zPEI, 46* 4:1 (w:w). The number above each lane signifies the mass of DS per mass of DNA.

3.3.5 Dynamic Light Scattering and ζ-Potential Measurements.

Dynamic light scattering was used to measure the effective diameter of the complexes formed at the polymer/DNA weight ratio that resulted in optimal transfection efficiency in HeLa cells (Table 3.1). In general, the succinylated polymers produced polyplexes with a larger diameter in comparison to unmodified PEI. zPEI 9, 14, and 25 produced complexes ranging from 174-287 nm.

Table 3.1: DLS and ζ-potential of complexes formed at the polymer/DNA weight ratio required for optimal transfection efficiency in HeLa cells (n = 3, error represents standard deviation).

	Polymer:pDNA (w:w)	Complex Diameter (nm)	ζ-Potential (mV)
PEI	1:1	61 ± 10	16 ± 1
zPEI 9	2:1	174 ± 10	15 ± 2
zPEI 14	4:1	287 ± 3	12 ± 2
zPEI 25	4:1	205 ± 3	10 ± 1
zPEI 55	4:1	670 ± 38	12 ± 2
zPEI 46*	4:1	61 ± 10	-1 ± 1

This is not unexpected, as these polyplexes contain 100-300% more polymer than the unmodified PEI polyplexes, which had a diameter of 61 nm. However, zPEI 55/DNA polyplexes were much larger, while zPEI 46*/DNA polyplexes exhibited a peculiarly small diameter at the same polymer/DNA ratio. This is a surprising result. We note that for zPEI 46*, weight ratios of 6:1 were required for complete DNA binding in the gel shift assay (Figure 3.4). This may suggest that zPEI46/DNA polyplexes exist as partially formed colloidal particles at 4:1 in the DLS resulting in an apparent decrease in complex diameter. Both DS competition experiments above show that very little DS is needed to induce unpackaging of zPEI 55 and zPEI 46* polyplexes, further supporting the notion that these complexes experience drastically decreased polymer-DNA interaction.

The ζ-potential of the polyplexes decreased only slightly with increasing succinylation from 15 mV for unmodified PEI/DNA to 10 mV for zPEI 25/DNA. As discussed earlier, the addition of the succinyl group is expected to reduce the charge

density of PEI by both neutralizing a positive charge while simultaneously adding a negative charge. Moderate decreases in ζ-potential were observed up until zPEI 46*, which produced a negatively charged particle, again consistent with this polymer not fully complexing DNA at this polymer/DNA ratio. Since zPEI 46* displays many characteristics, namely poor DNA binding strength and low buffering capacity, that make the polymer ill-suited to condensing and transporting DNA to cells, the polymer was not studied further.

3.3.6 In Vitro Cytotoxicity.

The high cationic charge density of PEI produces electrostatically favorable interactions between the polymer and the negatively charged surface of the cell membrane. However, these same interactions can produce irreversible damage to the cell membrane, causing lysis or necrotic death [157]. The reduced charge density of the succinylated polymers may alleviate these effects. As such, the cytotoxicity of the zPEIs was evaluated in three cell lines: HeLa, human cervical carcinoma; MDA-MB-231, human breast adenocarcinoma; and MC3T3-E1, murine preosteoblast (Figure 3.7). Unmodified PEI exhibited the highest cytotoxicity in all three cell lines. HeLa and MC3T3-E1 cell lines were minimally affected by the presence of zPEI 14-55. zPEI 14 exhibited significant cytotoxicity only at concentrations greater than 35 μg/mL, while zPEI 25-55 produced minimal or no toxicity, even at the highest concentration tested. Severe cytotoxicity was observed in MDA-MB-231 cells with unmodified PEI, but cytotoxicity was reduced with zPEI 9-25 resulting in a 50% or lower relative metabolic activity at 20 μg/mL.
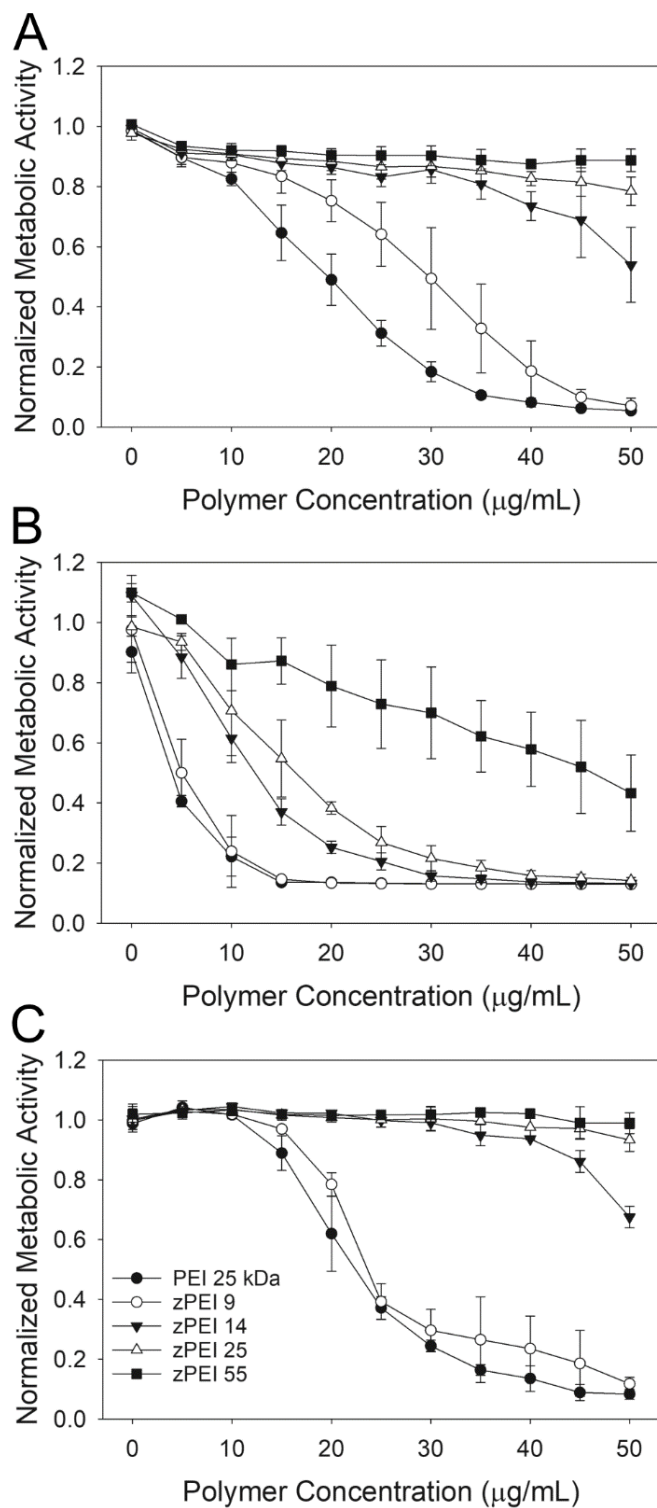
Figure 3.7: Cytotoxicity of unmodified 25 kDa PEI and succinylated PEI derivatives. HeLa (A), MDA-MB-231 (B), and MC3T3-E1 (C) cell lines were exposed to varying concentrations of polymer for 4 h. Metabolic activity was assessed 20 h post introduction of polymer and normalized to the activity of untreated cells (n = 3, error bars represent standard deviation).

3.3.7   Cellular Internalization of Polyplexes.

Electrostatic association of untargeted polyplexes with anionic constituents such as proteoglycan syndecan [96, 167] leads to cell-surface binding and subsequent endocytosis [108]. Cellular internalization of fluorescently labeled zPEI/DNA polyplexes was quantified by flow cytometry in three model cell lines (Figure 3.8). With the exception of zPEI 55/DNA polyplexes in MC3T3-E1 cells, cellular uptake of zPEI/DNA polyplexes decreased compared to unmodified PEI/DNA polyplexes. Since the main pathway into the cell requires non-specific interactions with negatively charged membrane proteins, the reduced uptake may be due to the presence of carboxyl groups on the surface of zPEI/DNA polyplexes, which may generate some electrostatic repulsion despite the relatively small decrease in zeta potential.
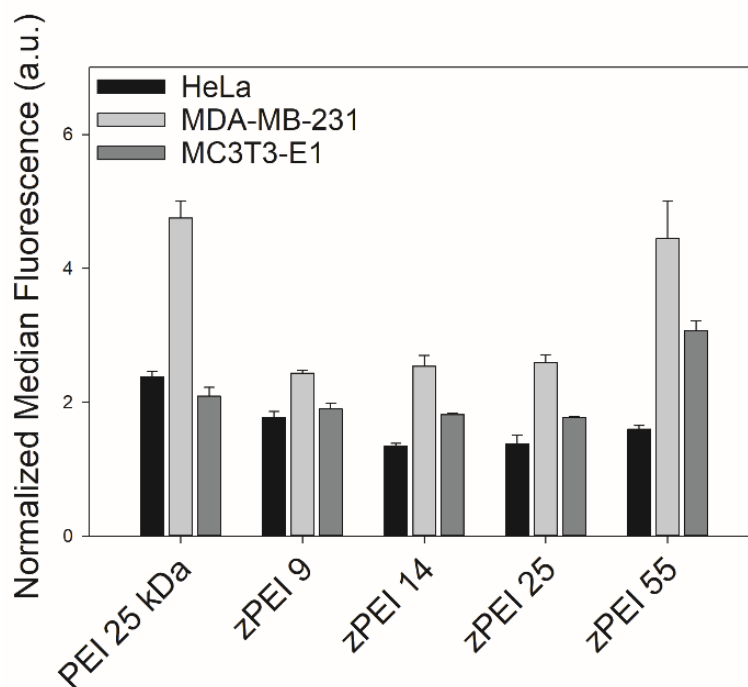
Figure 3.8: Cellular uptake of fluorescently labeled polyplexes. Polyplexes were formed with DNA incubated with the fluorescent intercalator YOYO-1 at the optimal polymer/DNA weight ratio for transfection in HeLa cells: PEI 25 kDa, 2:1 (w:w); zPEI, 9 8:1 (w:w); zPEI, 14 9:1 (w:w); zPEI, 25 9:1 (w:w); zPEI, 55 9:1 (w:w); zPEI, 46 9:1 (w:w). MDA-MB-231 cells: PEI 25 kDa, 1:1 (w:w); zPEI, 9 8:1 (w:w); zPEI, 14 7:1 (w:w); zPEI, 25 8:1 (w:w); zPEI, 55 10:1 (w:w). MC3T3-E1 cells: PEI 25 kDa, 2:1 (w:w); zPEI, 9 6:1 (w:w); zPEI, 14 6:1 (w:w); zPEI, 25 8:1 (w:w); zPEI, 55 6:1 (w:w). Polyplexes were used to transfect cells for 2 h in the presence of serum. Transfected cells were then subjected to FACS analysis to gauge the effect of succinylation on endocytosis. Fluorescence is normalized to the auto-fluorescence of each respective cell line (n=3, error bars represent standard deviation).

3.3.8   In Vitro Transfection Efficiency.

HeLa, MDA-MB-231, and MC3T3-E1 cell lines were transfected with polymer/DNA polyplexes comprising unmodified PEI or zPEI derivatives. In the absence of serum, zPEI 9, 14, and 25 exhibited 2- to 5-fold increased transfection efficiency compared to unmodified PEI, and further succinylation resulted in significantly reduced transfection in all three cell lines (Figure 3.9). While unmodified PEI exhibited maximum efficiency at polymer/DNA ratios 1-2 (w:w), polyplexes formed with succinylated PEI

required higher polymer/DNA ratios to achieve maximum gene expression. Specifically, the largest transgene expression in each of the three cell lines were zPEI 9/DNA at 2:1 (w:w) in HeLa; zPEI 25/DNA at 4:1 (w:w) in MC3T3-E1; and zPEI 14/DNA at 5:1 (w:w) in MDA-MB-231.

An important limitation to using cationic polymers as gene carrier systems is the significant reduction in transfection efficacy in the presence of serum proteins [30]. Complexes formed from cationic materials bind serum proteins through non-specific electrostatic interactions. These interactions can cause aggregation of polyplexes, which greatly hinders cellular uptake and can potentially encourage phagocytosis [91] In order for polymeric vectors to be a viable therapeutic agent in vivo, stability must be maintained in the presence of serum. Thus, transfections in the presence of serum were conducted on all three cell lines (Figure 3.9). Unmodified PEI/DNA polyplexes exhibited a characteristic decrease in transfection efficiency of 10- to 20-fold in all three cell lines compared to transfection in the absence of serum. Remarkably, polyplexes comprising zPEI 9, 14, and 25 exhibited up to 51-, 15-, and 10-fold higher transfection efficiency compared to unmodified PEI, respectively. In fact, the zPEI 9/DNA polyplexes mediated transgene expression in the presence of serum that was comparable to, or even surpassed, that of unmodified PEI/DNA in the absence of serum for each cell line. Interestingly, while the optimal PEI/DNA ratio was the same in serum-free and serum-supplemented transfections, zPEI 9, zPEI 14, and zPEI 25 achieved maximum transgene expression at polymer/DNA ratios of 6-8, 6-9, and 8-9 (w:w), respectively, which were all higher than optimized weight ratios in the absence of serum.
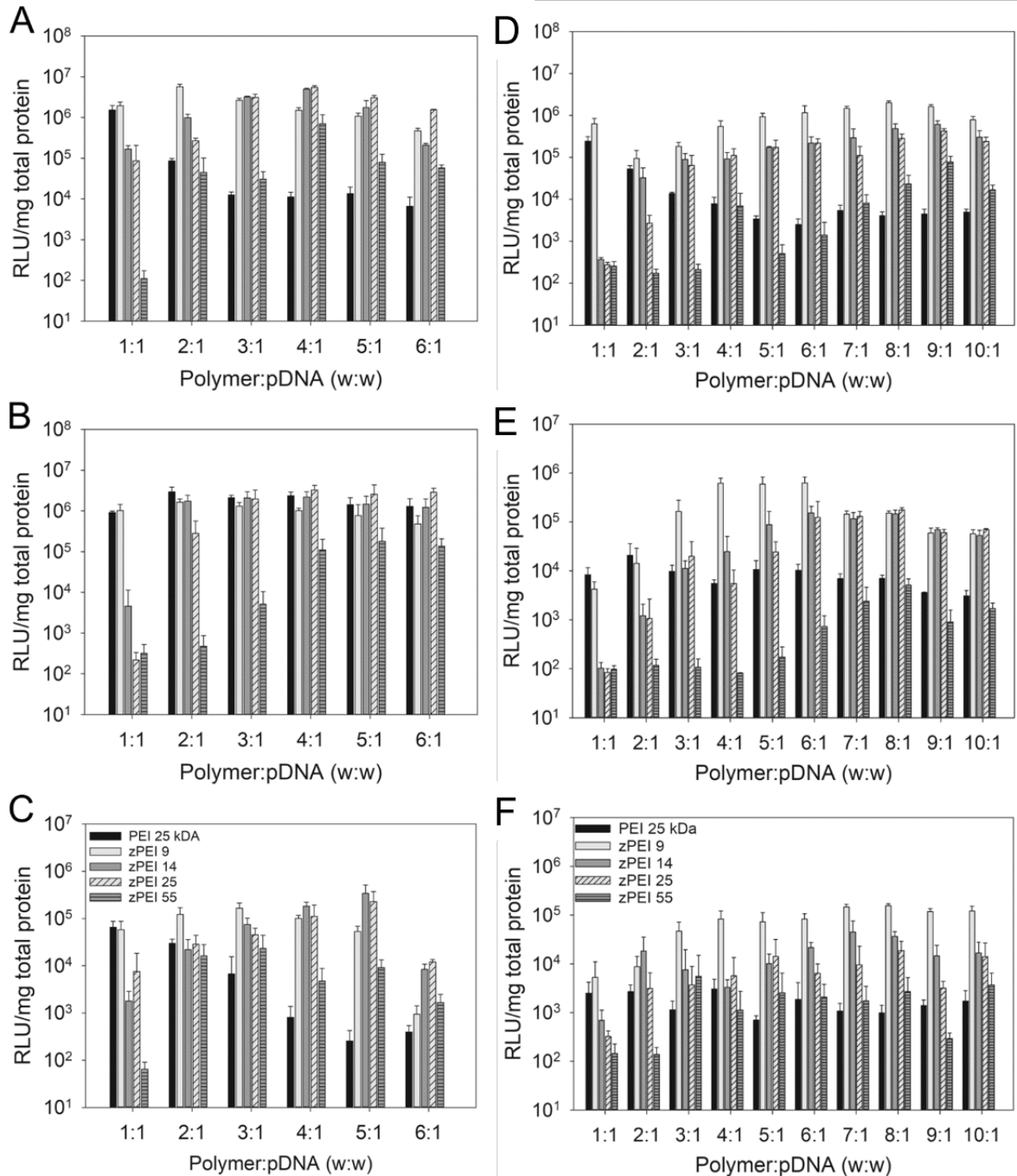
Figure 3.9: In vitro transfection efficiency of polyplexes of plasmid DNA (pGL3) with unmodified or succinylated PEI in (A) HeLa, (B) MC3T3-E1, and (C) MDA-MB-231 cells in the absence of serum and in vitro transfection efficiency of polyplexes of plasmid DNA (pGL3) with unmodified or succinylated PEI in (D) HeLa, (E) MC3T3-E1, and (F) MDA-MB-231 cells in the presence of 10% FBS. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. (n = 3; error bars represent standard deviation.)

