UNIVERSITY OF CALIFORNIA, SAN DIEGO

Nanoparticle Enabled Enzyme Delivery for Cancer

A dissertation submitted in partial satisfaction of the
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Electrical Engineering (Nano-Scale Devices and Systems)

by

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University of California, San Diego

2017

DEDICATION

To my parents, who have loved me unconditionally and longer than I have known myself, who believed in me in times that I have forgotten to believe in myself, and who have encouraged me to listen to my heart and to follow my dreams. And to my little brother, my biggest cheerleader.

EPIGRAPH

Your time is limited, so don't waste it living someone else's life. Don't be trapped by dogma - which is living with the results of other people's thinking. Don't let the noise of other's opinions drown out your own inner voice. And most important, have the courage to follow your heart and intuition. They somehow already know what you truly want to become. Everything else is secondary.

—Steve Jobs

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PUBLICATIONS

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PATENTS

Sadik Esener, Negin Mokhtari, Mukanth Vaidyanathan, Yasan Yeh, Bartu Ahiska, Ajay Sapre“Nano-Scale delivery device and applications thereof“, provisional patent 15/449,83

Sadik Esener, Mukanth Vaidyanathan, Negin Mokhtari,Yasan Yeh “Multiple enzyme cascading nanoparticle system“, disclosure accepted, provisional review

Abstract of the Dissertation

Nanoparticle Enabled Enzyme Delivery for Cancer

by

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Doctor of Philosophy in Electrical Engineering (Nano-Scale Devices and Systems)
University of California San Diego, 2017
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Foreign enzymes have shown great promise as therapeutic agents, owing to their natural specificity, substrate selectivity, and validated mechanism of action; however, since most of these enzymes have bacterial sources, they are rapidly cleared by the immune system. Their relatively large size and delicate nature complicates their encapsulation in most current delivery technologies, resulting in their poor pharmacokinetics and survival through biological barriers, as well inefficient therapeutic effect. Thus, there is an imminent need for a platform that protects these enzymes from the immune system and efficiently delivers them to the target site. This dissertation examines two different nanoparticle platforms and their applications in enzyme delivery: synthetic hollow enzyme loaded nanospheres (SHELS) and enzyme-loaded silica-coated PLGA nanoparticles (SiLGA). The porous silica coating on the nanoparticles will allow them to operate like nanosharkcages,

where an enzyme (the scuba diver) is trapped in the nanoparticles (the sharkcage) and are only accessible to small molecules like their substrate (small fish), but not bigger molecules like antibodies and blood proteins (sharks). The application of SHELS (previously developed) is examined in the depletion of the amino acid, methionine, using the enzyme, methioninase (MethSHELS). MethSHELS are clearly superior to bare methioninase in vitro in terms of protecting the enzyme from proteases and inactivation by albumin, as well as a more widespread and sustained methionine depletion in vivo. In an attempt to develop a simpler synthesis process using FDA approved materials, as well as to address challenges associated with current technologies (e.g. limitation of cargo encapsulation by size and burst release of protein cargo), a new delivery platform, SiLGA, is introduced. SiLGA was successfully synthesized and characterized its encapsulation capabilities were also tested with multiple enzymes in vitro. SiLGA shows superior protection capability compared to bare enzyme. SiLGA retains more than %83 of its cargo's enzymatic activity in the presence of protease enzymes in vitro and exhibits exceptional localization in vivo for more than 60 days. While SiLGA has shown minimal toxicity in mice and cells, our preliminary results in enzyme prodrug therapy shows successful and selective prodrug activation by enzyme-loaded SiLGA and cytotoxicty against cancer cells. SiLGA is a new delivery platform and can be employed in several different therapeutic and diagnostic applications. The inert nature of the FDA-approved materials used to fabricate these nanoparticles, as well as their mechanism of action, gives a high edge to the practical use of SiLGA for enzyme delivery for cancer and other diseases.

Chapter 1

Introduction

Although numerous efforts have been made in the area of drug discovery for cancer treatments, the delivery of those treatments to the target site remains a great challenge. This is not only true for cancer therapies , but for the therapies of many other diseases. This issue has its roots in evolution, as our survival as species was largely based upon our body's ability to fight and clear any foreign agents. This serves a great purpose when the foreign agents are bacteria or viruses, however when it comes to immune clearance of therapeutic drugs or enzymes, the fast clearance of these agents from the body becomes the main biological barrier to achieving therapeutic effect. In addition to delivery, the systemic and non specific toxicity of conventional therapies themselves has become a great issue to patients and as it is often heard in a clinical setting from doctors and patients, "sometimes the side effects of the chemotherapy drugs are worst than the cancer itself". To overcome this non specific and systemic toxicity effect, development of more targeted therapies is necessary. Enzymes have a natural specificity for their substrate and can be used as a therapeutic agent or be used in combination with conventional chemotherapy drugs. However most of the enzyme therapies utilize foreign enzymes which are mostly derived from bacterial sources giving them low half lives and poor pharmacokinetics in the body. In most methods, the enzyme or drug is either modified directly (like PEGylation) which not only will cause partial loss of enzymatic activity but also ultimately is met with limited success of protecting the enzyme from immune clearance, or by encapsulation of the payload

