Site-specific Nanoparticle Therapy for Treatment of Medial Arterial Calcification

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SITE-SPECIFIC NANOPARTICLE THERAPY FOR TREATMENT OF MEDIAL ARTERIAL CALCIFICATION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Tyler James Gibson
August 2021

Accepted by:
Dr. Naren Vyavahare, Committee Chair
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ABSTRACT

Vascular calcification is a disease associated with the cardiovascular system in which minerals (i.e. Calcium Phosphate) are deposited into the walls of arteries, veins, and valves of the heart. Although low amounts are common in most individuals, abnormally high deposition of these minerals, specifically calcium salts, reduces vascular elasticity and hardens the artery. Medial arterial calcification (MAC) occurs when minerals diffuse through the artery into the medial layer of the arterial wall. MAC causes hemodynamic disturbances by reducing elasticity in the artery and therefore hardens the artery. MAC can be intensified in combination with other diseases such as diabetes or chronic kidney disease (CKD). Specifically, patients with CKD have increased risk of cardiovascular diseases and are more likely to die due to cardiovascular diseases than end stage renal failure. Current treatments are more preventative and do not attempt to remove mineral deposition. In an attempt to remove mineral deposition, chelation therapy offers a unique approach to remove it, however, systemic delivery may induce hypocalcemia and bone resorption. Ethylene diamine tetraacetic acid (EDTA) loaded nanoparticles can offer a site-specific therapy to remove the mineral deposition found in the medial layer of an artery. This project attempts to create an EDTA loaded human serum albumin nanoparticles and attempts to remove vascular calcification in a CKD adenine mouse model.
DEDICATION

To my parents first and foremost, Holly and Jerry Gibson, your support, encouragement, and love have always pushed me to be the best person I can be. You guys have always been my rock, molded me into the man I am today, and I could not be here without both of you.

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CHAPTER ONE

INTRODUCTION

1.1 The Cardiovascular System

The cardiovascular system, more commonly known as the circulatory system, functions to transport blood throughout the body in order to maintain homeostasis. The heart, the primary muscular, propels blood through a closed system of vessels. The heart, during an average life, will beat more than 2.5 billion times. The flow of blood pushes nutrients and oxygen throughout the body through two major forms of circulation, pulmonary and systemic, seen in figure 1.1. Blood flows through two major vessels, arteries, which carry blood away from the heart to the body, and veins, which bring deoxygenated blood back to the heart to be reoxygenated in the lungs. Minor vessels known as capillaries, that are fine branching arteries or veins, link arterioles and venules. Both arteries and veins vary in size depending on their function, all have a similar structure. Arteries and veins both have three layers and a lumen, which is a hollow middle inner tube, that allows blood to pass through. The innermost layer of arteries, the tunica intima, is comprised of endothelial cells that are supported by the inner-elastic lamina. The internal elastic lamina is an elastic tissue that provides support to the cells throughout the entire arterial vascular system. The middle layer, the tunica media, is a smooth muscle rich layer supported by connective tissues, such as collagen and elastic fibers. This layer is essential to regulation of blood flow and blood pressure. The outermost layer, the tunica adventitia or tunica externa, is comprised mostly of connective tissue and contains collagen fibers that support the vessel and keep it in the same relative place. [2]
There are three main categories for size of arteries (Figure 1.2): elastic arteries, distributing arteries, and arterioles. Elastic, or conduit arteries, have a large inner diameter, typically larger than 10 mm. These arteries contain a thick tunica media and large amounts of elastic fibers, allowing them to withstand the high pulsatile pressure of blood caused by a heartbeat. Distributing arteries have an inner diameter that is typically between 0.5-10 mm. Distributing arteries contain a larger smooth muscle layer than the elastic fibers to help the artery vasoconstrict more effectively. Lastly, arterioles are arteries that are smaller than 0.5 mm. Arterioles walls progressively thin and these vessels contain only a
couple layers of smooth muscle that distribute blood into the capillary beds of organs to provide oxygen and nutrients to the organ. [4]

![Figure 1.2: Comparison of different types of arteries and arterioles](image)

### 1.1.1 ECM and Elastin Structure

The extracellular matrix (ECM) is a dynamic structure that provides support to organs and tissues. The components within the matrix that provide this support include proteins, such as collagen and elastin, and polysaccharides, known as glycosaminoglycans (GAGs). GAGs are glycosaminoglycan chains covalently linked to a protein core. [5] GAGs contain a repeating galactose polymerized sugar base linked to an amino sugar group. [6] This feature of GAGs provides the tissue both hydration features and compressive strength. There are four major categories of GAGs: hyaluronic acid (HA), heparan sulfate (HS), chondroitin sulfate/dermatan sulfate (CS/DS), and keratan sulfate (KS). These groups are differentiated by the specific types of sugars in the linear chain and the modification or absence of the sulfate groups. [7] Hyaluronic acid is the only GAG which is not sulfated. Due to the negatively charged sulfate groups and high polarity, these groups attract water into the ECM.
Of all the proteins found within the ECM, the most ubiquitous protein is collagen, which comprises roughly one third of the total proteins found in the human body. Collagen is a triple helical structure comprised of peptide chains wrapped around one another. The main sequence of amino acids in collagen is arginine, glycine, and aspartate. In this sequence, these three amino acids are known as integrins, and mimic receptors for cell adhesion molecules [8], which can provide binding support to many cell types within the body. Overall, collagen provides tensile strength to tissues.

Collagen synthesis mainly occurs within fibroblast cells. The intracellular portion of collagen synthesis begins with production of pre-procollagen. From there, the peptide chain is modified in the endoplasmic reticulum by a two-step process which hydroxylates the lysine and proline residues and further glycosylates the hydroxylated lysine with either galactose or glucose. This final structure is known as procollagen, and to become structurally mature it can be further processed extracellularly. Extracellularly, the procollagen molecule undergoes from procollagen to tropocollagen via collagen peptidases and then from tropocollagen to a collagen fibril by lysyl oxidase (LOX). The fibroblasts influence the overall alignment of the collagen fibers, turning individual fibers into collagen sheets. [9] To date, there have been 16 different types of collagen identified. Of the multiple collagen types, 90% is either Type I, II, or III [10], with Type I being most common in blood vessels.

Another component in the extracellular matrices of elastic tissues such as lungs, skin, and arteries is elastin. This protein is most abundant in arterial matrices, with elastin comprising of almost 50% of the matrix [11]. The arterial matrix needs a high concentration
of elastin to allow for appropriate load bearing in high-pressure situations. Elastin has two major components, an amorphous core that contains 90% of the fiber, and a scaffold of microfibrils, which are composed of Fibrillin-1 and Fibrillin-2 and microfibril-associated glycoproteins (MAGPs). [12] The structure of elastin is mostly composed of hydrophobic amino acids, glycine, valine, proline, and alanine, thus giving the overall protein a hydrophobic nature. [12]