3.3.9 Interaction of Polyplexes with Anionic Proteins.

In order to help elucidate the relative stability of the zPEI polymers in the presence of serum proteins, a bovine serum albumin (BSA) adsorption assay was performed for both the uncomplexed polymers and polyplexes formed at the optimum polymer/DNA ratios for transfection (Figure 3.10). Most serum proteins are anionic at physiological pH. BSA has an isoelectric point at 4.7, and thus serves as a model for serum proteins [98]. Polymers and polyplexes were incubated with BSA at 2 mg/mL, which is comparable to anionic protein concentration in growth media containing 10% serum. The succinylated polymers and zPEI/DNA polyplexes exhibited 64-76% and 46-77% decreased interaction, respectively, compared to unmodified PEI.
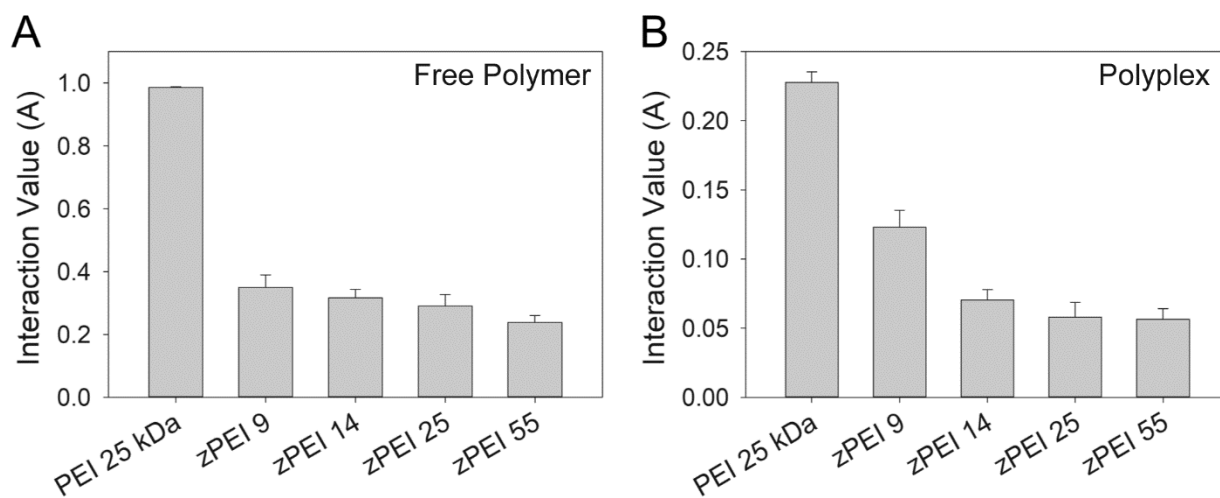
Figure 3.10: Protein interaction values for (A) free polymers at 1 mg/mL and (B) polyplexes at 1.0 µg DNA/mL formed at their respective optimum transfection weight ratios: PEI 25 kDa, 1:1 (w:w); zPEI 9 2:1 (w:w); zPEI 14 4:1 (w:w); zPEI 25 4:1 (w:w); zPEI 55 4:1 (w:w); zPEI 46 4:1 (w:w). Interaction values were expressed as the percentage of protein adsorbed per weight of polymer/polyplex determined through the difference in protein concentration before and after incubation.

3.3.10 Long Term Stability of Polyplexes.

With the increased prevalence of protein engineering, a method that can be used to consistently express certain enzymes over long periods of time is highly desirable. To test the long term stability of complexes comprising zPEI, polyplexes were lyophilized and stored for up to 8 weeks at -20 ˚C. Polyplexes were reconstituted and transfected alongside freshly made polyplexes in serum-supplemented conditions (Figure 3.11). Over the course of the test period, luciferase expression elicited by unmodified PEI complexes decreased nearly three orders of magnitude. Succinylated PEI derivatives performed remarkably better with zPEI 9 and zPEI 14 showing only minor efficiency losses of 3- and 7-fold, respectively. zPEI 25 performed the worst of the zPEIs, losing over an order of magnitude in relative expression, but still performing better than unmodified PEI.
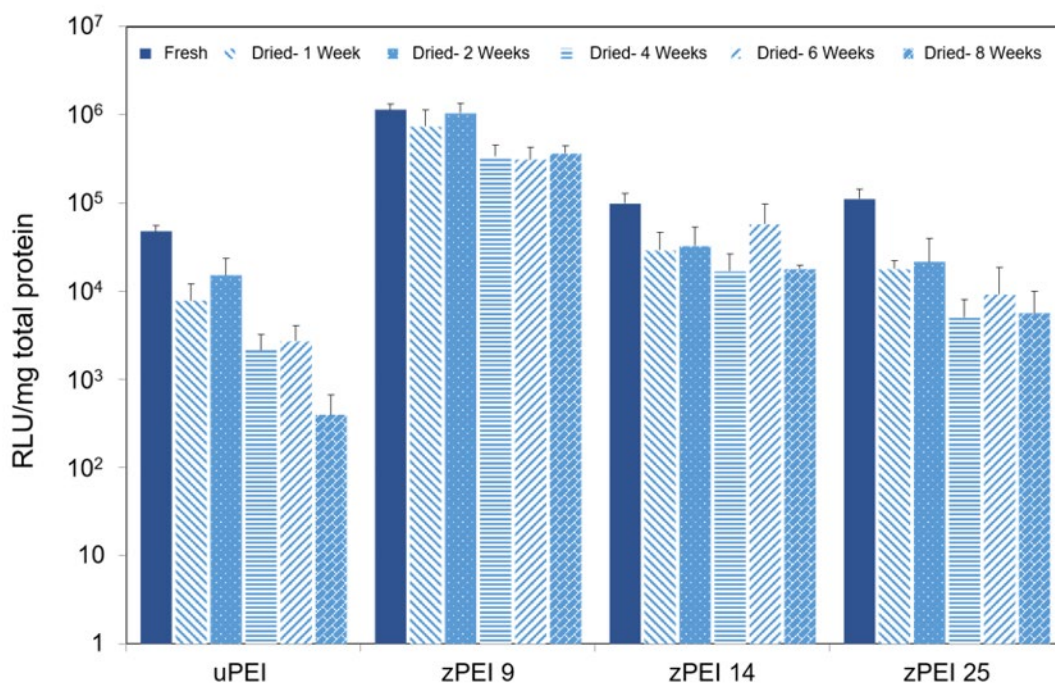
Figure 3.11: In vitro transfection efficiency of lyophilized and freshly prepared polyplexes of plasmid DNA (pGL3) with unmodified or succinylated PEI in HeLa cells in the presence of 10% FBS. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. (n = 3; error bars represent standard deviation.)

3.4   Discussion

Since the discovery and implementation of gene therapy as a viable treatment for genetic diseases, numerous polymers have been created in order to advance non-viral vectors as safe and effective gene delivery agents. Despite this large effort, of the nearly 2000 clinical trials initiated in the U.S. since 1989, only seven have employed polymeric vectors [31]. This is due to the fact that polymeric vectors translate poorly to in vivo environments, mainly in regards to reduced transfection efficacy [168]. PEI, the benchmark for polymeric vectors, produces a relatively high transfection efficiency in serum-free, in vitro conditions. In serum-supplemented conditions mimicking the in vivo environment,

however, PEI/DNA polyplexes aggregate with serum proteins, decreasing the overall transfection efficiency by one to two orders of magnitude.

Strategies to address this issue range from common modifications, such as conjugation of polyethylene glycol (PEG) or other "stealth" polymers [59], to more complex modifications, such as the formation of charge shielding polyanion-PEI-DNA ternary complexes [147] and pH-responsive charge-shifting polymers [162, 169]. Although these modifications reduce toxicity and non-specific protein interactions in comparison to unmodified bPEI, gene delivery and subsequent expression in branched PEI derivatives is typically reduced. Similar modifications of linear forms of PEI, however, have resulted in significantly increased levels of transgene expression [163]. This highlights the complexity of specific barriers to polymeric vectors. Polycations can be modified with PEG or similar groups to decrease non-specific protein binding interactions due to a lower effective charge, but the decreased surface charge of the polyplexes can be detrimental to association with the cellular membrane. Similarly, reducing the number of protonated cationic groups can decrease cytotoxicity, but may hinder escape from endocytic vesicles due to a lower buffering capacity.

By systematically modifying PEI through addition of succinyl groups, we generated zwitterion-like derivatives of PEI (zPEIs) containing 9-55% modified amines, which exhibited a high gene delivery activity while enhancing stability in the presence of serum proteins. With conversion of primary and secondary amines to secondary and tertiary amides, respectively, and the addition of a carboxyl group for every modified amine, the modified polymers exhibited a decreased buffering capacity in the biologically relevant pH range (Figure 3.3).

It is thought that cationic polymers facilitate escape from endocytic vesicles through osmotic swelling via the proton-sponge mechanism in which polymer buffering capacity is correlated with endocytic escape and gene delivery activity [64]. However, the effect of polymer buffering capacity on gene delivery efficiency has been inconsistent [170, 171]. For example, poly-L-lysine and beta-cyclodextrin-containing polymers have been found to be unable to buffer endosomes and result in low levels of gene delivery, but when paired with an endosomotropic agent such as chloroquine, transfection efficiency increased 2-3 fold. Conversely, poly(2-methyl-acrylic acid 2- [(2-(dimethylamino)-ethyl)-methyl-amino]-ethyl ester), a polymer specifically designed to exhibit high buffering capacity, provides poor gene delivery [171]. Thus, minor decreases in buffering capacity exhibited by the moderately succinylated polymers (zPEI 9-25) may be expected to negligibly impact gene delivery.

The introduction of succinyl groups was found to decrease the strength of electrostatic interactions between the plasmid and polymer. More polymer was required to completely complex DNA as succinylation increased (Figure 3.4). Furthermore, the reduced cationic charge density and introduction of negative charges reduced the binding strength between zPEIs and DNA (Figure 3.5). It is therefore plausible that anionic proteins or other biomolecules could displace the polymer from DNA more easily inside the cell.

The sizes of zPEI polyplexes formed from low to moderately succinylated PEI ranged from 174-287 nm, falling within acceptable range for non-receptor-mediated endocytosis [172]. The increase in size with increasing succinylation is most likely due to the decreased electrostatic interactions, producing a less condensed polyplex. In addition, the ζ-potential of the polyplexes remained positive but decreased slightly as succinylation

increased. Since only a fraction of the amines of PEI are succinylated, the introduction of negative charges and conversion of protonatable amines to amides do not completely negate the cationic charge of the PEI backbone. Hence, these derivatives are termed zwitterion-like since there still exists a net positive charge, but negatively charged moieties are contained within the branched network.

Polymers containing both positive and negative charges on the same monomer unit have been studied extensively for biomedical applications [160]. Biomaterials have long used the principal of surface hydration to prevent protein adsorption to implants and biosensors. Brash et al. showed that hydroxyl terminated PEG produced substantial increases in resistance to nonspecific protein binding [173]. It was hypothesized that water molecules were bound via hydrogen bonds to the terminal hydroxyl and ether oxygen groups, which formed a physical and energetic barrier to protein adsorption [174]. Later, it was discovered that polyzwitterions can achieve a similar effect through a stronger electrostatically induced hydration layer [161]. The zwitterion-like derivatives of PEI showed a significantly reduced affinity for non-specific protein binding (Figure 3.10). It is possible that the carboxyl terminal groups of succinate induce a hydration layer that provides protection from serum proteins. However, similar to PEG derivatives, when the degree of succinylation is increased, as in the case of zPEI 55 and zPEI 46*, it appears that the reduced interactions, whether from electrostatic repulsion or physical protection via hydrated branches, becomes detrimental to the ability of the polymer to associate with the cellular membrane, leading to reduced cellular uptake (Figure 3.8) and, ultimately, lower gene delivery activity.

Transfections showed moderate increases in efficiency for zPEI 9, zPEI 14, and zPEI 25 in all three model cell lines in the absence of serum (Figure 3.9). Zintchenko et al. reported similar results for the delivery of siRNA using modified PEI polymers, two of which were succinylated PEI derivatives [165]. In the present work, we show that larger degrees of succinylation on PEI appeared to reduce non-specific protein interaction and toxicity, however, DNA delivery decreases. Succinylation also appears to add some degree of stability to the polyplexes whenever they are dried and stored for long periods of time. PEI complexes traditionally do not respond well to freeze drying, due to minute changes in the condensed structure [175]. Where unmodified PEI exhibited three orders of magnitude loss in expression over eight weeks, zPEI 9 only observed a 3-fold decrease. Most surprisingly, however, transfections in serum-supplemented conditions (Figure 3.9) show the zPEI polyplexes mediated transgene expression to nearly the same degree as transfections performed in the absence of serum and up to 51-fold higher than unmodified PEI. We hypothesize this improved transfection efficacy in serum is a result of a hydration layer on the polyplex particle surface arising from the polyampholytic nature of the zPEI.