inside or on the surface of different types of nanoparticles like polymers. However specially in the case of payloads like enzymes, due to the relatively large size of some enzymes and their delicate nature, encapsulation of enzymes inside nanoparticles has had limited success. To address these issues, this dissertation will go over two different kind of nanoparticle delivery platforms. These nanoparticles will operate like nanosharkcages where a scuba diver (the enzyme) is trapped in the nanoparticles (the sharkcage). The bars of the shark cage are big enough for small fish (substrate and products) to get in, but too small for the scuba diver (enzyme) to get out and for sharks (antibodies, large serum proteins like albumin) to reach the scuba diver, thus keeping the scuba diver safe. The porous silica coating utilized on the surface of these nanoparticles is what gives it the shark cage quality. In addition to the porous nature of silica, it is an excellent candidate due to its biodegradability, bio-compatibility, low toxicity, adjustable porosity, and thermal and mechanical stability, making it suitable for *in vivo* applications [1, 2, 3, 4, 5, 6]. In comparison to other polymeric drug delivery vehicles, silica nanoparticles are more resistant to pH, heat, mechanical stress, and degradation by hydrolysis[6].

This dissertation will begin with an introduction to cancer, conventional cancer therapies including an introduction to enzymes and enzyme therapies, and the biological barriers for drug and enzyme delivery, followed by an overview of current enzyme delivery technologies and their challenges and shortcomings.This chapter will also briefly discuss the idea behind nanosharkcages.

Chapter 3 of this dissertation will focus on Synthetic Hollow Enzyme Loaded Silica nanospheres (SHELS), its application in amino acid depletion of methionine for cancerous tumors using the enzyme methioninase via methioninase loaded SHELS (MethSHELS). MethSHELS will be synthesized and characterized *in vivo* and *in vitro* and their future potential and shortcomings will be discussed.

In chapter 4 a new nanoparticle will be introduced: silica coated enzyme loaded PLGA nanoparticles (SiLGA). Beta Lactamse will be used as a mock enzyme and BLA-SiLGA's functional and structural characterization *in vitro* as well as its *in vivo* tissue residence time in mice will be discussed.

Chapter 5 will explore SiLGA's application for enzyme prodrug therapy pre-

liminary results using horse radish peroxidase encapsulated SiLGA (HRP-SiLGA) and indoleacetic acid (IAA) as the nontoxic prodrug. Successful synthesis of catalase loaded SiLGA (CAT-SiLGA) will be discussed and their potential applications in antioxidant therapy and diagnostics will be highlighted.

The dissertation concludes by a summary of results and discussions as well as future directions for MethSHELS and SiLGA nanoparticles.

Chapter 2

Background

2.1 Cancer

2.1.1 What is Cancer

Cells are the building blocks of the body. We have more than a hundred million cells in our bodies.New cells are constantly made and replace the old cells, while the old cells will go through programmed cell death (Apoptosis). This process in our bodies is highly regulated.

Cancer develops when the body's controlled mechanism of cell growth and death stops working properly. This abnormal cell growth and division forms a mass called a tumor. Some cancers like leukemia (blood cancer) do not form solid tumors.

Cancer is among the leading causes of death worldwide. In 2012, there were 14 million new cases and 8.2 million cancer-related deaths worldwide and it is projected that the number of new cancer cases will rise to 22 million within the next two decades [7]. According to the American Cancer Society, the probability of developing cancer during one's lifetime is one in two men and one in three women. For a cell to become cancerous, the cell goes through genetic changes. The nucleus of the cell contains our DNA, that is made of genes (figure 2.1) . The genetic information contained in the DNA is in the form of a chemical code, called the genetic code. This code tells the cell what proteins or RNA to produce and

holds instructions for the cell's life span,function, maturity, and death. Errors and mismatches can happen on gene as the cell is dividing. These errors are called genetic mutations. Among other things, mutations can cause a cell to undergo uncontrolled cell division and bypass checkpoints for programmed cell death and the cells will start to grow out of control. However mutations are common in our cells and are usually corrected and repaired. However overtime the mutation can build up and this effected can be intensified as more abnormal cells continue their fast growth rate and acquire mutations.Thus cancer in general is not a single mutation but an accumulation of mutations (figure 2.2. The most prominent genetic mutations in cancer are through mutations in proto-oncogenes, tumor suppressor genes, and DNA repair genes. These changes are sometimes called "drivers" of cancer [7]. A proto-oncogene is a normal gene that codes for proteins that helps to regulate normal cell growth and differentiation. Once mutations or over expression occurs in the proto-oncogene it becomes an oncogenes, allowing cells to grow and survive when they should not. Tumor suppressor genes are involved in cell control of cell division and once altered can result uncontrolled cell division. The DNA repair genes are involved in repairing damaged DNA. Mutations in these genes can cause a spiral effect of mutation in additional genes [7].