Figure 1.3: Illustration of elastogenesis intracellularly and extracellularly

Elastogenesis, the synthesis of elastin, can be broken down into three steps: secretion of tropoelastin, coacervation of tropoelastin into 1 um spherules, and cross-linking of elastic fibers onto microfibrils catalyzed by lysyl oxidase (LOX), as seen in
Figure 1.3. Synthesis of elastin begins with expression of the human tropoelastin gene (ELN) located on chromosome 7 and tropoelastin molecules are then moved intracellularly to the ECM. Tropoelastin is transported into extracellular space for cross-linking and assembly by elastin-binding protein (EBP), which additionally protects tropoelastin from intracellular aggregation and proteolysis [13]. In the extracellular space, the tropoelastin creates roughly 1 um spherules which bind to the surface of the cell prior to cross-linking [14]. Once tropoelastin is delivered to extracellular space and cross-linking has started, the EBP is recycled and returned intracellularly where it can assemble the next tropoelastin molecule. The hydrophobic domains include the non-polar amino acids: glycine, valine, proline, and alanine. The cross-linking domains are in areas rich in alanine and lysine. All lysine residues on tropoelastin are subjected to one of two reactions: oxidative deamination or cross-linking by lysyl oxidase [15]. The production of allysine is controlled by the copper-dependent enzyme lysyl oxidase which targets lysine for deamination, over the course of three steps. Three allysine react with one lysine to form desmosine or isodesmosine; both of which are markers of elastin cross-linking. Elastin assembly occurs at microfibrils that contain non-elastin components of the elastin fibers, such as fibrillin-1 and fibrillin-2 and MAGPs. These microfibrils provide a scaffold for tropoelastin deposition, alignment, and cross-linking with desmosine and isodesmosine [16]. After the organized deposition of tropoelastin at the microfibril, the final enzymatic cross-linking process ensues to create the elastin fiber. This final enzymatic process ensures stability and reversibility when the fiber undergoes mechanical stress, such as stretching seen in Figure 1.4 [17].
Cardiovascular disease (CVD), as defined by the World Health Organization (WHO), is the name for a group of disorders of the heart and blood vessels. These diseases include hypertension, coronary heart disease, cerebrovascular disease, peripheral artery disease (PAD), heart failure, rheumatic heart disease, congenital heart disease, and cardiomyopathies. CVDs are the number one cause of death globally, equating to 17.8 million deaths in 2017, an increase of 21.1% over the past decade [18]. Moreover, the prevalence of CVD was 485.6 million in 2017, an increase of 28.5% over the past decade [18]. The larger issue lies in the low and middle-income countries that do not have access to the technology higher-income countries do. Low and middle-income countries have significantly higher CVD deaths per 100,000 as compared to high-income countries that
can pay for treatment [19]. According to the American Heart Association (AHA), in 2012, the direct medical cost for CVDs was $396 billion, with a projected increase of over two and a half times to $918 billion by the year 2030.

In the United States, the risk of CVD, and therefore cost for treatment and resulting deaths, is not decreasing, but rather, increasing. There are many factors that can attribute to developing CVD including a poor and unhealthy diet, tobacco use, insufficient physical activity, and excessive use of alcohol. Obesity, measured through body mass index (BMI), shows a strong correlation for increased risk of CVD, and resulting mortality [20]. According to the Centers for Disease Control and Prevention (CDC), in 1999, the national rate of obesity was 30.5% compared to 42.4% in 2018 — an almost 12% increase [21].
Individuals that have cardiovascular disease or precursors to CVD are recommended to be screened annually for chronic kidney disease (CKD). Chronic kidney disease

Figure 1.5: Heart disease death rate across the United State circa 2016 [18].

Figure 1.6: Obesity rates across the United States in 2018 [21].

Chronic Kidney Disease

Individuals that have cardiovascular disease or precursors to CVD are recommended to be screened annually for chronic kidney disease (CKD). Chronic kidney disease
disease, as defined by Mayo Clinic, is a disorder impacts the either the structure or function of the kidney for longer than three months. This disease affects nearly 37 million Americans annually, and accounts for almost 87,000 deaths a year [22]. CKD is more common in certain populations, such as the elderly, who are typically Medicare beneficiaries. In 2018, the cost to treat Medicare beneficiaries with CKD was almost $82 million. In addition to CKD, Medicare beneficiaries with end-stage renal disease (ESRD) account for an additional $36.6 million [23].

The number of individuals with CKD is much larger in countries outside the U.S., an estimated 5-10 million individuals die annually from some form of kidney disease, either acute or chronic, with estimations upwards of 7 million people dying from advanced kidney failure annually [24]. However, this disease is very difficult to estimate due to the insufficient access to the necessary laboratory equipment needed to diagnose and treat CKD. Doctors have long discussed how to best evaluate and classify risk for individuals with CKD. Currently, focus is on an international guideline, known as the glomerular filtration rate (GFR), which assesses the current functionality of the kidney through urinalysis. In total, there are five stages of kidney disease. Those in Stage 1 have some form of kidney damage, but overall normal kidney function, with the GFR being 60 mL/min. Those in Stages 2 - 4 have significantly more loss of kidney function, with Stage 2 having a GFR of 60 – 89 mL/min, Stage 3 ranging from 30 – 59 mL/min, and Stage 4 15 – 29 mL/min. Stage 5, the final stage, is commonly known as end-stage renal disease (ESRD), where there is total kidney failure, and an inability to operate at any acceptable capacity. At this stage, the GFR is less than 15 mL/min [25]. Although dangerous when
combined with other progressive diseases, if kidney disease can be detected early enough and is not in combination with other diseases, there is possibility to treat it and to revert the kidney back to normal operating standards [26].

However, once later stages of CKD evolve, especially ESRD, quality of life is diminished to many side-effects and limited treatment options. The main form of “treatment” for patients with CKD, and especially those with ESRD, is dialysis. Dialysis, however, is not a treatment to improve kidney function, and is more so designed to keep the body in balance through removing waste, excess chemicals and liquids from your blood to help homeostasis and control blood pressure. Dialysis is used for patients with ESRD as a “lifeline” while they wait for a kidney transplant. These transplants are often difficult and there are not enough donors to meet the demand for the number of kidneys needed for individuals with CKD. According to the CDC in 2021, 786,000 individuals are living with ESRD, 71% are currently on dialysis and the other 29% are living with a kidney transplant [23]. Additionally, the National Kidney Foundation (NKF) states that, on average, over 3,000 people are added to the kidney transplant waitlist each month.

As destructive as CKD is alone, it only worsens when combined with CVD. In combinatorial disease pathologies, CVD is the leading cause of mortality in CKD patients. Many patients that have CKD also have some of the risk factors associated with CVD, including hypertension, diabetes, tobacco use, and obesity. However, recent studies are suggesting that there are two main theories regarding the of pathophysiology that contribute CVD into CKD. The first theory suggests that the kidney releases different hormones and cytokines in response to the kidney injury which then causes changes in
vasculature [27] [28]. The second theory suggests that the lack of proper kidney function leads to hemodynamic changes and overall cardiac damage within the cardiovascular system [29]. Oftentimes, many times patients with CKD are at increased risk for cardiovascular disease. In later stages of CKD, such as Stages 4 and 5, more than 50% of patients encounter some form of CVD. Furthermore, it is seen that CVD accounts for roughly 40 – 50% of deaths in patients with CKD as compared to 25% for those who have normal kidney function[30, 31].

1.2.1 Vascular Calcification

Vascular calcification is a disease associated with the cardiovascular system in which minerals (i.e. Calcium Phosphate) are deposited into the walls of arteries, veins, and valves of the heart. Although low amounts are common in most individuals, abnormally high deposition of these minerals, specifically calcium salts, reduces vascular elasticity and hardens the artery. This impairs overall cardiovascular hemodynamics as arteries stiffen. The systolic flow in the artery decreases because the artery cannot expand when calcified. This then causes an increase in the diastolic flow based on the same principle Figure 1.7 [32]. In CKD patients, the inability to filter chemicals, specifically ionic salts, out of their blood causes vascular calcification to accelerate. Complications from vascular calcification are the leading cause of death for patients with CKD.
There are four ways that calcification can occur within the body. Intimal and medial vascular calcification, as well as heart valve calcification, are the most common forms. Calciphylaxis is the final and least common form of calcification and occurs when calcium salt deposits into the capillaries of fat and skin tissues. Intimal calcification is the final step to atherosclerosis and involves lipid deposition, macrophage infiltration, and advanced glycation end products (AGEs) seen in Figure 2.4 [33]. AGEs are known to contribute to the development of atherosclerosis because the proteins and lipids in the walls of the vasculature become exposed to sugars and are subsequently glycated [34]. The buildup of these products, in combination with inflammation, cause occlusion of the lumen of the vessel. The narrowing of the artery causes disruption in hemodynamic properties by reducing blood flow within the vessel and could possibly cause the plaque to rupture and cause and embolism and possible stroke. By contrast, medial arterial calcification (MAC, Figure 1.8) occurs when minerals diffuse through the artery into the medial layer of the

Figure 1.7: Depiction of elastic arteries before and after calcification of the artery.
arterial wall. MAC also causes hemodynamic disturbances by reducing elasticity in the artery and therefore hardens the artery [35]. The elastic modulus, which measures the ratio of force to deformation, significantly increases in animal models of vascular calcification showing the impact of the artery stiffening [36]. Downstream effects of this hardening can be severe and can include increased systolic blood pressure and left ventricular hypertrophy, which ultimately can lead to heart failure [37]. Although CKD patients can have both intimal and medial calcification, it is more common for patients to have medial calcification, which has been associated with high mortality rates.