3.5   Conclusions

Succinylation of PEI produces a more efficient polymeric gene delivery vector in the presence of serum compared to unmodified PEI, likely due to increased serum stability. Introduction of negative charges and neutralization of primary and secondary amines led to a decrease in the strength of polymer/DNA interactions and buffering capacity. The reduction in binding strength resulted in larger polyplexes that dissociated more readily in the presence of a competing polyanion. Nonspecific protein binding was significantly

reduced in comparison to unmodified PEI, correlating with greater levels of transgene expression upon transfection in the presence of serum. It is possible that induction of a hydration barrier provides increased protection to the zwitterion-like polyplexes in addition to electrostatic shielding. However, it is evident from the higher degrees of modification that an optimal balance must be struck between shielding particles from proteins while maintaining favorable membrane interactions.

CHAPTER 4. APPLICATIONS OF NON-VIRAL GENE THERAPY

While recent advances have presented viable solutions to barriers that have prevented past success *in vivo*, the reality is that there are instances where viral vectors will most likely always remain superior, and similarly, there will be situations when polymeric vectors are the preferred option. Here we will discuss a few instances where polymeric vectors have displayed promising results in both *in-vivo* and *ex-vivo* applications.

4.1   In-Vivo Applications

4.1.1 Ocular

Nearly 300 million people worldwide suffer from severe low vision or complete blindness, with the majority stemming from genetic indications [176]. The difficulty with non-invasive delivery of nucleic acids to the eye is mainly due to two obstacles; the tear-film and tear drain [177]. Small changes in pH and detection of foreign material can cause tears to form and rapidly clear both viral and non-viral vectors from the eye, leaving less than 3% of the original dose. Fortunately, many polymers used in polymeric gene delivery inherently have bioadhesive properties that allow adherence to the tissue for increased residence time [178]. The second large barrier vectors have to navigate is the vitreous layer. This layer houses many anionic species such as glycosaminoglycans and collagen fibers, which can greatly restrict the movement of vectors due to electrostatic association. Ternary particles, such as pDNA/PEI/polyglutamic acid that have decreased cationic charge have displayed increased transfection efficiency compared to typical polyplexes [179]. The increased retention and charge shielding paired with the ability to carry large nucleic acids has led to recent success for a few polymeric nanoparticles. Normally, the gene needed to

correct Stargardt disease, ABCA4, is too large for conventional viral vectors, but can be condensed with relative ease using a PLL-PEG derivative. The polyplexes were delivered to ABCA4-deficient mice, and subsequent expression of the gene was observed for eight months post administration [180].

4.1.2 Pulmonary

Where ocular therapies demonstrate the advantageous carrying capacity of polymeric vectors, pulmonary gene therapy applications illustrates efficient targeting. Traditionally, pulmonary diseases have been targeted through intravenous injections to the lung tissue to circumvent the mucosal membrane, however the injection allows a significant population of vectors to enter circulation and potentially target tissues elsewhere in the body [177]. Polymeric vectors, like the EHDS particles, can be delivered by inhalation and decrease off-site targeting that accompanies injection. Successful delivery via inhalation requires transport through the mucus membrane to respiratory epithelial cells. To increase mucosal penetration, researchers have developed numerous methods. Two particularly successful strategies involve conjugating nonionic or anionic species to the cationic complex or coating the complex with a nonionic or anionic layer [181, 182]. The added moieties aid in shielding the polyplexes from anionic glycoproteins and increasing lipophilicity to allow vectors to access hydrophobic regions that contain pores that lead to increased delivery [183, 184]. PEGylated polyplexes carrying a gene encoding for thymulin, an anti-inflammatory compound shown to prevent airway remodeling during asthma incidents, were delivered to mice via a microsprayer [185]. Thymulin was detected for up to 27 days post administration, reduced lung inflammation, and prevented airway

obstruction during allergy-induced asthma incidents. A similar polyplex was administered to human cystic fibrosis patients that carried the cystic fibrosis transmembrane conductance regulator gene [186]. Of the 12 patients that received intranasal doses of polyplexes, eight had significant restoration in chloride channel function, which is disrupted by the mutation causing thick mucus secretions. Evidence of expression was observed for up to 28 days after administration in one patient, with the majority of corrections subsiding after six days.

4.1.3 Genetic Engineering for Diabetes Correction

Aside from correcting specific genetic mutations, gene therapy can also extend to producing proteins and biomolecules that restore functions such as glucose processing in diabetic patients. Both Type I and Type II diabetes have shown some progress in regards to non-viral gene therapy [187]. In Type I diabetes mellitus the cells responsible for producing insulin are incorrectly targeted by the host immune cells and destroyed, preventing glucose processing and leading to chronic hyperglycemia. The current method of treatment involves either complete organ transplant or transplant of islets. However, gene therapy provides a potential alternative by enabling insulin production in other cells besides pancreatic β cells [188]. In mice, a plasmid encoding for the human insulin gene was condensed with chitosan and subsequently injected into the peritoneum. Fasting blood glucose levels of transfected mice were significantly lower for four days post transfection indicating that insulin produced in locations other than the pancreas can have systemic effects.

In Type II diabetes, cells become resistant to insulin and thus cannot process glucose, leading to high blood sugar levels. Adiponectin is an adipocytokine that can regulate blood glucose levels similarly to insulin [189]. When Type II diabetic mice were injected with PEI complexes containing a gene encoding for adiponectin, sufficient expression was achieved to induce bioactivity for nine days [190]. The adiponectin-expressing mice showed improved ability to regulate blood glucose levels, similar to control mice that had no insulin resistance.

4.2   *Ex-Vivo* Applications

With the prominence of CRISPR/Cas9 and other gene editing technologies, the area of *ex-vivo* modification by non-viral vectors has vast potential. Where viruses can be time-intensive and expensive to produce and have an upper size limit to genetic cargo, polymers are relatively easy to produce and are inexpensive compared to viral reagents, and the time to prepare polyplexes is negligible in comparison to the time it takes to develop viral particles [191]. A co-transfection of two plasmids, one encoding for a cassette that expresses a guide RNA that targets a specific location on the genome with an editing enzyme such as Cas9 or transposase and one plasmid to encode for the genetic insert, can be packaged into one polyplex and delivered to cells. The transient expression of the editing enzyme is more desirable over stable expression obtained with viral transduction due to elimination of future possibility of genome excision. This concept has been demonstrated on human T cells for manufacturing specific CAR constructs using PEI derivatives [191]. The concept of modifying cells with the intent to re-implant for systemic therapeutic activity is an enticing notion due to the inherent simplicity of the transfections. It is easy to envision this methodology greatly expanding to include therapies other than CAR T-cell

manufacturing, such as implantation of human insulin-modified cells [192]. As shelf-stable polyplexes become more common, such as the one discussed in chapter 3, the access to genome editing machinery is becoming more readily available each year.

4.3   Curcuminoids

This leads us to the final concept explored within this work. What if cells can be programmed to produce their own supply of therapeutic compounds? Metabolic engineering and synthetic biology have made large contributions to isolating biosynthesis clusters involved in producing the compounds commonly explored as drugs. Many of these compounds naturally occur in plants, however they are usually expressed in small quantities and can be relatively difficult to cultivate [193]. Thus, researchers have moved enzyme clusters that result in the production of compounds of interest such as resveratrol, stilbenoids, and flavonoids to microorganisms such as bacteria to increase available supply [194]. The group of molecules called curcuminoids have recently undergone this transition. Curcuminoids have long been used throughout traditional medicine in the form of the common culinary spice, turmeric [195]. The vivid orange molecule, and two of its analogs, demethoxycurcumin and bisdemethoxycurcumin, are commonly used as food additives and coloring agents. The compounds are considered safe for ingestion by the FDA and can be consumed in upwards of 3 g daily with no adverse effects. The reason that curcuminoids have garnered so much attention in recent years is due to the expansive list of purported biological activities [196]. Curcuminoids have been shown to be potent antioxidants, readily scavenging radicals due to the central diketone and terminal phenol moieties [197-200]. Curcuminoids also exhibit anticancer and anti-inflammatory properties through inhibition of nuclear factor-κB and pro-inflammatory cytokines COX-2, iNOS, and TNF-

α [201, 202]. As curcuminoids are studied in more depth, the diversity of promising benefits to pathologies continues to grow. Fatty liver disease, Alzheimer's disease, and bacterial, fungal, and viral infections have been corroborated in practice to be impacted by the golden spice component [203-206]. However, despite the extensive list of benefits, clinical success of curcumin has been underwhelming, primarily due to poor oral bioavailability (<1%), rapid systemic clearance, low aqueous solubility, and degradation in physiological pH [207].

Many of the traditional encapsulation strategies for hydrophobic chemicals have been applied to curcumin in hopes to increase bioavailability, including polyelectrolyte, cyclodextrin, silica, casein, and chitosan nanoparticles [208-213] Liposomes, micelles, and phospholipids have also been deployed with some success, with some studies showing increased absorption and stability in comparison to the more traditional methods [214-218]. More recently, phytosomes have been utilized as a specialized system for herbal phytochemical delivery; again, these formulations have been successful in comparison to the free-form version of curcumin, but it is obvious that there is definitive need for modern solutions in order for this ancient drug to realize clinical potential [219-223].

4.4   Biosynthesis of Curcuminoids

Curcumin (CCM), and its analogs demethoxycurcumin (dCCM), and bisdemethoxycurcumin (ddCCM), are naturally occurring compounds extracted from the rhizome of the *Curcuma longa* plant. As discussed previously, extraction from plants can create supply problems, limiting the availability. Fortunately, Ramirez et al. isolated the enzymes responsible for curcuminoid synthesis and established the connection of synthesis

to the phenylpropanoid pathway [224]. Phenylalanine ammonia lyase was identified first, which indicated that synthesis likely proceeded under a cinnamic acid and diketide synthesis pathway. A p-coumaroyl transferase and cafeoyl-CoA methyltransferase were identified, as well as polyketide synthases. However, it was not until later that the distinction would be made between the various polyketide synthases: curcuminoid synthase (CUS) and curcumin synthase (CURS) [225]. Once a pathway was identified, advances in metabolic engineering have enabled researchers to move the biosynthesis clusters to microbial systems with relative ease [206, 226-229]. One of the first instances of microbial curcuminoid production involved the pathway reported by Ramirez as well as two new polyketide synthases, diketide-CoA synthase and CURS1 [230]. Results showed that the new polyketide synthases preferentially bind to the feruloyl-CoA structure, catalyzing the synthesis of curcumin over that of its analogs. This sparked a surge of studies exploring various polyketide synthases and how each can effect total production as well as selective preference for individual curcuminoid production [231]. Since then, many groups have tried to optimize each step of curcumin synthesis, which has resulted in the development of numerous biosynthetic pathways. Strategies include shifting the starting substrate from phenylalanine to the more soluble tyrosine and glucose, increasing the pool of the linking substrate malonyl-CoA, engineering new polyketide synthases, supplementing with cinnamic acid intermediates, as well as engineering specific strains of microorganisms [232]. Until now, curcuminoids have been produced only in strains of *E. Coli* and *Aspergillus oryzae*.

CHAPTER 5. GENETICALLY   ENGINEERING   HUMAN   CELLS   FOR
HETEROLOGOUS PRODUCTION OF CURCUMINOIDS

5.1   Introduction

The majority of medicinal compounds throughout medical history has primarily relied on naturally occurring therapeutic molecules isolated from flora [193]. Cultivating the plants that produce the compounds of interest can take a significant amount of time, and some compounds are only found in trace amounts in very specific sections of the plant limiting the availability of the therapeutic [193]. Advancements in chemistry have allowed researchers to make active pharmaceutical ingredients (APIs) through total chemical synthesis, decreasing both the time needed to acquire the API and increasing the yield [233]. However, some APIs, such as the chemotherapy drug paclitaxel, require a complex and difficult synthesis that can exceed 20 individual steps [234]. This can make industrial-scale production economically unfeasible.

More recently, metabolic engineering has emerged as an alternative to total chemical synthesis. Researchers can move genes responsible for the biosynthesis of therapeutic molecules in plants to various microorganisms, such as yeast and bacteria, to increase yield and decrease cost [235]. For example, paclitaxel has been successfully synthesized in bacteria, yeast, and other fungal systems [236]. Biosynthetic engineering of APIs has been limited to these simple microbial systems. That is because problems arise when attempting to express complex protein systems between prokaryotes, plants and animals, mainly due to differences in transcription, protein folding, and post-translational modifications, which can prevent enzyme expression, decrease bioactivity, or severely

reduce the half-life of enzymes responsible for synthesis [237-240]. These reasons are largely why researchers have not been successful, or have even attempted, expressing entire plant biosynthesis clusters responsible for these important biomolecules in mammalian cells. If these limitations can be overcome, it may be possible to engineer human cells to make their own APIs.

In this study we employ a gene cluster previously developed for biosynthesis of curcumin from tyrosine and cinnamic acids in *E. Coli* (Figure 5.1) [232]. Genes encoding for each enzyme involved in the pathway were synthesized and cloned into mammalian expression vectors and delivered to HEK293 cells through the use of the succinylated PEI derivatives described in Chapter 3 [72]. Production of curcuminoids was assessed via high performance liquid chromatography as well as mass spectrometry. To our knowledge, this is the first time a natural product has been synthesized in mammalian cells. Although curcuminoids are the focus of the present study, the biosynthesis cluster of curcuminoids are similar to many plant-derived biomolecules, such as stilbenoids, resveratrol, and flavonoids. Thus, we envision that this strategy could be extended to other compounds of interest [241]. We believe this study may represent the first step towards a fundamental shift in how diseases are treated, focusing not on the external delivery of therapeutic compounds, but instead engineering patients' bodies to produce their own supply of drugs.

5.2 Materials and Methods

5.2.1 Cell Culture and Plasmid Construction.

HEK293 human embryonic kidney cells were purchased from the American Type Culture Collection. Cells were cultured in DMEM (ThermoFisher Scientific, Waltham, MA) at 37 °C, 5% CO_2, and supplemented with 10% fetal bovine serum per ATCC recommendations. Genes encoding for the curcuminoid biosynthesis cluster (Figure 5.1) were developed from the pathway reported by Rodrigues et. al. using publicly published sequences from GenBank (NCBI, Bethesda, MD) [232]. Tyrosine-ammonia lyase (TAL-GenBank MG712805), 4-coumarate-coenzyme A ligase (4CL1- GenBank U18675), 4-coumarate 3-hydroxylase (C3H- GenBank HE804045), caffeoyl-CoA 3-o-methyltransferase (CCoAMT- GenBank JN849911), bisdemethoxycurcumin synthase (CUS GenBank- AK109558), diketide-CoA synthase 1 (DCS GenBank- KM880191), and curcumin synthase 1 (CURS1 GenBank- AB495007) were individually synthesized and cloned into pcDNA3.1(+) mammalian expression vectors by GenScript (Piscataway, NJ, USA).