Mutations can be caused by random errors, but the majority of cancers (%90-95) are caused by environmental factors such as tobacco (%25-30), diet and obesity (%30-35), infections (%15-20), radiation (both ionizing and non-ionizing, up to %10), stress, lack of physical activity and environmental pollutants [8]. And some people can inherit genetic mutations that make them more likely to develop a cancer (%5-10).

Not all type of tissue abnormalities leads to cancer. Some could develop to cancer, but others are just monitored and do not develop to cancer (figure 2.3). For instance, cells can grow fast within a tissue due to chronic irritation or other factors but look normal under the microscope (hyperplasia). Disyplasia is a more serious forms of hyperplasia where cells look abnormal under microscope but are not cancerous. When these abnormal cells are containced within the tissue they are called carcinoma in situ and if detected,are usually treated. Carcinoma in situ is sometimes considered as stage zero cancer. Even so, many carcinoma in situ will

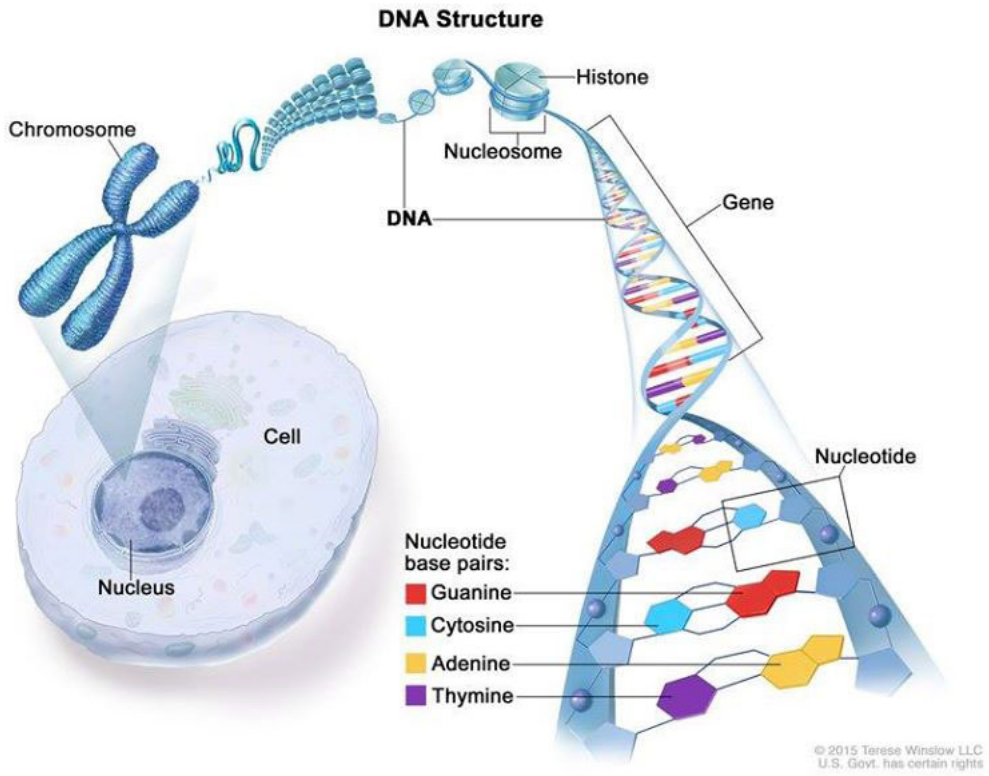


Figure 2.1: The cell and DNA [7]

not develop to cancer.

As the cancer cells continue its abnormal growth, a tumor is formed. A tumor can be benign (not cancerous) or malignant (cancerous). Benign tumors usually grow quite slowly and don't spread to other parts of the body (they don't metastasize). If these cells break through the basement membrane of the tissue the cancer is considered invasive. As the tumor gets bigger, its need for nutrients increases. This leads to tumors building their own blood supply from existing blood supplies (angiogenisis). Once this step happens, the tumor growth rate picks up. This is the reason why many drugs target angiogenesis (like bevacizumab (Avastin) that stops angiogenesis by blocking vascular endothelial growth factor (VEGF) from attaching to VEGF receptors that line the blood vessels).