When the calcium deposits into an artery, the extracellular matrix is disrupted, and elastin is damaged. There are two major issues occur due to vascular calcification. The first issue is that as we age the gene expression of ELN, which produces tropoelastin, significantly decreases and causes issues when attempting to repair elastin when it is damaged. Elastin’s half-life is 74 years and most elastin that is deposited into the ECM happens early in life. Elastin degradation occurs naturally over time, but in cases of MAC, this process is accelerated by both physical disruption from calcification and the initiated inflammatory process. Inflammatory cells can secrete three enzymes capable of damaging elastin and the ECM: matrix metalloproteinases, serine proteases, and cysteine proteases. As these enzymes degrade elastin, elastokines are released that exacerbate inflammation and lead to pro-degradation diseases, such as vascular calcification and AAA [38].
1.3 Animal Models of Vascular Calcification

Vascular calcification is a complex disease pathology that cannot be adequately studied using *in vitro* or organ culture models. Animal models offer a unique opportunity to evaluate the complex disease pathology *in vivo*. There are many ways to induce pathological vascular calcification in these models have been developed using many different methods to induce the pathology. Vascular calcification models can be generated with or without kidney damage, although newer models lean more to using some form of kidney damage to accelerate the arterial calcification. As previously stated, when kidney damage in the form of CKD presents itself in patients, vascular calcification is accelerated. Models that do not involve kidney damage instead use warfarin-induced calcification with vitamin K injections, vitamin D injections, or application of a low concentration calcium chloride patch.

The warfarin-induced calcification model is generated in rats through a combination of injections of warfarin and vitamin K [39]. In this model, warfarin induces
vascular calcifications similar to matrix Gla protein (MGP) deficient mice [40]. In healthy animals, carboxylation of MGP inactivates the protein, which helps inhibit calcification whereas in deficient animals this process is interrupted, and spontaneous calcification can occur. Vitamin K is used in this model to help restore the coagulation cascade in the animal. In the vitamin D induced models, high doses of vitamin D or vitamin D analogs are injected intravenously and induce vascular calcification by increasing the amount of calcium and phosphate circulating in the blood thereby stimulating vascular smooth muscle cells (VSMCs) that express pro-calcific proteins [41]. In rodent calcium chloride models, a calcium chloride soaked patch is applied directly to the abdominal aorta which creates severe medial calcification in the area of application [42]. This in turn causes the breakdown of elastin fibers.

Vascular calcification induced in models of kidney dysfunction are used more frequently in literature due to the metabolic abnormalities that compare well to human disease. The 5/6 nephrectomy is a model for mimicking kidney failure where portions of the kidney are removed surgically. In the classic 5/6 nephrectomy, one full kidney is removed in one procedure, and the other 2/3 of the second kidney is removed a week later [43]. There are many complications that can occur during both surgeries, including kidney infection and excessive bleeding, which ultimately can lead to animal death. However, newer methodology including cauterization of the arteries using electrocoagulation techniques can help reduce excessive bleeding. Moreover, a new method (1-PNx), that does not require any removal of the second kidney has been established by tying the upper
and lower poles of the kidney, seen in figure x.x have been introduced to reduce complications from the second surgery [44].

Figure 1.9: 1-PNx diagram describing the tying of upper and lower poles of the kidney to induce 2/3 kidney failure in the second kidney.

Diet-induced models create a CKD model that better mimics human CKD. These models create vascular calcification through diet, which is a more analogous process to humans. Adenine diets have been introduced to better mimic human systemic calcification. The enzyme adenine phosphoribosyltransferase (APRT) that degrades adenine becomes saturated due to high levels in the diet and therefore cannot degrade the adenine in the body. Excess adenine is oxidized to form 2,8 dihydroxyadenine (DHA), which has poor solubility in the kidney and cannot be easily filtered. Over time, precipitation of this product in the renal tubules commonly causes the formation of kidney stones [45]. If DHA builds up in the renal tubules for long periods of time, permanent renal damage can occur [45]. These models have become more active in current research because of the ease of creating and maintaining the model. Additionally, these models create changes within the body that are commonly seen in CKD patients, such as elevated levels of creatinine and blood urea nitrogen and the APRT deficiency in humans causes DHA buildup in the kidneys and eventually causes CKD which is directly what occurs in animals. However,
there are two major limitations of these models. Nearly 50% of the animals on the adenine diet do not calcify and the rigid diet routine and stiff pellets of the diet cause significant weight loss and increased mortality rate [46]. These models have been mostly created in rats, using 1.06% calcium and 0.92% phosphorus with 0.75% adenine to create medial calcification as published previously by our lab [47].

Figure 1.10: Morphological changes in kidney size and coloration between standard fed animals (b1) and adenine fed animals (b2) seen in [47].

1.4: Current Treatment Options

Patients with CVDs, especially those with MAC, have limited treatment options for revascularization of the calcified arteries. The current standard of care includes four surgical interventions that can be used to revascularize calcified arteries. The four surgical interventions are: balloon angioplasty, patch angioplasty, and bypass grafts, and endarterectomies. In theory, all the surgeries are safe, especially when surgeons that have years of experience are performing them. However, there are always general complications that come with surgery, such as infection, nerve damage, and organ damage resulting from potential scalpel nicking from the surgeon. In the case of vascular surgery, one major complication that can occur during active surgery is bleeding from the artery, and in the
In a case of femoral angioplasty, a possible infection of the artery that ultimately causes gangrene in the patient limb amputation. In patients with medial arterial calcification, the risk of these complications is increased.

Major invasive surgery is sometimes the only option available for correcting major vascular blockages. Patch angioplasty, bypass grafting, and endarterectomies are major surgeries that require a portion of an artery of interest to be clamped shut so the surgeon can operate on the artery. On occasions where patients show signs of MAC the artery cannot be properly closed and as result the operation cannot be performed. When attempting to clamp the artery shut, the medical calcification can stiffen the artery to the point where the blood flow cannot be completely occluded. In severe cases where the artery has lost elasticity or the artery can permanently deform from clamping causing permanent disruption of blood flow. The worst-case scenario is that a severely calcified artery can become brittle and “crack like an eggshell” leading to severe hemorrhage.

In an endarterectomy, a surgeon will surgically remove plaque buildup inside the artery by making a small incision in the arterial wall and scraping away unwanted plaque. Following the removal, the artery is stitched back, and the surgery concludes. In patch angioplasty, the inner arterial wall is exposed a patch, which is made synthetically or with venous tissues, expands the inner diameter of the arteries. When compared to primary closure of the artery, patch angioplasty has no higher complication risk[48], however, the amount of time the artery is clamped is significantly longer. Clamping the artery longer in MAC patients could have detrimental effects, such as deforming the artery and therefore changing overall hemodynamics. An artery bypass is the final extensive surgery used to
reroute the blood around the calcified artery or blockage. This procedure is most utilized when blockage of coronary arteries is present. The vessels that are used in this procedure are usually veins that are taken from a patient's own leg or wrist, or an artery taken from elsewhere in the chest, however the mortality risk one year after the procedure is high [49]. A study conducted by the Department of Cardiac Surgery in Iran examined patients over the age of 70 that had coronary artery bypass grafts to look at possible complications immediately following surgery. In these surgeries, complications such as stroke, hemorrhaging, and death are generally low. However, in their study, after surgery many patients experienced some form of acute renal failure, and many required ventilation after 48 hours [50].

Minimally invasive options pose less risk of complications to the patient. Balloon angioplasty does not require clamping of the artery and is one of the most used minimally invasive options for correcting reduced blood flow. In this procedure, a catheter is inserted near a blocked or narrowed calcified artery, where a balloon that is attached to the end of the catheter is inflated. When the balloon is inflated, the buildup of plaque and calcium in the artery are broken up and pushed against the arterial wall. In most cases, the balloon has a stent attached to it as the balloon is inflated and the stent is placed where the balloon has compressed the plaque. The stent keeps the vessel from reoccluding.