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Figure 5.1: Proposed curcuminoid biosynthetic pathway. P-coumaric acid is synthesized from tyrosine by tyrosine ammonia lyase (TAL). From here, the pathway diverges to either favor production of bisdemethoxycurcumin or curcumin. For bisdemethoxycurcumin, 4-coumarate-CoA ligase (4CL1) converts p-coumaric acid to p-coumaroyl-CoA, while 4-coumarate 3-hydroxylase (C3H) and caffeoyl-CoA 3- O-methyltransferase (CCoAMT) converts to feruloyl-CoA. The cinnamic acid-CoA structures are then converted to diketide-CoAs by condensation with malonyl-CoA via curcuminoid synthase (CUS) and diketide-CoA synthase (DCS). Finally, curcuminoids are synthesized by condensing the diketide-CoAs with cinnamic acid-CoAs through CUS and curcumin synthase (CURS1).

5.2.2 Polymer Mediated Transfections.

HEK293 cells were seeded in 100-mm plates at 2.0×10^6 cells/plate 24 h prior to transfection. Polymer/DNA complexes were formed by first diluting 200 μL of 0.1 μg/μL DNA solution with 800 μL of PBS in a 15-mL centrifuge tube. An equi-volume solution of a zPEI 9 (Chapter 3) of 0.12 μg/μL added dropwise to the DNA solution under constant agitation to achieve the desired optimum polymer:DNA weight ratio reported in our previous work [72]. Particles were allowed to incubate at room temperature for 20 minutes. Immediately before transfection, 2 mL polyplex solution was deposited into 8 mL of fresh serum-supplemented media. Conditioned growth medium was aspirated from cells and replaced with 10 mL of polyplex/growth medium solution (20 μg DNA/plate). After 4 h, the transfection medium was replaced with serum-supplemented growth medium devoid of phenol red in order to visually aid observation of curcuminoid production.

5.2.3 Curcuminoid Extraction.

To quantify the amount of curcuminoid produced from transfected cells, the conditioned media was collected 48 h post transfection and adjusted to pH 3 using 6 M HCL. An equal volume of ethyl acetate was added to pH-adjusted media and placed under constant agitation for 10 min. The resulting mixture was centrifuged to separate the organic and aqueous layers. The extracts were concentrated using a roto-vap, resuspended in 300 μL of acetonitrile and then subjected to either high-performance liquid chromatography or mass spectrometry.

5.2.4 HPLC and LC/MS Analysis of Products.

Analysis of curcuminoids were conducted on an Agilent 1200 HPLC system with an Agilent ZORBAX Eclipse Plus C18 column (4.6x150 mm, 5 μm). The samples were eluted with a gradient of acetonitrile–water from 40:60 v/v to 100:0 v/v over 16 min at a flow rate of 1 mL/min. Curcuminoids were detected at 425 nm, and concentrations were calculated based on standard curves of analytical grade compounds purchased from Sigma Aldrich (St. Louis, MO). The mean values from parallel experiments are reported. LC-MS analysis was conducted on an Agilent 1200 LC system coupled to an Agilent 6410 QQQ mass spectrometer in the positive mode using the same separatory column and gradient as the HPLC protocol described above.

5.2.5 Fluorescently Activated Cell Sorting.

To determine the percentage of cells actively producing curcuminoids, 48 h post-transfection HEK293 cells were rinsed twice with 0.001% SDS in PBS and PBS, respectively. Next, 250 μL of 0.05% trypsin in PBS was added to each well. The cells and trypsin were allowed to incubate for 5 min before 50 μL of FBS was added to each well. The cells were then collected and centrifuged at 6400 rpm for 5 min. The resulting supernatant was discarded and the cells were resuspended in 1 mL of fresh PBS. Samples were transferred into 15 mL polystyrene tubes and sealed. The polystyrene tubes were placed in a sealed container and then transported in a secondary container to an iCyt-Sony Cell Sorter System, where FACS analysis was performed. Data was processed using FloJo software (FlowJo LLC., Ashland, OR).

5.3 Results

5.3.1 Curcuminoid Production.

The main goal of this study was to produce three curcuminoids from naturally occurring tyrosine through polymer-mediated transfections. To test the feasibility of producing curcuminoids in mammalian cells, HEK293 cells were transfected with plasmid cocktails encoding the simplest pathways of the biosynthesis cluster: 4CL1/CUS, which converts p-coumaric acid to ddCCM, and 4CL1/DCS/CUS, which converts ferulic acid or ferulic and p-coumaric acids` to CCM and dCCM, respectively. The cells were allowed to grow for 48 h in media supplemented with the cinnamic acids, after which the conditioned media was extracted with ethyl acetate (Figure 5.2).
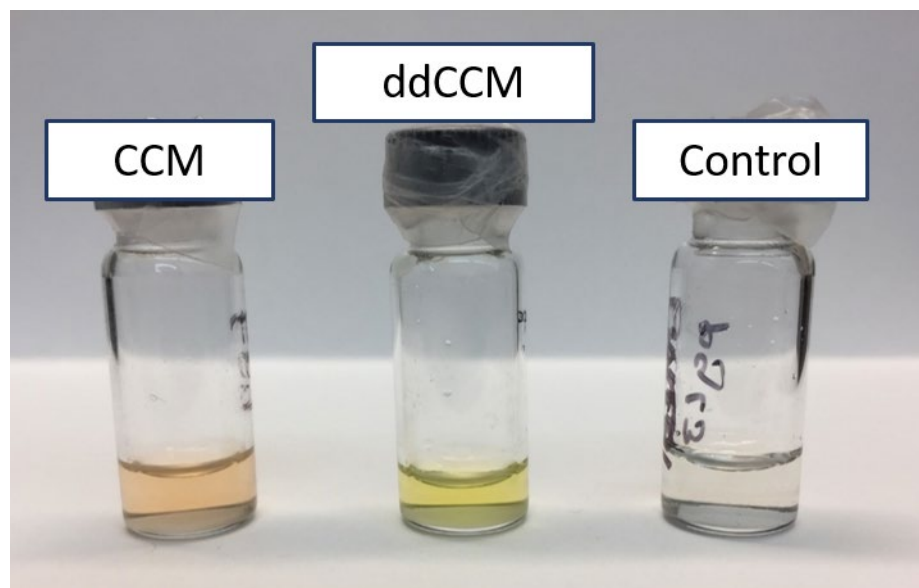
Figure 5.2: Ethyl acetate extractions from conditioned media collected 48 h post transfection with 4CL1/DCS/CURS1 for CUS, 4CL1/CUS for ddCCM, and pGL3 for Control. The yellow and orange pigmentation visually suggests the presence of curcuminoids.

HPLC confirmed the presence of the desired curcuminoids in the extracts (Figure 5.3). In addition, mass spectrometry of the extracts showed mass peaks of 309, 339, and 369, corresponding to ddCCM, dCCM, and CCM, respectively. Dose-dependent conversion of the cinnaimic acids to curcuminoids was confirmed by supplementing the growth media with 0.05, 0.15, and 0.30 mM p-coumaric acid or ferulic acid, which are similar to concentrations previously employed in bacterial fermentation [242, 243]. Of the three concentrations, 0.30 mM p-coumaric acid resulted in the highest ddCCM concentration (0.19 µM) and 0.05 mM ferulic acid resulted in the highest concentration of CCM (0.20 µM) (Figure 5.4). The two optimal doses of each respective cinnamic acid were used as supplementation for dCCM production, resulting in a concentration of 0.17 µM.
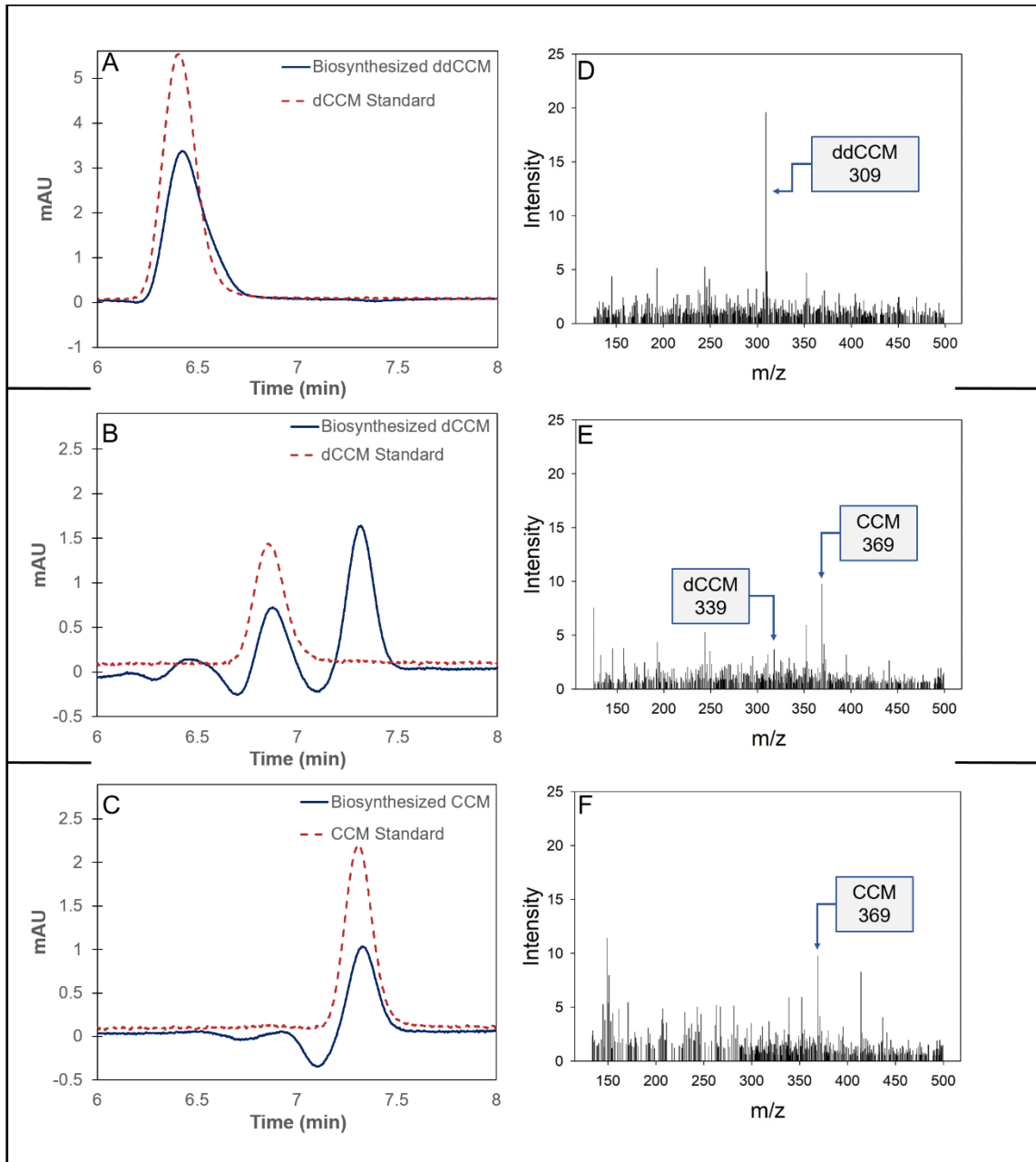
Figure 5.3: HPLC analysis of biosynthesized curcuminoids compared to analytical standards, and the corresponding MS spectra verifying the molecular weight of ddCCM (A, D), dCCM (B, E), and CCM (C, F). Cells were transfected with (A) 4CL1/CUS or (B, C) 4CL1/DCS/CURS1, and cell media was supplemented with the 0.30 mM of p-coumaric acid for ddCCM, 0.30 mM of p-coumaric acid and 0.05 mM ferulic acid for dCCM, and 0.05 mM ferulic acid for CCM for 48 h. Curcuminoids were extracted from the conditioned media 48 h post-transfection and subjected to HPLC and LC/MS analysis.
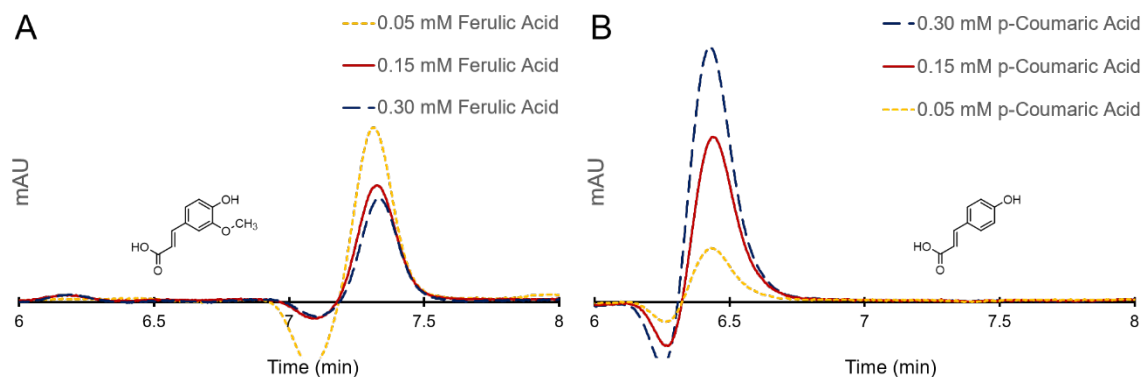
Figure 5.4: Dose response of supplementation of (A) ferulic acid and (B) p-coumaric acid on curcumin and bisdemethoxycurcumin production, respectively. Intermediate cinnamic acids where supplemented in the regular growth media of HEK293 cell post transfection at 0.05, 0.15 and 0.30 mM. Curcuminoids were extracted from the conditioned media 48 h post-transfection and subjected to HPLC and LC/MS analysis. Spectra shown is average of parallel experiments.

Once each of the three main curcuminoids had been successfully produced from the corresponding cinnamic acids, we introduced additional enzymes to allow production of each curcuminoid from endogenous tyrosine. Bisdemethoxycurcumin was produced from tyrosine (termed TddCCM) by transfecting cells with TAL, 4CL1, and CUS, while demethoxycurcumin (TdCCM) and curcumin (TCCM) were produced from tyrosine by transfecting cells with all six enzymes: TAL, 4CL1, C3H, CCoAMT, DCS, and CURS1. Curcuminoids are highly fluorescent and can be observed under FITC channels in flow cytometry, allowing for quantification of the number of cells successfully producing product (Figure 5.5). As the number of enzymes, and therefore plasmids, increased from three for TddCCM to six for TCCM, the fraction of cells producing curcuminoids decreased from 2% to nearly 0.02%.