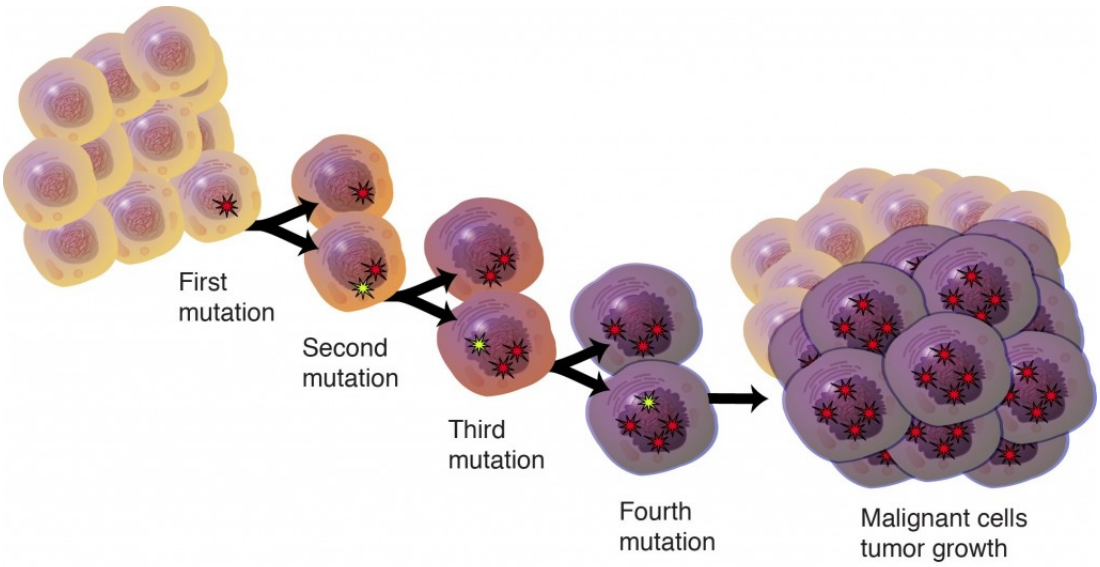


Figure 2.2: Cancer and mutations from http://kintalk.org/genetics-101/.

2.1.2 Metastasis

The original location of the tumor is referred to as the primary cancer. When cancer cells have the ability to spread to other places in the body, they are referred to as metastatic cancer and the process that they spread throughout the body is called metastasis (figure 2.4). As we will see later, cancers are named after the original tumor side, not the metastasis site. The metastasized tumor cells usually have similar traits as the primary tumor. Once the tumor cells start to metastasize, it becomes very difficult to treat cancer. Thus one of the main ways of tackling cancer is through early detection, where cancer can be detected at its early stages, specifically before metastasis.

The events leading to cell metastasis is commonly referred to as the invasion-metastasis cascade.The epithelial cells in primary tumors metastasis is as followed: (1) local invasion to neighbouring tissue (2) intravasate into blood vessels, (3) surviving circulation (4) arrest at distant organ sites, (5) extravasate into distant tissues, (6) adjust and survive the new microenvirnment and form micrometastases (7) Re initiate growth and proliferation (metastatic colonization [9](Figure 2.5)

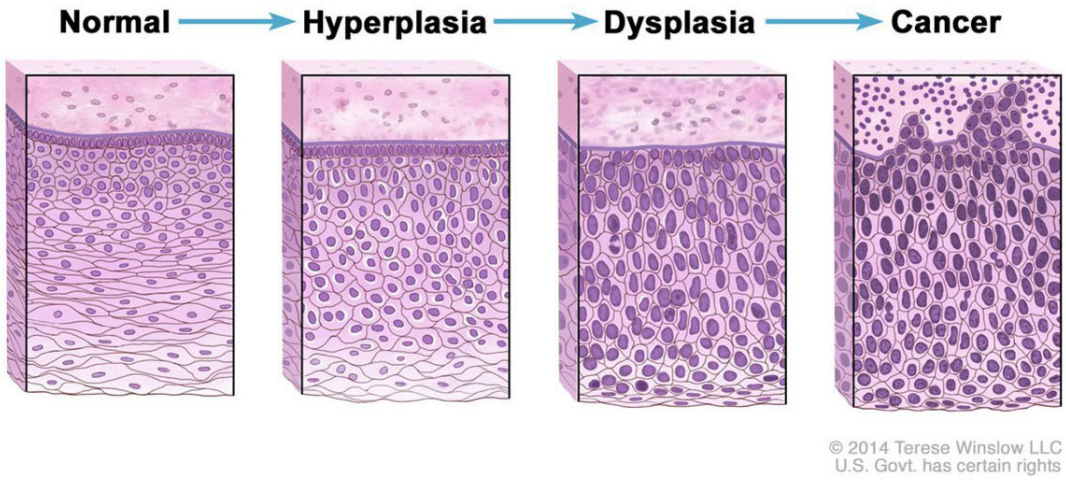


Figure 2.3: Normal and abnormalities in cell growth [7]

2.1.3 Types of Cancer

The heterogeneity of tumors as well as the individuality of each person's genetic mutations in cancer, is why cancer is no longer thought of as one disease, but a subtype of more than 100 diseases.