The two main classifications of stents used today are bare metal stents and drug eluting stents (DES). The main stents that are used today are drug eluting stents (DES). In 2018, studies were completed comparing bare metal stents to DES. Although the bare metal stent had no significant difference restentosis, primary patency rate, freedom from target
lesion revascularization, or stent fracture, there was a significant difference in terms of binary restenosis [51], which is considered when the artery has lost more than 50% of its diameter. With overall less restenosis, DES allowed for the stent to keep the vessel dilated and allow for adequate blood flow. The three major companies that manufacture DES are Medtronic, Abbott, and Boston Scientific. These three companies produce four different stent types and use three different drug types when coating their stents to offer a unique therapeutic option to help with restenosis in the artery that is being stented. The stent produced by Medtronic uses a synthetic polymer to attach the drug zotarolimus to the stent. When zotarolimus is attached their synthetic polymer, it allows for an extended release of the drug over 30 days that helps suppress neointimal hyperplasia [52, 53]. In reducing the amount of VSMC proliferation, the stent can reduce the restenosis in the artery. The stent that is produced by Abbott and one by Boston Scientific also uses a synthetic polymer on the surface of the stent to help adhere everolimus. Everolimus has a unique mechanism of action that inhibits the mammalin target of rapamycin (mTOR). Everolimus binds to an intracellular protein, FKBP-12, which is used in the mTOR complex 1 and renders the protein useless and inhibits kinase activity in the mTOR pathway[54]. In addition, everolimus can lower endothelial cell activation can be lowered by suppressing portions of the NF-kB pathway [55]. The final stent type produced by Boston scientific uses a biodegradable polymer (PLGA) to attach everolimus to the stent. Using biodegradable polymers improves biocompatibility by causing less inflammation, neoatherosclerosis, and late stent thrombosis than what occurs in stents coated in non-degradable polymers such as other synthetic polymers seen in other stents.
The largest issue with all these procedures is weighing the cost and benefits for patients. These surgeries are expensive and come with complications, and in patients that have diabetes or CKD, there are additional increased risks. Before surgical intervention, doctors will attempt to place the patient on statins, a lipid-lowering medication, in hopes to prevent further atherosclerotic plaque buildup within the arteries. However, if the medication does not seem to positively impact the patient, surgical intervention may be the only choice by current market options. To date, there is not a “drain-o” that can remove the calcification from the artery without major surgical operations. To further complicate matters, a majority of current research being done in vascular calcification focuses on preventative measures. With diagnosis of vascular calcification being particularly difficult, the preventative research often cannot be used once the patient is diagnosed. Instead, research done in the Vyavahare lab focuses on how to treat the disease in a responsive matter instead of a preventative one. Our research offers a unique opportunity to remove the calcium deposition without major vascular surgery and use nanoparticles through intravenous injections and create a “drain-o” type response to the disease.

1.5: Chelation Therapy

Chelation therapy is the utilization of chelating agents, such as ethylene diamine tetraacetic acid (EDTA), to remove divalent cations. The Food and Drug Administration (FDA) has only approved chelation for very specific cases, such as heavy metal poisoning. Chelation therapy has been used since the 1930s to treat people with heavy metal poisoning in both the U.S. navy and those impacted by chemical warfare. Today, chelation therapy is still the preferred method for treating many different forms of metal poisoning, especially
lead, arsenic, and mercury poisoning. EDTA forms a strong metal ligand complex with these elements and forms stable heterocyclic rings that then can be removed from the body through clearance by the kidneys.

![Figure 1.11: EDTA-Calcium complex formation](image)

Although good for removing toxic metals from the body, the side effects from chelation therapy are severe. EDTA has strong binding to these heavy metals, but also has binding affinity for any divalent cation in the body. This binding affinity can include metals that are necessary for the body to maintain homeostasis, such as calcium. EDTA chelation therapy can cause hypocalcemia, which in turn could cause bone resorption and remove the needed calcium for proper blood clotting. This treatment is not currently advised for individuals with kidney damage, which becomes a unique opportunity for research. As previously stated in section 1.2, vascular calcification is accelerated in patients with CKD, but chelation therapy, which may remove calcium deposition, could be useful if a therapy could be introduced that could reduce the overall amount of EDTA introduced into the kidneys, the therapy may be a viable strategy to remove calcium deposition from the arteries.
There are many different types of chelating agents that are used to chelate heavy metals. The various chelators have different binding affinities for each heavy metals, which is achieved by having different structures or mechanisms of action, making certain chelators more desirable than others. These chelators are mostly injected intravenously (IV), but some can be injected intramuscularly or taken orally [56, 57]. To date, only chelators that can treat lead, arsenic, or mercury poisoning are approved to be used in humans.

There have been two major studies that determined the efficacy of EDTA as a chelating agent for cardiovascular calcifications in atherosclerosis, PAD, and CAD. The Trial to Assess Chelation Therapy (TACT) and Trial to Assess Chelation Therapy 2 (TACT2) are NIH funded, stage 3 clinical trials, that assess the ability of EDTA to reduce atherosclerotic plaque and other possible mineral deposits from the arteries. All patients within this trial were over 50 years old and were more than six months removed from myocardial infarction (MI). There were four treatment groups within TACT to test the viability of chelation agents: IV EDTA with high dose vitamins, IV EDTA without high dose vitamins, IV EDTA without high dose vitamins, and IV EDTA placebo with and without high dose vitamins. The high dose vitamins did not reduce cardiovascular events, but the nonadherence of patients to the vitamin dosing schedule was high and may have impacted this result[58]. Although not significant from the placebo group, EDTA treatment

<table>
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<th>Cu</th>
<th>Hg</th>
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</thead>
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<td>8.79</td>
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<td>12.7</td>
<td>13.6</td>
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<td>16.5</td>
<td>18</td>
<td>18.4</td>
<td>18.8</td>
<td>21.5</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Table 1.1: Formation Constants for metal-EDTA Complexes
showed a moderate ability to reduce plaques with coronary revascularization occurring in 15% of patients [59]. This result was different in patients that had diabetes mellitus. In the diabetes group, there was a reduction in cardiovascular events, such as reinfarction or hospitalization for chest pain, and the chelation increased coronary revascularization [60]. This study also suggests that EDTA chelation is not significant enough to routinely use as a treatment to remove plaque and reduce cardiovascular outcomes. TACT 2 is a follow-up to the positive outcomes of the TACT 1 clinical trial. In TACT 2, the NIH is attempting to recreate the reduction of reoccurring cardiovascular events in diabetic patients post-MI. The participants in this clinical trial are modified from patients that have coronary artery disease to patients that have diabetes and a history of myocardial infarction. This study is to be completed in 2023 and seeks to compare the results from the two trials, as well as to make a final decision on the use of intravenous chelating agents to remove calcium deposition and minerals from arteries.

Despite the outcome of this trial, chelating agents have significant side effects to both metabolism and homeostasis. Intravenous EDTA can cause a negative calcium imbalance. Calcium is important not only for bone health, but also for proper nerve and muscle function. Calcium ions are released at neuromuscular junctions, which allows for muscle contraction and relaxation. A low amount of calcium in the body can cause muscle twitching and neuromuscular irritability [61]. Another major issue with intravenous EDTA is that calcium is removed from the blood, which then leads to bone resorption. In the TACT 1 clinical trial, almost 15% of patients had symptoms of hypocalcemia, which could indicate this side effect. Although the body is constantly remodeling bone, the removal of
calcific bone due to EDTA is quicker than normal and causes bones to become brittle and more likely to break. Therefore, to allow EDTA to be used for treatment of cardiovascular disease it is necessary to target the drug to a specific area of cardiovascular disease. This could provide a higher dose in the area of interest, whilst also reducing negative side effects of chelation therapy by reducing the proportion of circulating EDTA.