Designated Letter	Plasmid	Percent Expressing
A	None (Control)	0.00%
B	GFP (Control)	13.54%
C	TddCCM	1.95%
D	ddCCM	2.32%
E	dCCM	0.04%
F	CCM	0.79%
G	TCCM	0.02%
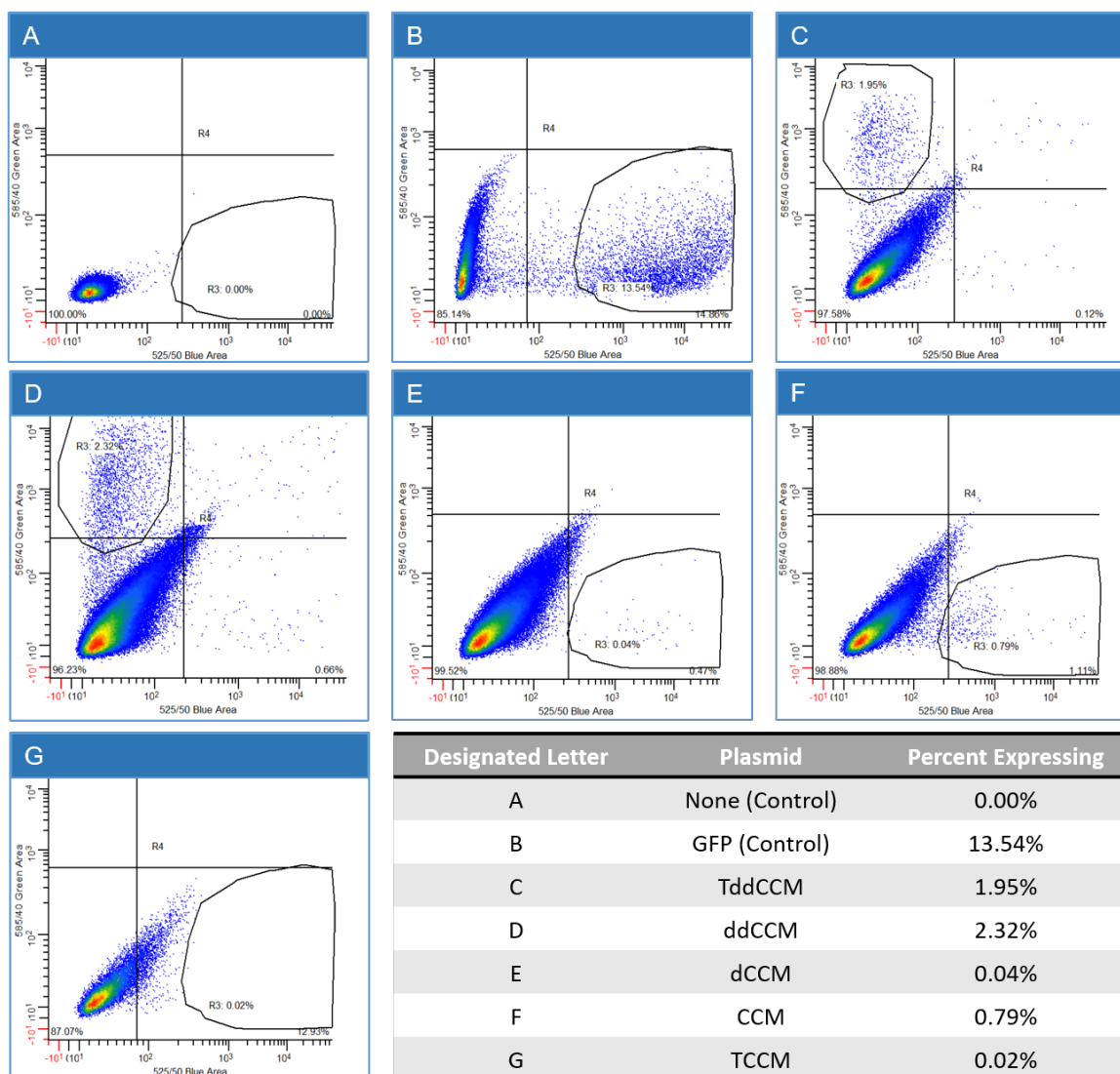
Figure 5.5: Fluorescently activated cell sorting of curcuminoid producing HEK293 cells. Cells were collected 48 h post transfection and sorted using FITC (495 nm/519 nm ex/em) channel on a iCyt-Sony Cell Sorter System. Reported percentages are the percent of cells expressing curcuminoids as determined by fluorescent intensity. Sorting population has been gated to exclude cell debris.

In the experiments described above, plasmid cocktails contained equal masses of plasmids encoding each enzyme. Thus, we investigated the effects of relative enzyme expression levels by transfecting HEK293 cells with the plasmid cocktails described above while varying the mass of a single plasmid at 0.5, 1.0, and 2.0 (w/w) relative to the other

plasmids (Figure 5.6). In general, pathways that depend on intermediate supplementation for curcuminoid production (4CL1/CUS and 4CL1/DCS/CURS1) produced the most curcuminoids when the plasmids were present in equivalent ratios. However, differing plasmid ratios had a noticeable effect on production of TddCCM (TAL/4CL1/CUS) and TCCM (TAL/4CL1/C3H/CCoAMT/DCS/CURS1). Higher quantities of curcuminoids were produced when the TAL-encoding plasmid was present at the lower ratio, and the CCoAMT-encoding plasmid was present at the higher ratio. Although, this is an elementary examination of relative expression and rate-limiting steps, it suggests that there may be benefit in expressing certain enzymes in higher or lower relative ratios, which will be paramount when moving from a transient expression system to a stable system.
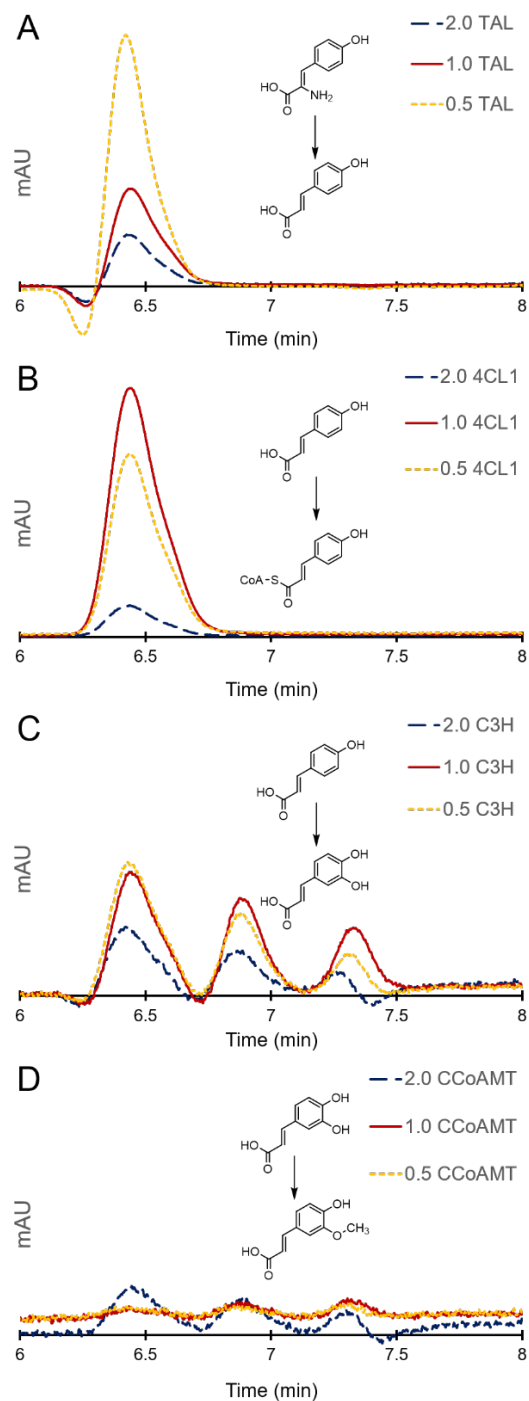
Figure 5.6: Enzyme optimization of (A) TAL with 4CL1/CUS, (B) 4CL1 with TAL/CUS, (C) C3H with TAL/4CL1/CCoAMT/DCS/CURS1, and (D) CCoAMT with TAL/4CL1/C3H/DCS/CURS1. Enzymes were delivered in relative ratios of 0.5, 1.0, and 2.0 in comparison to the polyketide synthase CUS for TddCCM production and DCS/CURS1 for TCCM production. Curcuminoids were extracted from the conditioned media 48 h post-transfection and subjected to HPLC. Spectra shown are averages of parallel experiments.

Once the supplementation and relative enzyme expression had been optimized for each pathway, the maximal curcuminoid concentration for each was obtained (Figure 5.7). The highest curcuminoid media concentrations resulting from the biosynthesis pathways depending on supplemented intermediates were 0.19 μM ddCCM using 4CL1/CUS, 0.17 μM dCCM using 4CL1/DCS/CURS1, and 0.20 μM for CCM using 4CL1/DCS/CURS1 at equal plasmid ratios. Optimal curcuminoid production from tyrosine was 0.05 μM for TddCCM using TAL/4CL1/CUS at 0.5:1:1 (weight:weight:weight), 0.02 μM TdCCM using TAL/4CL1/C3H/CCoAMT/DCS/CURS1 at 0.5:1:1:2:1:1, and 0.01 μM TCCM using TAL/4CL1/C3H/CCoAMT/DCS/CURS1 at 0.5:1:1:2:1:1.
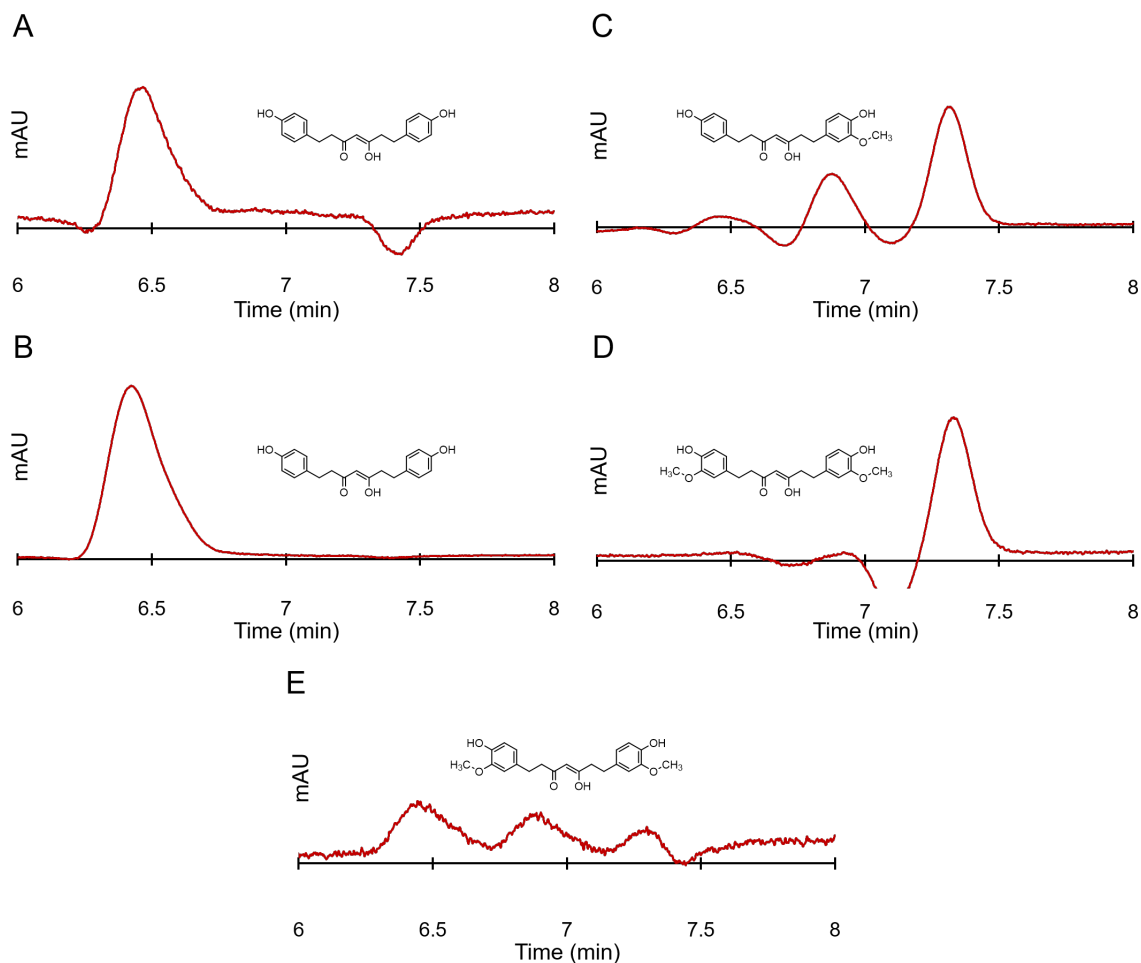
Figure 5.7: HPLC analysis of curcuminoids produced from optimized conditions for (A) TddCCM, (B) ddCCM, (C) dCCM, (D) CCM, and (E) TCCM. Results show successful production of all three main curcuminoids directly from tyrosine (E). Curcuminoids were extracted from the conditioned media 48 h post-transfection and subjected to HPLC. Spectra shown is average of three parallel experiments.

5.4   Discussion

Support for the pharmacologic effects of curcumin and its analogs continues to rise, as evidenced by over 200 clinical trials currently registered or completed [244]. To satisfy the ever-growing demand for curcuminoids, researchers turned to synthetic biology and metabolic engineering to increase the availability. One of the first major discoveries, and

the foundation of this study, was the isolation of CUS from *O. sativa* that allowed the production of curcuminoids in *E. coli* [230]. CUS catalyzes three steps in the biosynthesis pathway that are catalyzed by two different polyketide synthases, DCS and CURS1 in *C. longa* (Figure 5.1). This allowed for a decrease in the complexity of producing curcuminoids from tyrosine by decreasing the required number of enzymes needed to synthesize the end product. However, CUS was found to prefer p-coumaroyl-CoA as substrate over feruloyl-CoA, leading to higher production of bisdemethoxycurcumin [245]. Conversely, DCS and CURS1 preferentially catalyzed the steps involving feruloyl-CoA leading to higher production of curcumin and demethoxycurcumin [231]. For these reasons, CUS was chosen as the main curcuminoid polyketide synthase for bisdemethoxycurcumin, and DCS/CURS1 were employed in the curcumin and demethoxycurcumin pathways.

Using the two different pathways, all three curcuminoids were produced in HEK293 cells from endogenous tyrosine, albeit at low concentrations. Bisdemethoxycurcumin was produced in the highest concentration, 0.05 μM, with only trace amounts of demethoxycurcumin and curcumin being observed. This most likely occurred for two reasons. First, plant enzymes often exhibit poor expression, decreased stability, and impaired functionality when expressed in mammalian cells due to differences in codon utilization, protein-folding chaperones or post-translational modifications [237, 240]. Second, the enzymatic pathway to produce bisdemethoxycurcumin was less complex, comprising only three enzymes, compared to demethoxycurcumin and curcumin which require six. While total plasmid content was kept consistent due to constraints of transient transfections, individual expression levels may be expected to decrease as the

number of plasmids increased. To highlight the effect of the increased number of plasmids on curcuminoid production, flow cytometry revealed that the total fraction of cells producing curcuminoids dropped from 2% to nearly 0.02% with the additional plasmids.