Cancers are usually named after after the organ where it originated or the type of cell the formed the cancer cells. Some of the main types of cancers are mentioned below [7]:

Carcinomas are cancer of the epithelial cells and are the most common type of cancer. Adenocarcinomas are cancer of the epithelial cells that produce fluids (like breast, prostate), squamous cell carcinoma originates in squamous cells, basal cell carcinoma originates in the basal layer of the epidermis, and transitional cell carcinoma originates transitional epithelium.

Sarcomas are cancers the originate in the bone and soft tissues such as muscle, fat, blood vessels, lymph vessels,nerves and fibrous tissue (like tendons and ligaments).

Blood cancer, are cancers that originate in the blood cells. The most common blood cancers are multiple myeloma, leukemia and lymphoma. Multiple myeloma is a cancer that originates in a type of white blood cells known as plasma

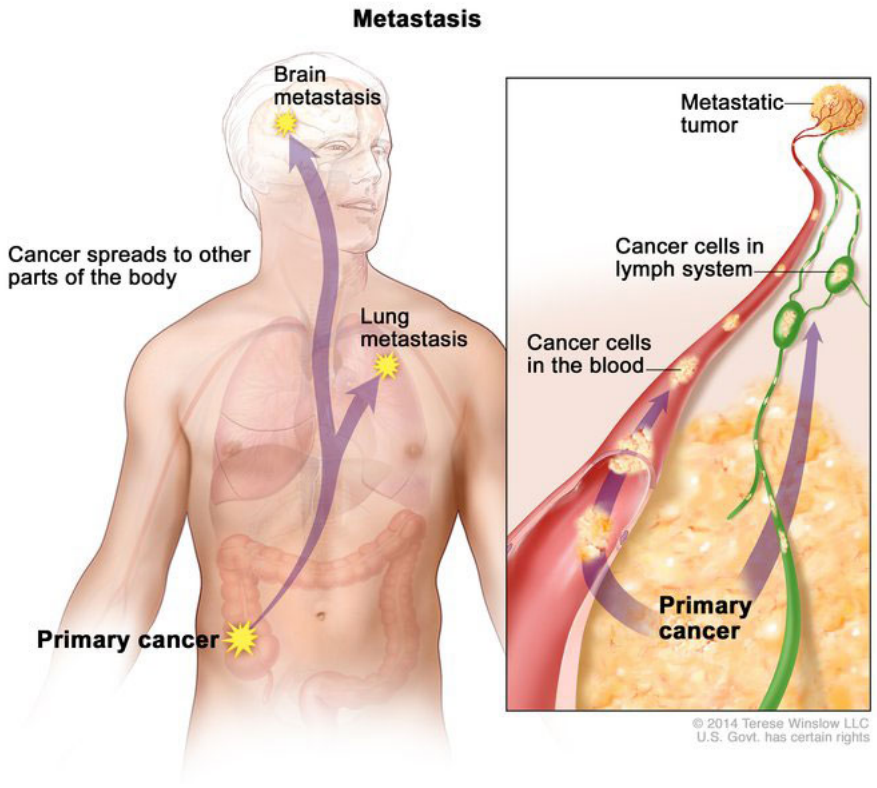


Figure 2.4: Cancer metastasis [9]

cells that can accumulate and overcrowd the bone marrow.

Leukemia originates in immature blood cells that overcrowd the bone marrow and normal blood cells. Leukemias are grouped based on how fast the disease grows (chronic and acute) as well as the blood cell it originates in (lymphoblastic or myeloid).

Lymphoma (or non-hodgkin lymphoma) is a cancer that begins in lymphocytes (T cells or B cells). Hodgkin lymphoma, is marked by the presence of an abnormal lymphocyte called the Reed-Sternberg cell (derived from B lymphocyte). About %90 of lymphomas are non-Hodgkin lymphomas

Melanoma is cancer that begins in cells that become melanocytes (cells that make melanin).

Germ cell tumors are tumors that originate in the germ cells.

Brain and Spinal Cord Tumors, are cancers that originates in the cells of the brain and spinal cord like astrocytic tumors that originate in astrocytes.