1.6: Nanoparticles as drug delivery systems

Nanotechnology offers a unique opportunity for developing systems that can target drugs to a region of interest thereby and specifically targeting the disease. Nanoparticles (NPs) have three distinct features provide advantages these goals. Firstly, the size of nanoparticles can be controlled to achieve a specific size that cannot be filtered by the reticuloendothelial system [62]. Secondly, the surface of nanoparticles can be modified, by attaching targeting molecules onto them such as antibodies and ligands [63]. Finally, nanoparticles can be prepared with varying degrees of stability in biological systems [64], making them advantageous to use as drug delivery systems. These features allow nanoparticles to target specific areas, focusing treatment to those areas and thereby lowering potential side effects. For application in patients with CVD and CKD, using EDTA-loaded NPs instead of free-EDTA would allow specific delivery of EDTA to the site of calcification using a novel targeting antibody and lower the needed dosage of EDTA put into the body. Lowering the circulating EDTA concentration will in turn reduce the chances of bone desorption and hypocalcemia in-vivo.

There are many different types of nanoparticles including dendrimers, polymeric, liposomal, and albumin nanoparticles that are attractive as drug delivery platforms due to
their high biocompatibility, biodegradability, and ability to encapsulate large amounts of drug. Despite these strengths, there are some challenges to successful use of nanoparticles in vivo, such as protein opsonization and variable durability. In the case of albumin nanoparticles, using a protein-based nanoparticle provides stable drug encapsulation and desirable surface chemistry for simple attachment of antibodies. These protein-based nanoparticles are still vulnerable to opsonization and subsequent removal from circulation by the mononuclear phagocyte system (MPS) [65]. However, surface modifications, such as PEGylation, shield against opsonization, reduce immunogenicity, and improve length of circulation which improves the success of reaching the desired target.

*Albumin based nanoparticles*

Albumin is one of the most abundant transport proteins found in the blood. Albumin is a highly investigated candidate for drug delivery because it is known to be non-toxic, relatively non-immunogenic, biocompatible, and biodegradable. Additionally, the albumin protein has multiple binding sites that allow for a high loading efficiency of many drugs. This protein is divided in literature by three major groups based on extraction source: ovalbumin (OVA), bovine serum albumin (BSA), and human serum albumin (HSA). To date, BSA is the most used and accepted form of albumin due to its ease of purification and low cost of production. Although more expensive, HSA may further reduce the immunologic response when use in humans since it is a more allogenic source. HSA particles are currently available on the market, such as Albunex™ and Abraxane™, and
have shown high drug delivery profiles and are overall well-tolerated in clinical trial patients.

There are many methods for synthesizing nanoparticles, such as desolvation, thermal gelation, stirring, emulsification, and nanospray drying. Each mode of synthesis attempts to optimize the size and loading to create the particles with characteristics ideal to the intended application. In the Vyavahare lab, desolvation and stirring methods have been used to make nanoparticles. In the desolvation method, albumin is coacervated in an organic solvent, such as 200 Proof ethanol, to phase-separate the albumin out of solution under constant agitation and create nano-sized particles. To prevent dissolution during purification in water, the particles must be hardened to stabilize the albumin nanoparticles. There are multiple methods for achieving this including the addition of chemical crosslinker or heat-induced permanent denaturation of the albumin. For chemical crosslinking there many available protein crosslinking chemical including glutaraldehyde, polyethyleneimine (PEI), methyl polyethylene glycol modified oxidized dextran (Dextranox-MPEG), formaldehyde, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). In our lab, glutaraldehyde is the preferred crosslinker to harden and stabilize our particles and is typically added immediately following desolvation.

**Antibody Targeting**

Targeting can be achieved both by passive and active mechanisms depending on the intended target. Passive mechanisms take advantage of how the cellular environment in
cancer works. NPs typically accumulate near tumor sites because blood vessels tend to leak into these environment and limit lymphatic drainage [66]. Active targeting mechanisms are more common since these strategies rely on targeting molecules or specific particle chemistry to direct particle accumulation and concentrate drug release at the target site. Surface modifications, such as antibodies or ligands, are applied that target to sites with specificity to a specific biological molecule with higher expression at the target location. In collaboration with Dr. Charles Rice, our lab has designed novel antibodies capable targeting degraded elastin fibers. By conjugating albumin-based nanoparticles with these antibodies we can target nanoparticles to sites of degraded elastin. In calcified arteries, the glycoproteins that are encapsulating the amorphous core of elastin are degraded and lost. When this amorphous core is exposed, it serves as the target for the anti-elastin antibody conjugated nanoparticles to bind to, seen in figure 1.12. In the case of calcified arteries, we use these targeted nanoparticles will release EDTA in close proximity to these vascular calcification sites. We have performed multiple studies in disease models incorporating pathological degradation of elastin, such as abdominal aortic aneurysms (AAA) [67], emphysema [68], chronic obstructive pulmonary disease (COPD) [68], and vascular calcification. In each of these models we have shown the capability of these nanoparticles to target the damaged elastin fiber.
Figure 1.12: Elastic fiber degradation and targeting of nanoparticles to the exposed amorphous core.
Current treatment for vascular calcification is significantly limited. There are four current clinical options that are approved to treat vascular calcification: endarterectomy, balloon angioplasty, patch angioplasty, and bypass grafts. Although all are viable options, they require surgical interventions that could cause complications and expenses to the patient. Moreover, patients with diabetes or CVDs are at increased risk for vascular calcification, and complications from these surgeries. The most unfortunate aspect is that most research being conducted for cardiovascular disease focuses on prevention and suppression of calcification rather than attempted removal. In most cases, patients with vascular calcification go undiagnosed and by the time they are diagnosed, preventative strategies and treatments are no longer a viable option. Therefore, the reversal of vascular calcification should be incorporated after diagnosis and resorb the mineralization that occurs within the artery. Resorption of calcium deposits must occur only at the site of mineralization in the artery and not in places where the mineralization is needed for healthy metabolism, such as the bones. The use of EDTA NPs conjugated with a custom anti-elastin antibody will allow for targeted release release the chelating agent to remove vascular calcification while reducing system effects of chelation therapy.

The Vyavahare lab has primarily focused on the preparation of nanoparticles using probe sonication-based methods for use in animal models of vascular calcification. Although this method works for animal models, sonication is not scalable to the industrial processes that would be used for clinical use with humans. This is in part due to the limitations on volume
that can be processed by probe sonication. **Aim 1** of this project seeks to conduct optimization of HSA nanoparticle synthesis using a stirring method that is compatible with scale-up for clinical application. **Aim 2**, focuses on the application of HSA nanoparticles in a mouse model of adenine-induced CKD and MAC. The goal is to be able to utilize this mouse model to evaluate a EDTA chelation therapy to remove calcium deposition.
CHAPTER THREE

AIM 1: OPTIMIZATION OF HSA EDTA NANO PARTICLES

3.1 Introduction

Patients with CKD have an increased risk for developing CVD. The leading cause of mortality in CKD patients is comorbidity with CVD which accounts for roughly 40 – 50% of deaths in patients with CKD. Comparatively, CVD only accounts for 25% of deaths in those for those who have normal kidney function[30, 31]. In many patients with CKD, vascular calcification occurs which reduces vascular elasticity and hardens arteries. This hardening can be severe and can lead to serious consequences including increased systolic blood pressure and left ventricular hypertrophy, which ultimately leads to heart failure [20].

Current therapies that are used clinically are limited. Often patients that have significant calcification in their arteries require surgical intervention. Besides the general complications that accompany major surgery, many forms of vascular surgery often lead to intimal hyperplasia, which could re-occlude the artery post-operation. Chelating agents offer an alternative approach for removing calcification via resorption of calcific deposits. The delivery of these chelating agents can be accomplished by injectable nanoparticle delivery. Previous studies in our lab have shown success in using bovine serum albumin (BSA) to create many different nanoparticles for delivery of therapeutics. When translating this to humans, there care several different issues that arise from using bovine-based nanoparticles including possible immunological response from repeated injections or even patients rejecting the treatment for religious reasons. Human albumin-based nanoparticles
can avoid these complications in clinical application of these nanoparticles. Here we attempt the optimization of a preparation method for human albumin-based nanoparticles. We also attempt to scale the nanoparticle preparation into a clinical setting regarding yield. The goal is to use these optimized nanoparticles for future treatment studies in an adenine mouse model.