To elucidate potential rate-limiting steps of the biosynthesis pathway, the relative ratios of each plasmid delivered to the cells was modulated between three weight ratios, 0.5, 1.0 and 2.0 (w/w) relative to the other plasmids. Maximum curcuminoid production resulted in a delivery ratio of 1.0 for most enzymes. However, TAL produced a significant increase in curcuminoid production when delivered at a 0.5 ratio. This is may be in part due to the catalytic efficiency of TAL. TAL has been substituted for a similar enzyme, phenylalanine ammonia lyase (PAL), in numerous phenylpropanoid biosynthesis pathways due to a 90- to 160-fold increase in catalytic efficiency over PAL [246]. The higher activity of TAL potentially means expression of TAL can be lowered in favor of increasing expression of 4CL1 or CUS to facilitate higher curcuminoid production. Conversely, delivery of CCoAMT-encoding plasmid at a 2.0 ratio resulted in the highest production of curcuminoids. CCoAMT is an enzyme commonly involved in cell wall reinforcement in plants through the phenylpropanoid pathway. Interestingly, the activity of CCoAMT is greatly influenced by the concentration of its product, feruloyl-CoA [247]. If relatively high levels of feruloyl-CoA are present, CCoAMT activity can be inhibited. Thus, to keep CCoAMT active, the enzyme must be present at higher concentration in comparison to steady-state concentration of feruloyl-CoA. This is most likely why the highest concentration of curcuminoids were obtained at a 2.0 CCoAMT delivery ratio.

5.5 Conclusion

We have designed a system that provides for biosynthesis of bisdemethoxycurcumin, demethoxycurcumin, and curcumin from endogenous tyrosine in mammalian cells. Simple pathways that employ the use of cinnamic acid intermediates produced higher concentrations of curcuminoids. However, we have shown that altering the relative expression of certain enzymes can increase the production of curcuminoids from tyrosine. This study serves as a proof-of-principle that not only can we produce plant-derived enzymes in mammalian cells, but it is also possible to produce a plant biomolecule entirely from naturally occurring starting materials in mammalian cells. To our knowledge, this is the first instance of such a phenomenon, potentially providing a platform for initiating focus on internal self-produced drug systems, versus traditional external administration.

CHAPTER 6. BIOACTIVITY OF CURCUMINOIDS PRODUCED IN MAMMALIAN CELLS

6.1   Introduction

Curcuminoids have been used in traditional medicine in the form of turmeric to treat headaches, wounds, skin diseases, gastrointestinal inflammation, the common cold, and other ailments for centuries [248]. More recently, a multitude of pharmacological activities of curcuminoids have been attributed to the regulation of transcription factors, growth factors, inflammatory cytokines, and other oncogenic molecules [195]. Interestingly, these effects are largely restricted to cancer cells with little to no deviation in basal expression levels being observed in normal cells. Many explanations have been proposed for this phenomenon. First, it has been shown that cancer cells exhibit an increased uptake of curcumin by a factor of two or more [249]. Second, the cytosolic concentration of glutathione (GSH) , which scavenges free radicals and protects the cell from damage by reactive oxygen species, is higher in cancerous cells [250]. Curcumin has been shown to inhibit glutathione production, as well as reduce GSH levels by forming CCM-GSH conjugates, resulting in accumulation of oxidative species and inducing apoptosis [251]. Finally, curcumin is known to inhibit the activation of nuclear factor-κB, a transcription factor that regulates the expression of a multitude of pro-inflammatory cytokines and growth factors [252].

While increased internalization and depletion of glutathione occur in the majority of cancer cells, the effects of curcumin on specific molecular targets have been elucidated recently. One such case is the downregulation of 26S proteasome (26Sp) in triple negative breast cancer (TNBC) and multiple myeloma cells (MM), which have been shown to

heavily rely on the activity of 26S proteasome [253, 254]. In MM, 26Sp degrades damaged or misfolded proteins that allow the disease to further progress, while in TNBC 26Sp systemically degrades proapoptotic factors and increases the survival and progression of the cancer. 26Sp is activated through dual-specificity tyrosine phosphorylation regulated kinase 2 (DYRK2) [255]. Curcuminoids are a highly specific and potent inhibitor of DYRK2 [253]. In the TNBC cancer cell line MDA-MB-231, curcumin inhibited DYRK2 at nanomolar concentrations. The inhibition of DYRK2 led to decreased proteasome activity, as witnessed by protein accumulation and induced apoptosis in the cells.

Since curcuminoids can elicit responses from cancer cells with little effect on proximal normal cells at relatively low concentration, they are excellent candidates for intracellular production with the intent to provide local or systemic therapy (Figure 6.1). Having successfully produced curcumin and its two main analogs in mammalian cells, we will establish the grounds for using intracellularly produced natural products versus exogenously delivered molecules as a therapy. Once a protocol was established that elicited consistent production of the desired curcuminoids (Section 5.3.1), potential therapeutic applications were explored via radical scavenging assays, and viability and migration assays of cancer cells in the presence of curcuminoid-producing HEK293 cells.
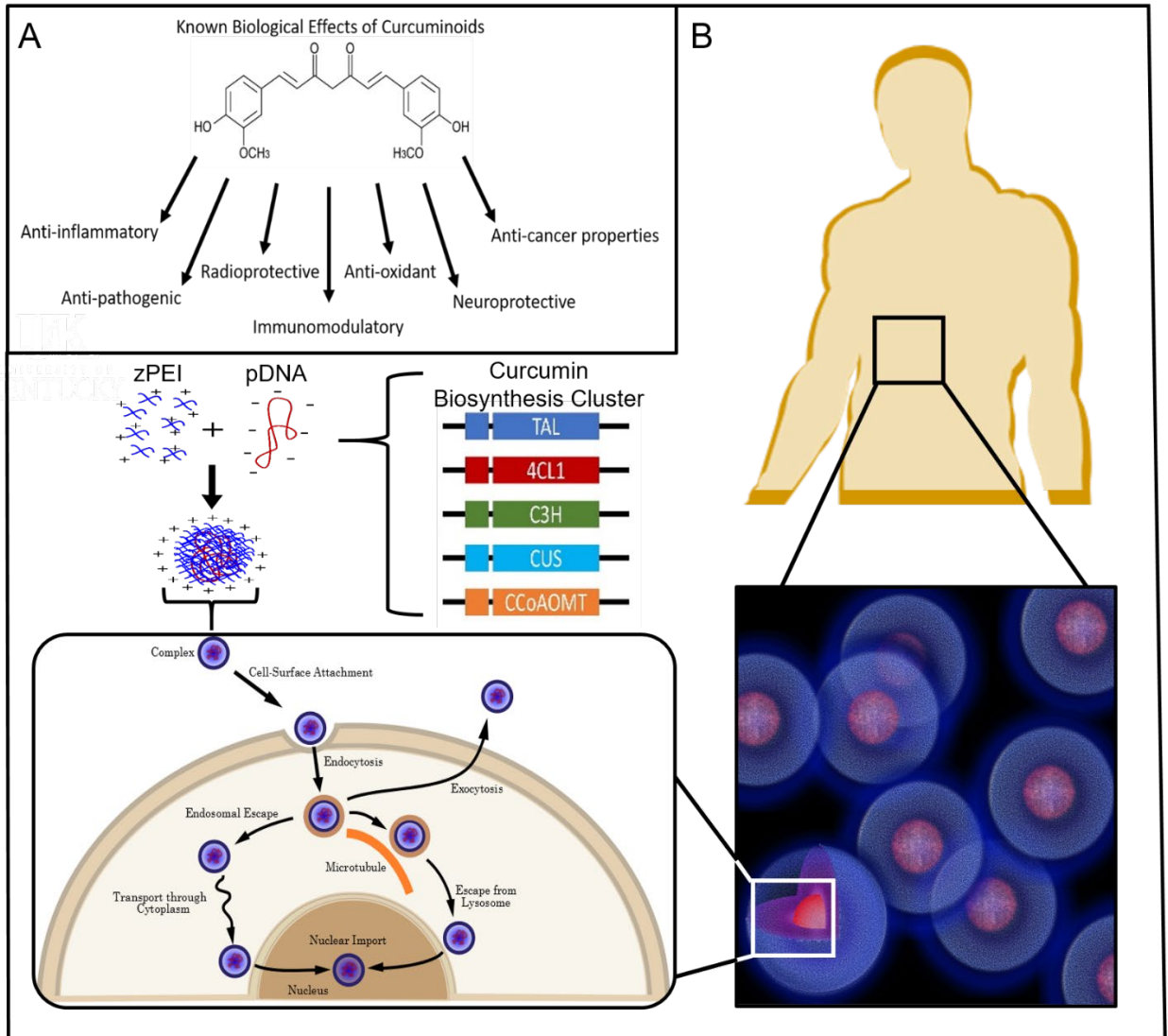
Figure 6.1: A.) Diagram summarizing many of the known biological effects of curcuminoids. B.) Schematic of in vivo curcuminoid biosynthesis therapy. A cluster of genes encoding enzymes of the curcuminoid biosynthesis pathway are introduced via polymer-plasmid DNA complexes, resulting in the patient's own cells producing curcuminoids, which may exhibit therapeutic effects locally or systemically.

6.2 Methods and Materials

6.2.1 Cell Culture and Plasmid Construction.

HEK293 human embryonic kidney cells were purchased from the American Type Culture Collection. Cells were cultured in DMEM (ThermoFisher Scientific, Waltham, MA) at 37 °C, 5% CO_2, and supplemented with 10% fetal bovine serum per ATCC recommendations. Genes encoding for the curcuminoid biosynthesis cluster (Figure 5.1) were developed from the pathway reported by Rodrigues et al. using publicly published sequences from GenBank (NCBI, Bethesda, MD) [232]. Tyrosine-ammonia lyase (TAL-GenBank MG712805), 4-coumarate-coenzyme A ligase (4CL1- GenBank U18675), 4-coumarate 3-hydroxylase (C3H- GenBank HE804045), caffeoyl-CoA 3-o-methyltransferase (CCoAMT- GenBank JN849911), bisdemethoxycurcumin synthase (CUS GenBank- AK109558), diketide-CoA synthase 1 (DCS GenBank- KM880191), and curcumin synthase 1 (CURS1 GenBank- AB495007) were individually synthesized and cloned into pcDNA3.1(+) mammalian expression vectors by GenScript (Piscataway, NJ, USA).

6.2.2 Polymer-Mediated Transfections.

HEK293 cells were seeded in 100-mm plates at 2.0×10^6 cells/plate 24 h prior to transfection. Polymer/DNA complexes were formed by first diluting 200 μL of 0.1 μg/μL DNA solution with 800 μL of PBS in a 15-mL centrifuge tube. An equi-volume solution of 0.12 μg/μL zPEI9 was added dropwise to the DNA solution under constant agitation to achieve the desired optimum polymer:DNA weight ratio reported in our previous work

[72]. Particles were allowed to incubate at room temperature for 20 minutes. Immediately before transfection, 2 mL polyplex solution was deposited into 8 mL of fresh serum-supplemented media. Conditioned growth medium was aspirated from cells and replaced with 10 mL of polyplex/growth medium solution (20 μg DNA/plate). After 4 h, the transfection medium was replaced with serum-supplemented growth medium devoid of phenol red in order to visually aid observation of curcuminoid production.

6.2.3 Radical Scavenging Assay.

Modified cells were wounded with an oxidation agent to assess the ability of intracellularly produced curcuminoids to scavenge radicals. Twenty-four hours post initial transfection with plasmid cocktails encoding various curcuminoid biosynthesis clusters as described in Chapter 5, cells were seeded into 96 well plates at a density of 20,000 cells/well. Cells were allowed to grow and produce curcuminoids for an additional 48 h under optimal supplementation conditions stated in the previous chapter. Radical formation was induced by exposing cells to 1000 μM H_2O_2 for 1 h. After wounding, the redox dye CellROX Red was added to each well. CellROX fluoresces upon oxidation, signifying the presence of oxidative species. Cells were lysed and fluorescence was quantified via a Synergy 2 plate-reader at 644/655 excitation/emission. The fluorescence of both modified and control cells was normalized to unmodified HEK293 cells under normal growth conditions.

6.2.4   Inhibition of MDA-MB-231 Luciferase Proliferation.

To probe the anti-cancer activity of curcuminoid-producing cells, luciferase expression from MDA-MB-231-Luc, a cell line stably expressing firefly luciferase from Cell Biolabs (San Diego, CA), was monitored after being co-cultured with curcuminoid-producing cells. HEK293 cells were transfected as previously described in a 100-mm dish and allowed to incubate for 24 h. After the incubatory period, MDA-MB-231 and modified HEK293 cells were seeded together in 12-well plates at a density of 150,000 total cells/well. Co-cultures were comprised of an equal number of each cell line (75,000 cells). Luciferase expression was quantified every 48 h using a Promega luciferase assay kit (Madison, WI) in relative light units (RLU) using a Synergy 2 plate-reader (BioTek, Winooski, VT). Results were normalized to luciferase expression of normal HEK293 cells co-cultured with MDA-MB-231 Luc cells.

6.2.5   Transwell Viability Assay.

Effects of proximal curcuminoid production by HEK293 on cancer cell metabolic viability were tested on two model cell lines. MDA-MB-231 and MCF7 cells were seeded in 12-well plates at 150,000 cells/well. Previously transfected and control HEK293 cells were seeded in transwell (0.4 μm pore) inserts at 75,000 cells/insert 24 h post transfection. Cells were allowed to incubate for 24 h at 37 °C and 5% CO_2 separately. HEK293 containing transwell inserts were then transferred to wells containing cancer cells. Media was replenished and supplemented with cinnamic acids for optimal curcuminoid production and allowed to incubate for 48 h. To assess the viability of cancer cells,

transwells were removed, 150uL of CellTiter Blue Reagent (Promega, Madison, WI) was added to each well plate, plates were incubated for an additional 4 h, and fluorescence was read using a BioTek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT) at 530/590 excitation/emission. Fluorescence was normalized to control cells of unmodified HEK293 cultured with each cancer line.

6.2.6   Transwell Migration Assay.

Effects of proximal curcuminoid production by HEK293 on cancer cell migration were tested on two model cell lines. MDA-MB-231 and MCF7 cells were seeded in 12-well plates at 150,000 cells/well. An Ibidi (Planegg, Germany) wound-healing insert was used to create a 500 μm gap. Previously transfected and control HEK293 cells were seeded in transwell (0.4 μm pore) inserts at a cell density of 75,000 cells/insert 24 h post-transfection. Cells were allowed to incubate for 24 h at 37 °C and 5% CO_2 separately. HEK293 containing transwell inserts were then moved into wells containing cancer cells. Media was replenished and supplemented for optimal curcuminoid production and allowed to incubate for 48 h. The wound healing insert was removed after 48 h. Cells were then allowed to grow for an additional 72 h. Growth of the cell front was imaged at 0, 36, and 72 h using a Dino-Eye USB Digital Eye-Piece (Torrance, CA).