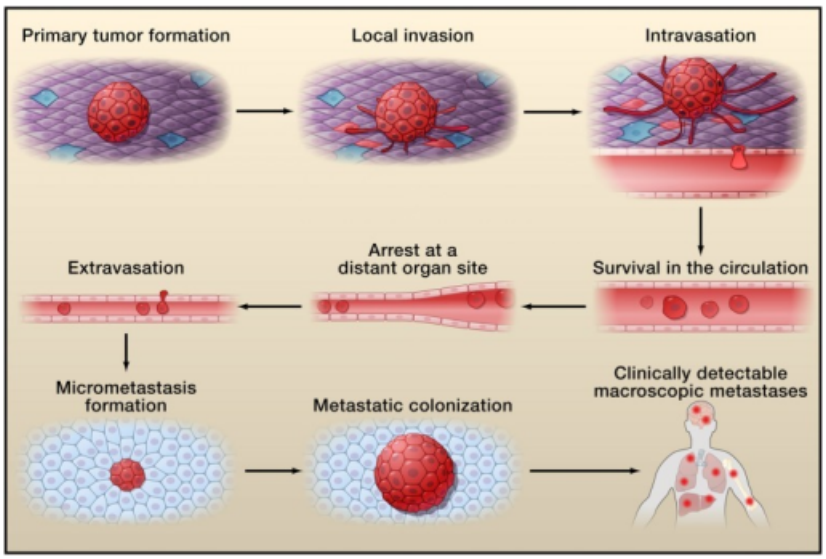


Figure 2.5: Metastasis of tumor cells. Tumor cells initially leave the primary cite and locally invade and intravesate through neighbouring tissue, transport and survive circulation, arrest at a distant tissue and extravegate, form micrometastasis and later metastatic colonization [9].

Endocrine tumor originate in hormone releasing cells. The tumor cells developed are able to release hormones as well. A neuroendocrine tumor originate in cells that are a combination of hormone-producing endocrine cells and nerve cells.Carcinoid tumors are a type of neuroendocrine tumor. They are slow-growing tumors that are usually found in the gastrointestinal system

2.2 Treatments for Cancer

2.2.1 Conventional Therapies for Cancer

There are many different approaches to treat and kill cancerous tumors. The most conventional ways are : surgical removal of the tumor, radiation therapy where high doses of radiation is used to shrink or kill tumor cells and chemotherapy where chemical drugs are used to treat tumors. Hormone therapy can also be used in cancers like breast and prostate (where hormones promote cancer growth) by blocking the body's ability to produce hormones or interfere with hormonal functions.There are many newer areas for cancer therapies such as immune therapy

that uses the bodies own immune system to recognize and destroy cancer cells.

2.2.2 Limitation of Conventional Cancer Therapies

While many of these treatments have been the standard of care for a very long time, they have had limited success in the past [10]. Many tumors are non-operable and even after operation require additional adjuvant chemotherapy. Chemotherapy itself is limited by several drawbacks, including systemic toxicity, lack of specificity and selectivity for cancer cells vs normal cells, immune clearance and low circulation half lives, insufficient drug concentrations in tumors, solubility and stability *in vivo* (specially for hydrophobic drugs),inability to pass through the blood brain barrier (BBB) for applications in brain tumors, development of major and unwanted side effect and development of drug resistance due to repeated administration and dosing [11, 12, 13, 14, 15, 16]

2.3 Enzyme Therapy for Cancer

Enzymes have been utilized in many different therapies. For instance in enzyme pro-drug therapy for tumors, an inactive and non toxic form of a drug (pro-drug) is systemically administered and activated to its toxic and active form by an enzyme at the tumor site [11, 17, 18]. Another example is depletion of amino acids that are essential for tumors growth but their depletion does not effect normal cells. For instance L-aspargin depletion using Asparaginase has for treatment of childhood acute lymphoblastic leukemia [19, 20, 21, 22],methionine depletion using Methioninase e for tumors with p16 deletions that also affect methylthioadenosine phosphorylase (MTAP) [23, 24] and arginine depletion using arginine deiminase for treatment for hepatocellular carcinomas and melanomas and prostate cancer tumors [25, 26, 27].

The natural specificity and selectivity that enzymes have for their substrates makes them a highly specific tool for targeted therapy. More over the high growth rate of cancer cells and their increase need for amino acids, provides a unique opportunity to deplete amino acids using enzymes.

2.3.1 Enzymes

To gain a better understanding of enzyme therapy for cancer and its advantages and challenges, a deeper knowledge of enzyme-substrate interaction is needed. Enzymes are proteins that accelerate and catalyze reactions while they are not used in the reaction themselves. Different enzymes can be active in different temperatures and PH. While increased temperatures can lead to increased enzymatic reaction rates, dramatic changes in PH, high temperature and salt concentration, can lead to enzyme denaturation and thus lead to enzyme inactivation. Some enzymes, like Methioninase, have cofactors. A cofactor is a nonorganic molecule that are essential for enzymatic function. This means that if this cofactor is sequestered from the enzyme, the enzyme will be inactivated.