3.2 Methods and Materials

*EDTA-Loaded Albumin nanoparticle preparation and conjugation*

EDTA-loaded NPs were made by dissolving HSA (Seracare Life Sciences, Milford, MA, 1850-0028) and disodium EDTA (Sigma-Aldrich, St. Louis, MO, 6381-92-6) in 4 mL of deionized water. The pH was adjusted with 6 N NaOH. The aqueous HSA+EDTA solution was added dropwise at 1 mL/minute to a set volume of 200 Proof ethanol in a 50 mL beaker on ice under probe sonication (Omni Sonic Ruptor 400 Ultrasonic Homogenizer, Omni International, Kennesaw, GA). Upon completion of HSA+EDTA solution addition, 8% glutaraldehyde was added for nanoparticle crosslinking. The solution was then sonicated for 1 hour on ice. The solution was then centrifuged, and the particles redistributed in water to wash. Nanoparticles were purified through two separate centrifugation washes.

For preparation of nanoparticle by stirring, EDTA-loaded NPs were made by dissolving 800 mg HSA and disodium EDTA (400 mg or 200 mg) in 16 mL of deionized water. The pH was adjusted to 8.5 with 6 N NaOH. The aqueous HSA/EDTA solution, was added dropwise at 1 mL/minute into 100 mL 200 Proof ethanol under constant stirring at
800 rpm. After desolvation, 100 µL of 8% glutaraldehyde was added by dropwise addition. The solution mixed for 2 hours to allow for adequate particle fixation and the particles were washed and purified through centrifugation (2000 rpm for 3 minutes, 6000 rpm for 10 minutes).

10 mg of fixated particles were incubated with 2.5 mg Methoxypolyethylene glycol maleimide (Maleimide-PEG-NHS ester, MW 2000 Da) to PEGylate the surface of the particles, seen in figure 3.1. Traut's reagent (34 µg, G-Biosciences, Saint Louis, MO) was used to thiolate 10 µg of humanized elastin antibody (Clemson University, Clemson, SC), and the mixture was incubated in PBS buffer for an hour at room temperature. Thiolated antibodies were rinsed with PBS buffer and were added to PEGylated NPs (4 µg antibody per 1 mg NPs) and incubated overnight for conjugation.

![Figure 3.1: Depiction of elastin antibody conjugation to nanoparticle.](image)

Figure 3.1: Depiction of elastin antibody conjugation to nanoparticle.
Characterization of HSA EDTA nanoparticles

The sizes of the HSA EDTA NPs were measured using a dynamic light scattering system (DLS) (Zetasizer Nano ZS, Malvern, Westborough, MA). For sizing, 20 uL of each sample were diluted in 1 mL of DI water in a disposable cuvette at room temperature. The sample was then triturated through a pipette tip to mix the solution. Three measurements were subsequently recorded and average. The absorbance setting for the material (i.e. protein) was set as 0.001 with a refractive index of 1.450.

Quantification of EDTA Loading and Release

Purified nanoparticles were dried by lyophilization and digested at in 6 N HCl at 50 °C for 2 hours. The resulting solution was neutralized with NaOH and diluted for analysis. To measure EDTA concentration 25 µL of 0.5% ferric chloride (Fisher Scientific, Waltham, MA, 7705-08-0) solution was added to 100 µL of sample or standard solution in a well plate and the absorbance was read at 257 nm using a plate reader (BioTek Synergy 2, BioTek, Winooski, VT). Alternatively, HPLC analysis was completed through using a mobile phase consisting of a 95/5 ratio of 0.01 M tetra-n-butylammonium at pH 3.0 (with acetic acid) and acetonitrile. The analytical column is a Zobrax RX-C18 analytical guard column (4.6 x 12.5 mm 5-micron) and ran at 40 °C and ran off for 15 minutes. Analysis was conducted by a poly-diode array detector at an absorbance of 255 nm.

Release of EDTA was evaluated using 10 mL of nanoparticle solution (2 mg NPs/ 1 mL of DI water). Nanoparticles were centrifuged and supernatants collected at the time points of 1, 2, 4, 8, 12, 24, and 48 hours. The pellet was redistributed in a fresh 10 mL of
solution at each time point. The EDTA concentration was measured by the previously described plate reader method use a BioTek Synergy 2 plate reader at 257 nm.

SEM imaging

HSA EDTA NPs were lyophilized and milled into a fine powder. The powder was taken from the lyophilized vial and smeared across a glass coverslip mounted to a SEM stub using double-sided carbon tape. The sample was taken to the Advanced Materials Research Lab (Clemson, SC) and sputter-coated with platinum for visualization by SEM. Images were captured using a Hitachi SU5000 Scanning Electron Microscope (10.0kV).

3.3 Results

Optimization of the preparation of EDTA-loaded HSA nanoparticles by sonication

Nanoparticles were synthesized using a previous BSA EDTA method and resulting particle size was 757.9 nm seen in figure 3.2, 3.5 times larger than the original method. Coacervation ratio was changed from a volume ratio of 4:1, to 5:1 and 6:1 (Ethanol:ddH2O). Figure 3.2 shows the decrease in particle size according to the literature and the polydispersity index. The coacervation ratio of 6:1 produced the best particle size at 458.6 nm, however, this size was over double the size that previously published BSA method and non-reproducible over multiple batches suggesting further optimization was needed.
The addition of an organic solvent DMSO was also evaluated to help improve particle size. Different reagents such as copper sulfate, sodium bicarbonate, and ferric...
chloride, were also used after sonication to attempt to remove excess EDTA from the surface. Neither of these two methods impacted particle size significantly enough to continue using them in the synthesis.

A range of pH’s were tested from 5.5 to 9. At pH 5.5, the particle size was almost near optimized in terms of size, being 351 nm seen in figure 3.4. The yield was as low at nearly 10% when using pH 5.5. We decided to keep the pH basic at 8.5 to 9, which is best for crosslinking by glutaraldehyde.

Several glutaraldehyde concentrations were evaluated based on previously published preparation methods including 5 µg/mg, 6 µg/mg, 7.5 µg/mg albumin were selected for trial. At 5 µg/mg, the particles came to 135 nm smaller than expected and yield was poor to 3.7%, moved up to 9.9% for 6 µg/mg but size did too, up to 389 nm, and at 7.5 µg/mg the particles were back at 494 nm (Figure 3.5).

Figure 3.4: Size distribution by intensity by DLS when changing the pH of aqueous solution before coacervation.
When 50 mg of EDTA was used instead of 100 mg, the particle size was not impacted a lot, moving from 458 to 451, but the PDI decreased from 0.499 to 0.247 suggesting that the solution was more monodisperse than the previous 100 mg EDTA NPs. The 50 mg of EDTA method was chosen as the optimized variable for subsequent optimization as seen in Figure 3.6.

Figure 3.5: Size distribution by intensity by DLS when changing glutaraldehyde concentration in the system after coacervation. Less glutaraldehyde decreased particle size.

Figure 3.6: Size distribution by intensity by DLS when changing the concentration of EDTA in the synthesis. 50 mg of EDTA produced smaller particles.
The method that was set for optimization was dissolving 200 mg HSA and 50 mg disodium EDTA in 4 mL of deionized water and adjust with NaOH to pH 8.5. While on ice and using a 50 ml beaker, the aqueous HSA/EDTA solution was added dropwise at 1 mL/minute into 24 mL of 100% ethanol while under sonication at 40 % power and 30 pulse. Upon completion of the dropwise addition, 8 % glutaraldehyde was added at 10 µg glutaraldehyde per mg of HSA powder. The solution sonicated for 1 hour on ice. The nanoparticles were washed and purified through centrifugation (6000 rpm for 10 minutes). This method created reproducible particles at 450 nm, seen in figure 3.7, with 40 % yield and 13 % loading (n=5 preparations). The nature of encapsulating drugs into nanoparticles allows for EDTA to release over time, with 40% of the drug released in the first six hours, and the entirety of the drug was released in 72 hours, as seen in figure 3.8. Following fixation, the particles were conjugated with a novel humanized elastin antibody capable targeting degraded elastin fibers. By conjugating our nanoparticles with these antibodies, we can target nanoparticles to sites of degraded elastin. The conjugation did not significantly increase the particle size, which remained at an average of 450 nm, seen in Table 3.1. The conjugated nanoparticles were suspended in 5% sucrose solution, frozen, and lyophilized to be used in the adenine mouse model.