6.3   Results

6.3.1   Anti-Oxidant Capacity of Curcuminoid-Producing HEK293 Cells.

Curcuminoids are known scavengers of free radicals and provide some degree of protection to wounded cells [195]. To confirm a similar bioactivity of biosynthetic curcuminoids, their ability to protect cells against reactive oxygen species was probed using a redox-responsive dye (Figure 6.2). Cells were wounded with 1 mM hydrogen peroxide, inducing formation of free radicals. Control cells that include unmodified cells with and without p-coumaric and ferulic acid, as well as cells modified with a pGL3 control vector, with and without p-coumaric acid and ferulic acid, were included to ensure radical scavenging activity was not a byproduct of the supplemented cinnamic acids or transfection process. When compared to unmodified cells and control cells, the curcuminoid-producing cells exhibit a significant decrease of free radical levels (lower relative fluorescence). The lowest relative fluorescence (65%) was observed in the CCM-producing cells, which characteristically produce the highest concentration of curcuminoids (Section 5.3.1). ddCCM and dCCM also showed increased efficacy at radical scavenging in comparison to controls. TddCCM, which characteristically is present at the lowest concentration of the tested systems, exhibited only 16% decrease in relative fluorescence, indicating the increased curcuminoid concentration benefits scavenging capabilities.
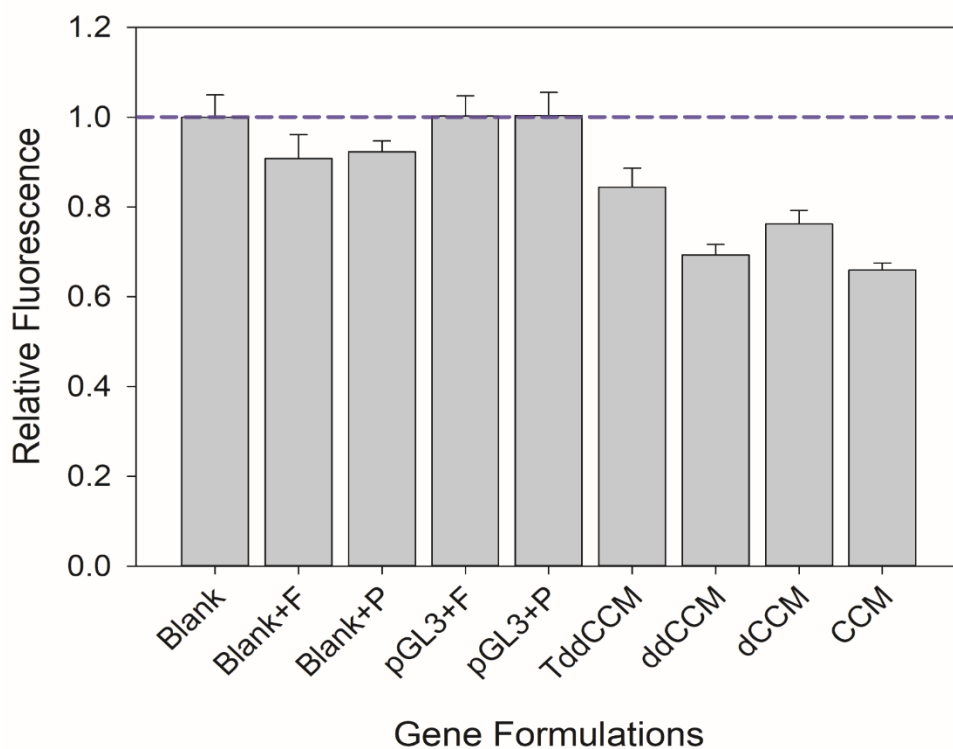
Figure 6.2: Anti-oxidant capacity of curcuminoid producing HEK293 cells. HEK293 cells were modified using polymer-DNA complexes and allowed to grow undisturbed for 48 h. After incubation, cells were treated with 1000 μM H_2O_2 for 30 min to induce radical formation. Antioxidant activity was assessed using the redox fluorescent dye CellROX Red. Upon oxidation, CellROX will fluoresce in the deep red spectrum. HEK293 cells modified with ddCCM, dCCM, and CCM showed upwards of 35% decreased in radical species over that of unmodified control cells (n = 3, error bars represent standard deviation). Nomenclature: Blank- Co-culture with unmodified HEK293 cells, Blank+P- co-culture of unmodified HEK293 cells with 0.30 mM p-coumaric acid, Blank+F- co-culture of unmodified HEK293 cells with 0.05 mM ferulic acid, pGL3 + F- co-culture of HEK293 cells modified with pGL3 control vector with 0.05 ferulic acid, pGL3 + P- co-culture of HEK293 cells modified with pGL3 control vector with 0.30 mM p-coumaric acid.

6.3.2   Anti-Cancer Activity of Biosynthetically Produced Curcuminoids

Anticancer properties of intracellularly produced curcuminoids were first investigated

using a co-culture system of MDA-MB-231 Luc cells and the modified HEK293 cells. This

particular line of MDA-MB-231 cells stably expresses luciferase, allowing for quantitation

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of the cancer cell population, separately from the HEK293 cells, by monitoring the luciferase activity. After being co-cultured with curcuminoid-producing cells, the luciferase activity consistently decreased over the course of 8 days in comparison to control cells (Figure 6.3). CCM, which produced the highest concentration of curcuminoid (Section 5.3.1), decreased the relative luciferase expression by 93%. dCCM and ddCCM, the next highest producing curcuminoid lines, resulted in a decrease of 91% and 70%, respectively. TddCCM, which produced only a fourth of the bisdemethoxycurcumin content as ddCCM still decreased the relative luciferase expression by 54%. CCM again performed best, reducing luciferase expression to 20% in comparison to luciferase in co-cultures of MDA-MB-231 Luc and normal HEK293. Indicating significant inhibition of cancer cell growth.
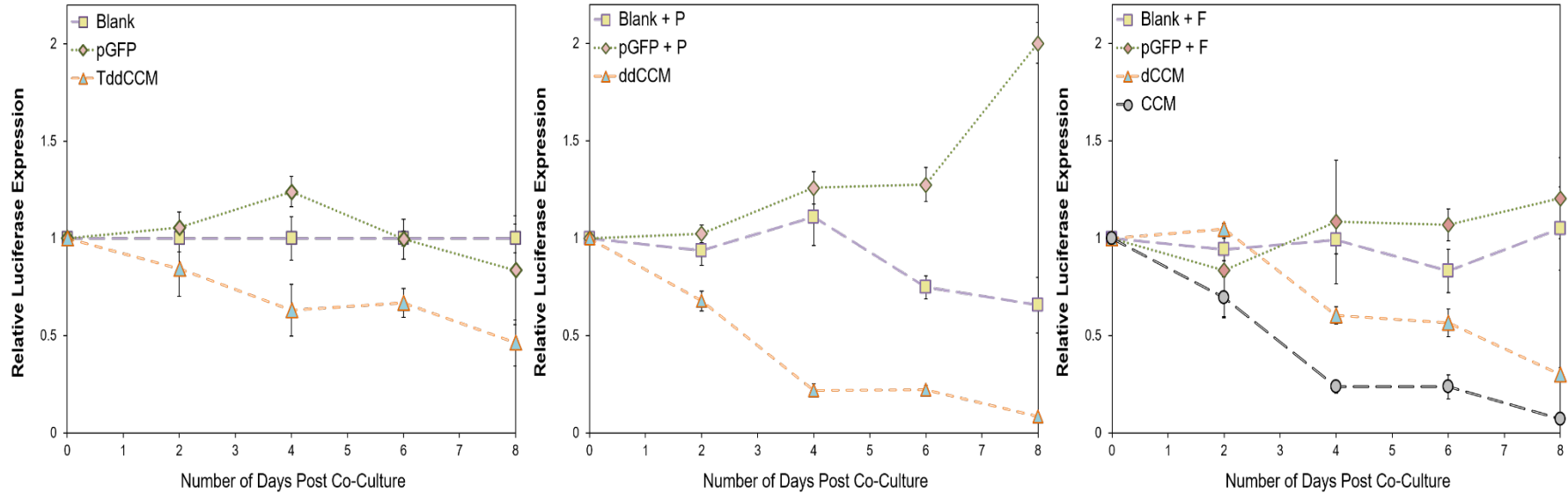
Figure 6.3: Co-culture of MDA-MB-231-Luc cells and HEK293 cells. HEK293 cells were modified via transfection 24 h before being co-cultured with luciferase producing MDA-MB-231 cells. Luciferase levels were measured over the course of 8 days. Curcuminoid producing HEK293 cells reduced luciferase expression by up to 80% indicating significant inhibition of MDA-MB-231 growth in comparison to co-cultures of unmodified HEK293 (n = 3, error bars represent standard deviation). Nomenclature: Blank- Co-culture with unmodified HEK293 cells, Blank+P- co-culture of unmodified HEK293 cells with 0.30 mM p-coumaric acid, Blank+F- co-culture of unmodified HEK293 cells with 0.05 mM ferulic acid, pGFP + F- co-culture of HEK293 cells modified with GFP control vector with 0.05 ferulic acid, pGFP + P- co-culture of HEK293 cells modified with GFP control vector with 0.30 mM p-coumaric acid, pGFP- co-culture of HEK293 cells modified with GFP control vector.

To further corroborate these results, a new set of experiments was designed around a transwell framework that enabled model cancer cells, MDA-MB-231 and MCF7, to grow in the presence of curcuminoids secreted by modified HEK293, while still allowing for separation of the individual populations during assaying and imaging. Viability of cancer cells incubated with modified HEK293 cells for 48 h was assessed via CellTiter-Blue (Figure 6.4). Both cancer lines showed minor deviations from normalized metabolic activity when cultured with cells in media supplemented with cinnamic acids, or cells modified by a control GFP vector, indicating little to no effect as a byproduct of supplementation or transfection. Cancer cell lines that were co-cultured with curcuminoid-producing lines showed significant decreases in metabolic activity, even in lines where the curcuminoids were produced from tyrosine. Relative decreases in metabolic activity were 18, 26, 26, and 35% for TddCCM, ddCCM, dCCM, and CCM, respectively, in MDA-MB-231 and 5, 7, 33, and 40% for TddCCM, ddCCM, dCCM, and CCM, respectively, in MCF7. Due to the relatively small volume of media present in the transwell plates, we were unable to directly determine the curcuminoid concentration in each well. Instead, we compared the decreases in metabolic activity to that observed with known concentrations of free curcuminoids supplemented into the growth media (Figure 6.5). TddCCM reduced metabolic activity of cancer cells equivalent to 0.31 μM supplemented bisdemethoxycurcumin in MCF7 and 0.16 μM in MDA-MB-231, ddCCM produced results equivalent to 0.35 μM supplemented bisdemethoxycurcumin in MCF7 and 0.17 μM in MDA-MB-231, dCCM produced results equivalent to 0.36 μM supplemented demethoxycurcumin in MCF7 and 0.30 μM in MDA-MB-231, and CCM produced results equivalent to 0.41 μM supplemented curcumin in MCF7 and 0.39 μM in MDA-MB-231.

Generally, larger decreases in metabolic activity corresponded to HEK293 lines that produced a higher curcuminoid concentration.
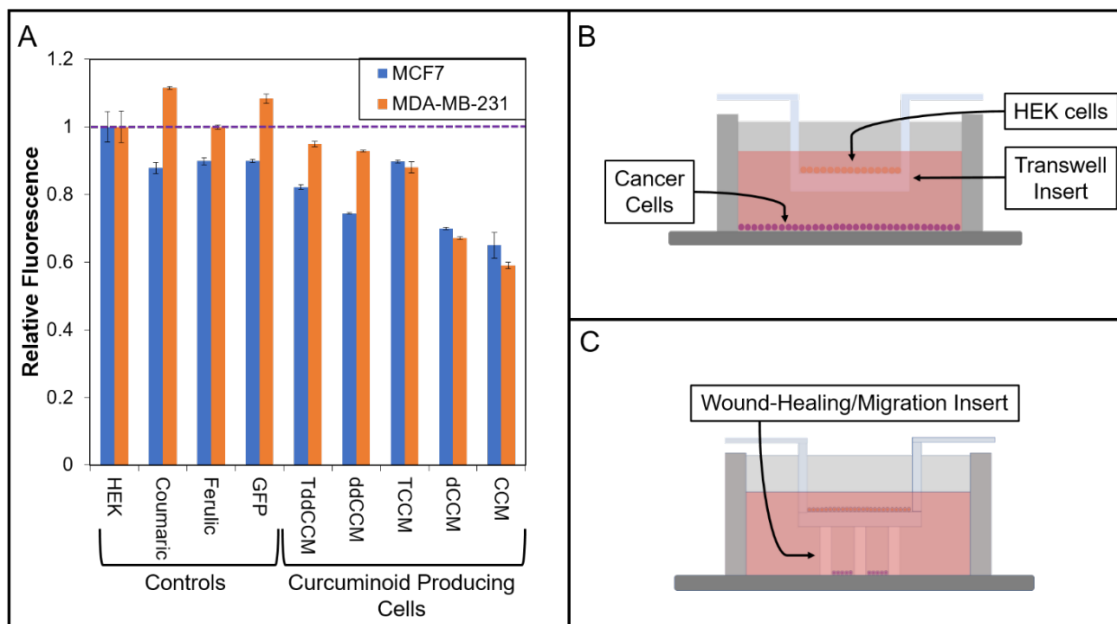
Figure 6.4: A.) Metabolic viability of MCF7 and MDA-MB-231 cells exposed to curcuminoids produced from varying enzymatic pathways in modified HEK293 cells. Metabolic activity was assessed 48 h post introduction of transwell insert inoculated with transfected HEK293 and normalized to fluorescence of cancer cells co-cultured with unmodified HEK293 cells. Controls that were employed include unmodified-HEK293 with normal growth media (HEK), unmodified-HEK293 with 0.30 mM p-coumaric acid supplemented growth media (Coumaric), unmodified-HEK293 with 0.05 mM ferulic acid supplemented growth media (Ferulic), HEK293 transfected by a GFP control vector with 0.30 mM p-coumaric acid and 0.05 mM ferulic acid supplemented growth media(GFP) (n = 3, error bars represent standard deviation). Schematic of the transwell framework developed for (B) metabolic viability and (C) migration assays. The compartmental transwell designed allowed curcuminoids produced from HEK293 to interact with each cancer cell without cross contaminating the populations. Allowing for easy separation to assay the individual metabolic activity and migration tendencies.
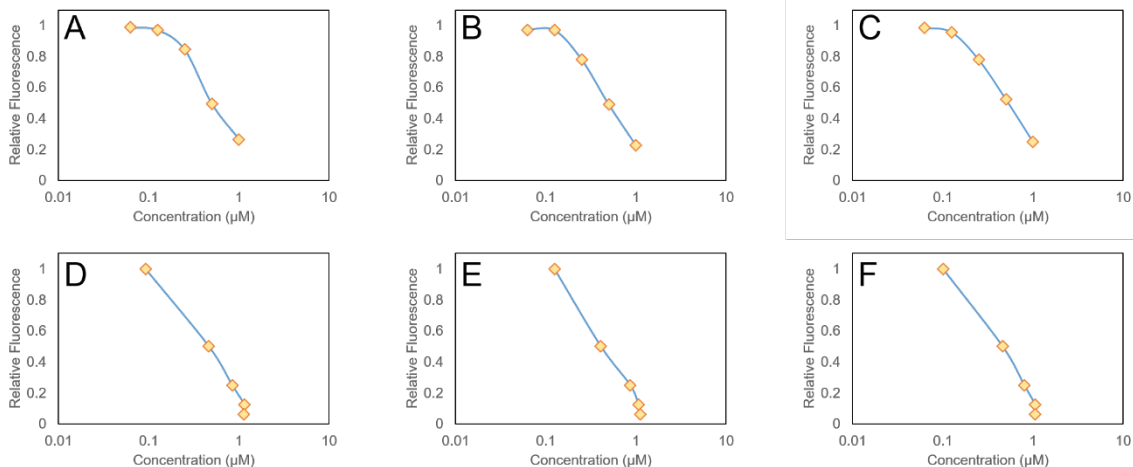
Figure 6.5: Relative fluorescence of MCF7 cells dosed with free (A) curcumin (B) demethoxycurcumin and (C) bisdemethoxycurcumin and MDA-MB-231 cells dosed with free (D) curcumin (E) demethoxycurcumin and (F) bisdemethoxycurcumin. Relatively fluorescence of the viability assay using the transwell design were compared to the free dosed curcuminoid curves to determine an equivalent concentration produced within the transwell compartments.