When an enzyme binds to its substrate it forms an enzyme-substrate complex. After the reaction is completed, the enzyme will return to its original state. Enzymes are very specific to their substrates. In the past the enzyme-substrate binding was though of as a lock and key model while nowadays this binding is thought of more as an induced fit model where when the enzyme and substrate interact there is a mild shift in the enzymes structure to accommodate the substrate and have an optimum fit (figure 2.6).

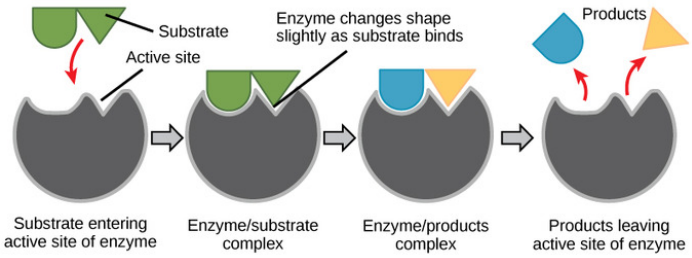


Figure 2.6: Enzyme-substrate interaction (from OpenStax College, Biology, CC BY 3.0)

One of the best known model for enzyme kinetics is described by Michaelis-Menten kinetics. Many enzymes follow the Michaelis-Menten kinetics where the rate of catalysis (which is defined as the number of moles of product formed per second) increases linearly as the substrate concentration [S] is increased and then

reaches a plateau where the active site of the enzyme is saturated with substrates (figure 2.7. The Michaelis constant (KM) is the substrate concentration yielding a velocity of Vmax/2 and is a measure of enzyme-substrate affinity.

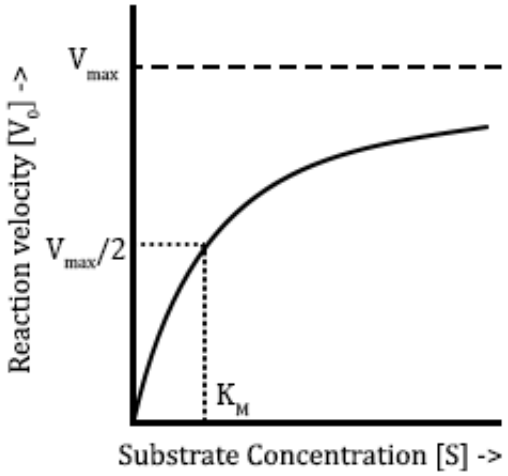


Figure 2.7: A generic example of an enzyme following michaelis-menten kinetics. In this plot of the reaction velocity (V0) changes as a function of the substrate concentration [S]. Vmax is the maximal velocity reached by the system and the michaelis menten constant (Km) is where the substrate concentration yields a velocity of Vmax/2.

2.3.2 Limitations of Enzyme Therapy

Recombinant enzymes have a foreign nature, and this fact by itself results in rapid immune clearance from the body. Hence even though recombinant enzymes are highly specific for their substrates and have shown great promise for cancer therapy applications, their clinical use in the past have been limited to their poor pharmacokinetics and rapid clearance by the immune system [28, 29, 30, 16]. For many enzymes that have cofactors this issue becomes even more challenging. Even though methioninase showed great promise in clinical trials it was later discovered that its cofactor (pyrodoxal phosphate) was rapidly sequestered by albumin in the blood, causing enzyme deactivation [31, 32]. The most common attempt at increasing enzymatic half life is the use of poly(ethylene glycol) (PEG) [33, 31, 34].

Although increased circulation half lives and decreased clearance is observed in this method, enzymatic activity is significantly reduced due to direct modification of the enzyme with PEG. This method also can result in antibody generation against PEG, weaken retention at the target site, degradation of PEG itself and increased costs and optimization due to direct and specific enzyme modification and ultimately might not result in complete enzyme protection [35, 36, 31].

There for there is an great need for a platform that protects the enzyme from antibody neutralization, immune clearance and cofactor loss *in vivo* without directly modifying the enzyme itself and at the same time allow substrate access to the enzyme.