Table 3.1 Characterization of Conjugated Nanoparticles

<table>
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<tr>
<th>Preparations</th>
<th>Z Avg. Size (nm)</th>
<th>PDI</th>
<th>Intensity Peak (nm)</th>
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</table>
Figure 3.7: (A) SEM imaging of humanized antibody-conjugated HSA/EDTA nanoparticles. (B) Higher magnification images of individual nanoparticles.

Figure 3.8: EDTA release profile from HSA/EDTA nanoparticles.
Discussion

Optimization of HSA/EDTA NPs at both laboratory and small industrial scaled volumes is a vital step when preparing for the commercialization of this technology. Several parameters were considered for optimization of the HSA/EDTA NP preparation method including coacervation ratio [69], solution pH [70], glutaraldehyde concentration [71], addition of organic solvents prior to coacervation, and EDTA concentration [72]. Adjusting the coacervation ratio was considered the easiest parameter to adjust without major losses to loading or particle integrity. When changing the coacervation ratio, nanoparticles lowered in size considered acceptable for in vivo application. As pH became more acidic the particles were smaller but yield significantly decreased due to reduced crosslinking efficiency by glutaraldehyde at the acidic pH. The crosslinking action of glutaraldehyde occurs via a Schiff-base reaction with imine formation. At low pH the nucleophilic nitrogen on HSA becomes protonated and will not react with the aldehyde. This was the most likely explanation for the low yield demonstrated in acidic pH nanoparticle preparations.

The next parameter evaluated was the concentration of glutaraldehyde added for crosslinking. The hypothesis for changing the glutaraldehyde concentration was to reduce the amount of unreacted glutaraldehyde and evaluating the amount of glutaraldehyde as the limit for crosslinking density. A similar result to the acidic pH method was observed when lowering the glutaraldehyde concentration, indicating that neither of these methods was desirable for preparation of HSA/EDTA nanoparticles.
In other nanoparticle syntheses in the Vyavahare lab, organic solvents such as DMSO and acetone are added to help the drug dissolve into solution [47]. We hypothesized that adding DMSO in low amounts would help the EDTA stay in solution and allow the HSA to encapsulate it while maintaining a lower particle size. When we attempted nanoparticle preparation with the addition of DMSO to the aqueous phase prior to coacervation. In this instance the particles were under 50 nm which suggests inadequate particle fixation and poor EDTA entrapment. A possible explanation is that DMSO solubilized the glutaraldehyde and prevented interaction with EDTA and HSA. Finally, we attempted to use different reagents to attempt to remove EDTA from solution by reacting it with a cation that EDTA had affinity for, however, this did not work and many of the attempts aggregated the entire solution in a solid ball of protein and drug.

EDTA seemed to create many difficulties when attempting to synthesize the optimal nanoparticle. The drug itself is a chelating agent and can impact the ionic solution by both binding to cationic ions in solution and act as a buffer. Changes in ionic strength can cause significant changes in nanoparticle size by either causing large aggregates of nanoparticles or the ionic strength can interfere with glutaraldehyde crosslinking [70]. In addition, disodium EDTA has relatively poor solubility in water in comparison to other EDTA salt solubilities. This inability to solubilize could cause lower loading and create the possibility for the EDTA to solubilize into the ethanol and not load into the nanoparticle. Therefore, the EDTA concentration was the last parameter considered for optimization of the preparation method because it was possible that this would result in reduced loading. However, since the targeted particle size of approximately 300 nm was not reached during
optimization of other parameters, this was the next logical parameter to evaluate. When the EDTA amount was lowered to 50 mg, the size was more acceptable at approximately 450 nm with approximately 13 % loading, as compared to previous methodologies seen in the Vyavahare lab [72]. The final parameter that was evaluated was zeta potential. The negative charge on the nanoparticles is crucial, so that they are not taken up by cells and remain extracellular to release drug into the ECM and not intracellularly where it would not have significant impact. The negative zeta potential on these particles matches other zeta potentials for particles from the Vyavahare lab and suggest that the particles will still release the drug extracellularly [72] [47]. Although the loading was not consistent with BSA preparations, this method provided acceptable size, loading, and zeta potential for continuing towards in vivo application.

Conclusion

We successfully optimized a human albumin-based nanoparticle to use both in an adenine mouse model scaled the nanoparticle preparation into a clinical setting using a stirring method. Through optimization, we were successfully able to create nanoparticles that were 450 nm in size with 10 % drug loading and 40 % yield.
CHAPTER FOUR

AIM 2: ADENINE INDUCED CHRONIC KIDNEY DISEASE IN MICE TO DEVELOP MEDIAL ARTERIAL CALCIFICATION

4.1 Introduction

Adenine diet animal models are routinely used to better mimic human systemic calcification. Precipitation and buildup of 2,8-Dihydroxyadenine (DHA) in the renal tubules for long periods of time can cause permanent renal damage if left untreated. Adenine animal models have become more active in current research because of the straight-forward methods for creating and maintaining the model. Additionally, the models create changes within the body that are commonly seen with human CKD patients, such as elevated levels of creatinine and blood urea nitrogen. One major limitation of this study is that 50% of the animals on the adenine diet do not calcify which makes diagnosing and treatment of the vascular calcification that can occur highly variable. These models have been mostly created in rats, using 1.06% calcium and 0.92% phosphorus with 0.75% adenine to create medial calcification as published previously by our lab.

In the current aim, we attempt to create an adenine model in mice that uses two different diet stages: one containing adenine 0.2%, Ca 0.6%, P 0.9% to induce CKD followed by a second diet containing adenine 0.2%, Ca 0.6%, P 1.8% to induce MAC. Following generation of vascular calcification, we will attempt to remove the vascular calcification via HSA EDTA NPs configured in Aim 1.

4.2 Materials and Methods
Renal failure was induced by feeding adenine diets having high P and Ca levels. High adenine feeding results in crystallization of DHA in renal tubules. Male C57Bl-6 and female DBA/2 mice were fed a customized adenine diet (Harlan Teklad, Madison, WI, USA) made with 0.2% adenine, 0.6 % Ca, and 0.9 % P. Mice were maintained on this diet for 42 days while their body weights were measured twice a week. If the weight loss of the animal exceeded a critical point of 20 %, animals were given a high glucose gel (DietGel Recovery, ClearH2O, Westbrook, ME) in conjunction with the adenine diet to recover lost weight.

*Micro CT imaging*

Micro CT imaging was used when the animals first arrived, at the end of each custom fed adenine diet, and the aorta was imaged after exsanguination ex-vivo to determine calcium deposition in the aorta. The animals were placed under light isoflurane anesthesia and imaged (Al 0.5 mm filter, 50kV, 500uA, 70 ms exposure) with a high-performance micro-CT (Skyscan 1176, Bruker, Billerica, MA). Post-processing reconstruction and imaging completed using the Skyscan Nrecon software based on the Feldkamp algorithm and Bruker’s CT-Vox software respectively.

*Treatment with EDTA nanoparticles*

For the treatment study, after mice were fed adenine diets for 84 days the mice were randomly separated into treatment groups for injection of saline, blank NPs without drug, or EDTA-loaded NPs. All injections were given twice a week for two weeks at a dose of 10 mg/kg body weight. All animals were euthanized by cardiac perfusion by saline while
under isoflurane and all organs being harvested along with the aorta from the heart to the iliac bifurcation and fixed in 10 % neutral buffered formalin (NBF). Serum from the blood sample was sent to AnMed Health Medical Center for biochemical analysis.