Breast cancer cell migration assays were developed using a transwell framework that included a wound-healing insert that imparted a 500 μm gap after removal (Figure 6.6). Each cell line was grown for 24 h separately to allow the cells to attach and return to normal growth conditions. After the initial 24 h window of separated incubation, the wound healing inserts were removed from the cancer cell wells, and the cell fronts were imaged as a baseline. The transwell inserts containing HEK293 cells were then moved into the wells containing cancer cells. The cancer cells and curcuminoid-producing lines were then allowed to incubate together for an additional 72 h. The cell front was imaged at 36 h and 72 h. Migration mirrored the results of the viability assay. Control cells showed no discernible impact on growth, resulting in a complete confluency after 72 h. TddCCM and ddCCM showed moderate inhibition of migration, while dCCM and CCM showed severe

inhibition and evidence of some degree of cell death. This further corroborates that elevated

concentrations of curcuminoids are more potent to both MDA-MB-231 and MCF7.
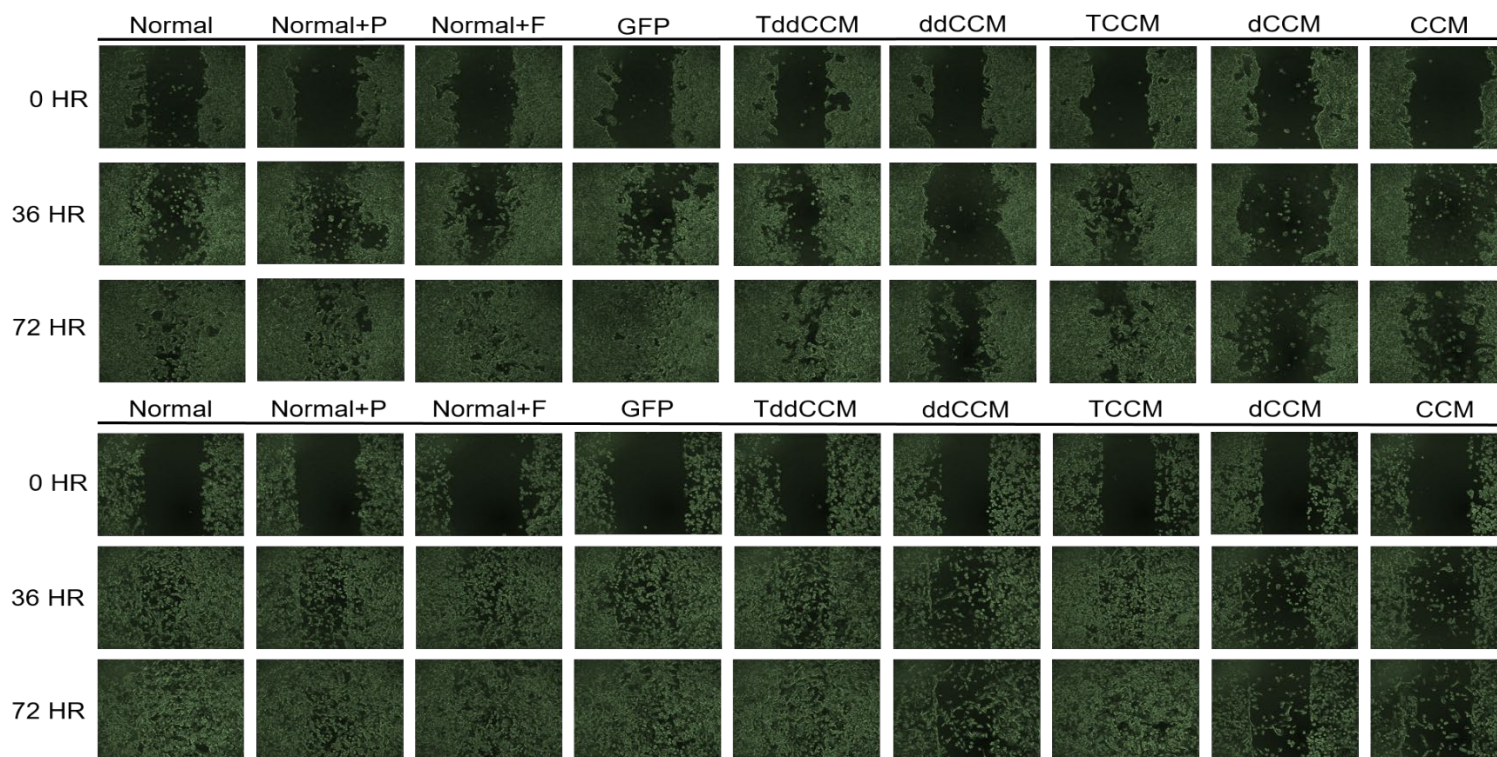
Figure 6.6: Wound-healing assay for evaluating the inhibitory effects of intracellularly produced curcuminoids on (Top) MCF7 and (Bottom) MDA-MB-231 cell migration. Cancer cells were deposited into an Ibidi wound healing insert and allowed to grow to confluency, before removal of the insert, leaving a 500 μM gap. Cells were then co-cultured with a transwell insert inoculated with HEK293 cells modified to produce curcuminoids. Cell migration front was monitored microscopically at 0, 36, and 72 h. The representative photographs showed the same area at time zero and after 72 h of incubation with or without curcuminoid producing cells. Nomenclature: Normal- unmodified HEK293 cells, Normal+P- unmodified HEK293 cells with 0.30 mM p-coumaric acid, Normal+f- unmodified HEK293 cells with 0.05 mM ferulic acid, GFP- HEK293 cells modified with GFP control vector with 0.05 ferulic acid and 0.30 mM p-coumaric acid.

6.4 Discussion

One of the main motivations for this study was to investigate the feasibility of modifying mammalian cells to produce sufficient concentrations of therapeutic compounds to stimulate bioactivity. Intracellular biosynthesis of curcuminoids *in vivo* could potentially circumvent the traditional problems of delivering curcumin by providing a long-term systemic source through either implantation of *ex-vivo* modified cells or direct transfection of host cells. Recently, many cancer lines have been shown to have a dependency on proteasomes due to changes in protein homeostasis [255]. Curcuminoids are an attractive biomolecule to exploit this weakness considering the ability of curcuminoids to act as potent inhibitors to dual-specificity tyrosine-regulated kinase (DYRK2), a direct positive regulator of proteasomes [253]. Even in nanomolar concentrations, curcumin has been demonstrated to induce apoptosis in 'proteasome-addicted' cell lines [256].

To investigate the anti-cancer properties of intracellularly produced curcuminoids, three sets of co-culture experiments were designed around probing the effect of proximal curcuminoid production on MCF7 and MDA-MB-231 cancer cells. As an initial first pass, luciferase-producing MDA-MB-231 cells were co-cultured with various modified HEK293 lines. Over the course of eight days, a significant decrease in luciferase levels of co-cultures containing curcuminoid lines was observed, with CCM decreasing levels to 20% of that of control populations. To validate these results, two transwell experiments were designed around exploring the effect of proximally produced curcuminoids on both MDA-MB-231 and MCF7 cancer cells. First, the viability of each cancer cell was assessed using the metabolic dye CellTiter-Blue after being co-cultured with modified and control HEK293 cells for 48 h. Surprisingly, even with only a small fraction of the transfected cells

successfully producing curcuminoids (Section 5.3.1), cancer cell growth inhibition was observed in nearly every tested enzymatic pathway. The simpler pathways, ddCCM, dCCM, and CCM, which depend on intermediate cinnamic acid supplementation, decreased relative metabolic activity by the greatest degree. This is not unexpected since these pathways also produced the highest concentration of each of the curcuminoids (Section 5.3.1). TddCCM, which produces bisdemethoxycurcumin from tyrosine, also produced a modest decrease in metabolic activity in MCF7, indicating that this method can induce anti-cancer effects by synthesizing curcuminoids from only naturally occurring starting materials. Bisdemethoxycurcumin produced from both tyrosine and p-coumaric acid produced a smaller response in comparison to demethoxycurcumin and curcumin in both cell lines. TddCCM and ddCCM in general produced less curcuminoid content than dCCM and CCM, but some studies suggest that bisdemethoxycurcumin may be a less potent inhibitor than curcumin or demethoxycurcumin [257].

Interestingly, all of the bioactivities of curcuminoids produced intracellularly were equivalent to free curcuminoid concentrations greater than the curcuminoid concentrations measured in the growth media. The equivalency results suggest higher concentrations of curcuminoids were produced in the transwell plates than the 100-mm dish transfections. Because the number of cells seeded was scaled proportionally to the cell culture area of the plates, this seems unlikely. One potential explanation to account for this discrepancy is an effect of both intracellularly produced curcuminoids and degradation products, such as vanillin. Studies have shown that 50% of free curcumin administered to cultured cells is decomposed within 8 h. Many of the degradation products of curcuminoids are known to elicit anticancer responses similar to curcuminoids themselves. What we may be observing

is a combined response of the accumulated degradation products with the curcuminoids that are resulting in the higher bioactive responses.

The co-culture experiment was modified to include a 500-μm gap to investigate effects on migration after 72 h of co-culture. In general, the visual results of the migration front corroborate the results from the metabolic assay, which are in line with the results of the original co-culture with luciferase-producing MDA-MB-231. Simpler pathways that produced higher concentrations of curcuminoids demonstrated inhibited migration and even some degree of cell death in both cell lines. Taking into consideration the results from all three assays, it is apparent that intracellularly produced curcuminoids induce therapeutic effects to the surrounding environment.

6.5   Conclusion

Biosynthetically produced curcuminoids can elicit bioactive responses in cancer cells and protect cells from oxidative stress. Generally, biosynthesis pathways (Chapter 5) that characteristically resulted in higher concentrations of curcuminoids demonstrated better antioxidant capacity and increased anti-cancer properties. CCM consistently proved to the be the highest performing curcuminoid, resulting in significant reduction in oxidative species as well as inhibiting production of luciferase in MDA-MB-231, decreasing the viability of MDA-MB-231 and MCF7, and inhibiting the migration of MDA-MB-231 and MCF7. Although not as potent as CCM, bisdemethoxycurcumin produced from endogenous tyrosine (TddCCM) displayed the same abilities. With the compelling results described within this work, we plan to further the concept of internally produced drug

systems by moving to stable expression of curcuminoids and extending production to other

natural products of interest.

CHAPTER7. PERSPECTIVES AND FUTURE WORK

The work and corresponding findings presented throughout this dissertation lay the foundation for several exciting paths to further non-viral gene therapy and potentially revolutionize the delivery of natural products. A vector that retains its transfection efficiency in the presence of serum proteins is a critical step towards providing a non-viral delivery mechanism that retains efficiency *in vivo*. How the polymer is able to produce such a striking phenomenon with such small modifications is an interesting question. We hypothesized the primary driving force behind efficiency retention is decreased non-specific protein interactions. However, from the literature we also know complex charge systems of cationic polymers with negatively charged moieties can target endocytic pathways that are more advantageous, specifically the caveolin pathway. Trafficking and co-localization studies would help elucidate if the enhanced performance of zPEI is primarily due to one mechanism or a synergistic combination of multiple mechanisms.

One of the more attractive features of the succinylated polyethylenimine derivatives is ease of access. zPEI was developed using off-the-shelf polyethylenimine and succinic acid. The reaction itself is straightforward, with both a quick reaction time and simple clean up, large quantities of the vector are 'transfection ready' in within 24 h. Furthermore, this particular vector has shown promise in withstanding the stresses of long term storage. Although non-viral vector preparation is simpler in comparison to viral packaging, a vector that can be prepared beforehand, stored for long periods of time, and shipped without any impact of transfection capability is paramount when discussing possibilities of clinical application. zPEI potentially could serve as a general-use transfection system where vectors can be premade in designated labs, lyophilized, packaged, and shipped as a ready-

to-use transfection agent. This system would work particularly well with the increasingly popular CRISPR/Cas9 gene editing technology. A plasmid encoding for the Cas9 editing enzyme with guide RNA could be packaged with a plasmid encoding for donor DNA using zPEI, creating a relatively cheap, efficient, and shippable system for producing stable transgenic cell lines. *Ex-vivo* therapies currently rely on viral vectors for transgenic cell production, which can take a significant amount of time. The impact zPEI could have on this particular field I feel warrants more investigation and could help streamline development of more *ex-vivo* therapies.

Natural products are used in everyday life, and represent a large part of our current medical practice. Countless hours and resources are spent developing new delivery technologies that increase bioavailability and controlled release of natural products. With the proof-of-principle that human cells can synthesize natural products, we may see the beginning of a paradigm shift in how some natural products are used as treatment. Instead of focusing on exogenously delivered therapeutics, we can instead focus on programming cells to make these products through endogenous means.

While we previously only mentioned curcuminoids, we believe this technology can be extended to other molecules of interest. Curcuminoids are a product of the phenylpropanoid pathway, which many natural products share. Flavonoids and stilbenoids are two classes of compounds that share many of the same therapeutic activities as curcuminoids and can be produced using a similar enzymatic biocluster.

Before extending this technology, it would be best to optimize the current framework. First and foremost would be to move the expression from transient to stable. As we saw in chapter 5, the fraction of cells producing curcuminoids is small, ranging from

a few percent to less than one percent. Raising this population to 100% could theoretically give rise to greater than 100-fold increases in curcuminoid concentration. With this we would better be able to elucidate therapeutic properties and potential side effects. Stable integration of the biocluster would also allow us to manipulate the enzymes to a greater degree than relative plasmid delivery. Enzymes could be tied to specific promoters, increasing or decreasing the relative expression. From here, rate-limiting steps from the synthesis pathway would be much more easily discernible due to a more controlled expression and large sample population. The genetic code for the enzymes themselves could be manipulated to be better suited for mammalian expression, such as commercial codon optimization. Finally, it may not be desired to have constant natural product synthesis. Thus, we could insert responsive expression system that is only activated in the presence of some external stimuli. With this 'smart' promoter approach, cells could be programmed to synthesize natural products under certain conditions and remain dormant otherwise. All of the above improvements could be culminated into a strategy that modifies cells *in vitro* and then implants the modified cell into a specific location within a patient's body, similar to how human islets are encapsulated in alginate and implanted in the body to restore insulin functionality. For example, with encapsulation and implantation of CCM producing cells one could provide a moderate systemic source of curcumin that could act as a biological countermeasure to radiation by implanting cells throughout the body, or a local high concentration of curcumin that could help prevent detrimental effects from radiation therapy by implanting cells around a tumor site that would protect healthy cells during irradiation of the tumor. I intend to pursue these interests in full as I continue in my career

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