*Whole-mount aorta alizarin red S staining*

To complete whole mount alizarin images, the tissue was fixed in 95% ethanol for 18 hours at room temperature. The resulting tissue was cleaned thoroughly under a stereomicroscope in a PBS to remove excess fat that could not be removed when harvesting the aorta. In a 20 mL scintillation vial, the tissue was placed in an Alizarin Red S solution (0.003 % Alizarin in 1 % KOH) for 24 hours. After 24 hours the aortas were rinsed in 2 % KOH twice for five minutes each. The aorta was then rinsed with PBS. The aortas imaged using a stereomicroscope by compressing the artery between two glass slides.

4.3 Results:

Mice were fed two different custom adenine diets containing higher amounts of calcium and phosphate for 84 days. During this time, mice were weighed twice a week to determine overall weight loss. In this study, there were mortalities, possibly due to the low initial weight of the mice in conjunction with the rigidity of diet. However, most animals survived through the diet to the complete the treatment phase.
Figure 4.1: Timeline representation of diet schedule and treatment following end of diet feeding

MicroCT analysis was used at the end of the first 42 days on the diet to determine CKD. Figure 4.2 shows that the adenine formed crystalline DHA in the renal tubules of the kidneys and could be seen via microCT analysis. However, when blood serum analysis was run on these mice, the GFR was greater than 60, which suggests the kidneys are properly operating and no CKD is present. In larger animals, enough blood can be taken from the species to do blood serum analysis when the animal is still alive. Due to the necessary volume that is needed for analysis (>100 uL blood serum), this cannot be done in mice and analysis had to be completed via micro-CT analysis to check for CKD which was seen in-vivo. At the end of the 42 days, in coordination with the micro-CT results showing crystallization in the kidneys, the second diet was given to the mice to induce vascular calcification.
Upon completion of both diets, animals were subjected to twice weekly to injection of nanoparticles or saline for 3 weeks. Repeated injections of nanoparticles, especially at 450 nm, proved difficult. Many animals’ behavior changed upon injection. Some appear to return to baseline immediately after injection, however, a short while after, the animals become lethargic and struggle to regain balance after injection. It was seen if this pattern lasted for longer than 30 minutes, many of the animals died. There was also high variability how each individual box of mice took the injections. In one group of injections, many mice seemed to have issues with the injections and lost a portion of their tails or occurred necrotic tissue in their limbs. Ex-vivo imaging of the aorta in corn oil showed high variability with less than 50% of animals showing calcification via imaging. In addition, the very few animals showed calcification when completing the alizarin whole mount, meanwhile a couple animals’ aortas showed heavy calcification. Treatment of the aortas with EDTA NPs seemed to not work either. Of the 3 groups within the study, the group that received EDTA treatment were the only group to show calcification, while the blank

Figure 4.2: Micro CT analysis of the abdomen of the mice. A. Image of baseline abdominal reading before adenine diet. B-D. Images after 42 days on the adenine diet showing varying DHA formation in the kidneys.
NP and saline injected groups showed no calcification either detectable by micro-CT or whole mount alizarin red.

Figure 4.3: Representative whole mount alizarin red S staining. #73, #76, #78, #79, and #80 show the result after twice weekly injected EDTA-containing NPs for three weeks. #81, #82, #83, #102, #103 show whole aortas in alizarin red S staining when no drug was encapsulated inside the nanoparticles.

4.4 Discussion:

In this aim, we attempted to create an adenine model in mice using two different diets, to induce CKD and MAC [73]. Following the vascular calcification given from the second diet, we attempted to remove the vascular calcification via HSA EDTA NPs configured in
Aim 1. In general, this model was relatively poor in creating vascular calcification. The animals often struggled with their weight and had to be given the DielGel Recovery in order to help restore weight. We believe that the animals may have been eating minimal amounts of the adenine diet and knew that the DielGel would come eventually, and they would eat that instead of the adenine diet and not develop calcification. In addition, the strain of female mice was not ideal. Although DBA/2J mice in literature state the females develop calcification at an 80% rate [74], the animals had high variability from group to group when developing calcification only showed significant calcification on the exterior of the heart. Another factor that may affect this data is the amount of elastin fibers in a mouse aorta. In a mouse aorta, there 3-5 elastin fiber layers in the aorta. When the elastin fiber is damaged the amorphous core of elastin is exposed and targeting can occur. In other animal models, there are more than double the number of elastic lamina seen in mice. With so few elastic lamina in mice, the calcification that occurs in mice may destroy all elastin and targeting with EDTA NPs cannot occur as there is no elastin left to target. Finally, the COVID-19 pandemic caused many logistical challenges when attempting to do this research. Due to longer shipping times, both diets and DietGel Recovery were significantly delayed and caused the animals to change which diets they were eating at a certain point. This caused the animals to not consistently be on the adenine diet and the result to be inconsistent.

4.5 Conclusion:

In this aim, it appears that a mouse adenine model is not the ideal model for inducing dietary CKD and MAC. Although, in our hands the mice had a lower incidence rate than
what was recorded in literature. Additionally, there were complications from injections. It appears to be advisable to return to the adenine rat model that has been completed in our lab previously and has shown targeting in-vivo.
CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion:

Vascular calcification is a common form of cardiovascular diseases that affects many patients throughout the world, especially those that are elderly, have diabetes, or chronic kidney disease. The leading cause of mortality in CKD patients is comorbidity with CVD which accounts for roughly 40 – 50% of deaths in patients with CKD. Comparatively, CVD only accounts for 25% of deaths in those for those who have normal kidney function [1, 2]. In many patients with CKD, vascular calcification occurs which reduces vascular elasticity and hardens arteries. This hardening can be severe and can lead to serious consequences including increased systolic blood pressure and left ventricular hypertrophy, which ultimately leads to heart failure [20]. A novel therapeutic to remove vascular calcification is needed in healthcare to aid these patients to offer a better quality of life and attempt to restore vascular elasticity. The Vyavahare lab has developed a specific antibody that targets degraded elastin and can be conjugated to a nanoparticle for site-specific drug delivery to these sites. Delivering a chelating agent, such as ethylene diamine tetraacetic acid (EDTA), allows for removal of calcium deposition at these targets to help restore vascular elasticity.

In Aim 1, we were able to optimize nanoparticles using human serum albumin instead of bovine based albumin and upscale the batches for possible clinical application.
These particles were 450 nm in diameter, containing 13% drug released over 72 hours, and 20% yield on the synthesis. This model creates the ability to use these nanoparticles both in animal models and clinical applications. In Aim 2, we were unable to show the ability to create an adenine-diet induced CKD model of vascular calcification. Due to the inability to adequately track the kidney function throughout the model, the kidneys were never able to be stated if there was proper kidney function. The inability to create kidney damage in the model, although seen by microCT analysis, proved difficult in developing vascular calcification. Additionally, the treatment with EDTA NPs seemed to not properly work, by not removing vascular calcifications in the aorta, although it has been seen in other rodent work in our lab.

### 5.2 Recommendations for Future Work:

Although 450nm is acceptable for all forms of animal models and clinical application, to have better smaller particles for rodent models to have less side-effects that we saw in our mouse model. Therefore, reducing the overall size of the nanoparticles down to 200 nm, like other nanoparticles synthesized in our lab, would be the most ideal. New methodologies such as heat denaturation[75], high-speed homogenization, or high-pressure homogenization [76], offer the ability to create nanoparticles in ways that were not attempted, but could significantly impact the size, loading and yield characteristics of the nanoparticles. In addition, the crosslinker and drug are optimal at different conditions, EDTA at acidic and glutaraldehyde at basic. One suggestion could be changing the drug to a basic chelator or acidic crosslinker. An acidic crosslinker [77] such as adipic acid dihydrazide or pimelic acid dihydrazide to help stabilize EDTA in an acidic condition while
also being optimal for crosslinking may offer the ability to lower the size of the particles. The final recommendation would be moving back to the adenine rat model that has been shown successful in our lab previously. The mouse model not only was unsuccessful but there is minimal literature on adenine diet induced CKD models in mice whereas adenine rat models can be found frequently and abundantly in literature. If an adenine model was not necessary, there are multiple versions of the 5/6 nephrectomy that offer similar pathologies as the adenine diet models in quicker time, although two surgeries would be needed to remove both kidneys.
References