2.4 Nanoparticles are the Trojan Horse of the War Against Cancer

Many current therapeutic agents and chemotherapy drugs are very potent highly cyto toxic to cancer cells, however the main issue is that they are non specific. Meaning that they have the same effect and toxicity to normal cells. Thus while new drug discoveries are necessary, safe and targeted delivery of these agents is of high importance as well. Delivery is a very important issue, not only in cancer therapeutics or diagnostics, but also in any disease or condition that relies on availability of a therapeutic agent to a specific organ or site. For this purpose, nanoparticles are excellent candidates. Nanoparticle delivery platforms have shown to overcome pharmacokinetic limitations associated with conventional drug formulations [37]. Nanoparticles are though of as the "Trojan Horse" for cancer drug/enzyme delivery. In the historic sense of the word, the Trojan horse was a hollow wooden horse constructed by the Greeks to gain entrance to the city of Troy during the Trojan war. The Greeks pretended to desert the war, while some of their soldiers were hiding inside the wooden horse. The Trojan's saw the horse as a defeat offering and it was taken into the city. At night the Greek warriors emerged from the horse and opened the gates to let the Greek army in and overtake the city. The therapeutic cargo can be delivered in the

same manner. The goal of delivery via nanoparticles is for the nanoparticle to act like a Trojan horse and hide the cargo from the immune system and other barriers that might destroy the cargo and safely deliver the cargo from the site of injection to the specific target. Depending on the therapeutic end goal, the goal can be to stay in circulation, or delivery to the interstitial space, intracellular, etc. Therapeutic agents as well as their delivery platforms can be administered via different routes such oral, intravenous (IV) , intraperitoneal (IP), intramuscular (IM), subcutaneous or direct intra tumoral injections (IT).

2.4.1 Biological Barriers for Drug Delivery

The journey of a therapeutic agent, or nanoparticle containing the therapeutic agent to their target side is indeed a long one. Even though nanoparticles do offer protection for their cargo, they themselves must overcome different obstacles to reach their target. Thus nanoparticle platforms need to be designed in such a way to maximize their chances of overcoming or bypassing these barriers and allow for achievement of the desired therapeutic effect. Of course there are scenarios in diseases when one can take advantage of the altered physiology resulted by the disease to improve delivery to the disease site, such as the Enhanced Permeation and Retention (EPR) effect.Cancer cells have increased need for nutrition due to their fast rate of growth thus they will build their own blood supply from existing blood supplies (angiogenisis). Since this process is done very fast, the tumors are known to have "leaky vasculature", hence the enhanced permeation. In addition to this, tumor tissues usually lack effective lymphatic drainage, hence the enhanced retention effect. Nanoparticles can exploit this effect to accumulate more in the tumor tissue compared to the normal tissues (also called passive targeting)[38]. An example is Doxorubicin, a very well known and potent chemotherapy drugs, limited by its major cardiotoxcity. The FDA approved liposomal doxorubicin and Pegylated liposomal doxorubicin (doxil), is nowadays widely used in the clinic and has shown reduced cardiotoxicty compared to patients who use conventional doxorubicin [39]. Even though these improved results have been shown with these delivery platforms compared to the conventional form of the drugs, the results

are still not optimum and overcoming biological barriers is still a great challenge for many forms of delivery vehicles. It is important to bare in mind the route of administration and the therapeutic end goal can significantly alter the biological barrier that the nanoparticles will go through. For instance upon systemic injection of nanoparticles, they will encounter opsonization and formation of the protein corona. Upon administration of the nanoparticles, biological molecules such as plasma proteins like serum albumin, lipids, apolipoproteins, complement components and immunoglobulins will adsorb to the surface of the nanoparticles, this is known as the protein corona [40, 41, 37]. The biomolecules with higher affinity will have a tighter bound to the nanoparticles will form the hard corona while the ones with lower affinity and looser bounds will form the soft corona (figure 2.8 from [42]) the composition of the protein corona is unique to each nanoparticle and is a function of the nanoparticle properties such as size, surface charge, shape, curvature, composition, surface functional groups, hydrophobicity as well as the physiological environment that the nanoparticle is exposed to such as blood, interstitial fluid, intracellular environment as well as the nanoparticles duration of exposure to those environments [40]. Moreover, the composition of the corona changes according to the disease status and it could differ for each person [43, 44]

Upon opsonization and formation of the protein corona, the nanoparticles become recognizable and are taken up by the reticuloendothelial system (RES). The RES system is a part of the immune system and is made of phagocytic cells primarily monocytes and macrophages accumulate in lymph nodes,spleen, liver and tissue resident histiocytes. Nanoparticles, particularly liposomes , are very prone to serum mediated opsonization and recognition by the phagocytes of the RES [45]. This uptake can results in non specific distribution where high accumulation of nanoparticles can be seen in organs, such as the spleen and the liver.There are many strategies that are used to camouflage the nanoparticles to prevent or decrease opsonization. The most known way is by PEGylation, where nanoparticles have poly ethelyn glycol as a functional group on their surface. PEG will attract water molecules and create a hydration layer around the nanoparticle [46, 47].However, the PEG effect is transient, so eventual opsonization and macrophage clearance still occurs [48]. Attachment of CD 47 peptides ('self' peptides) [49], coating

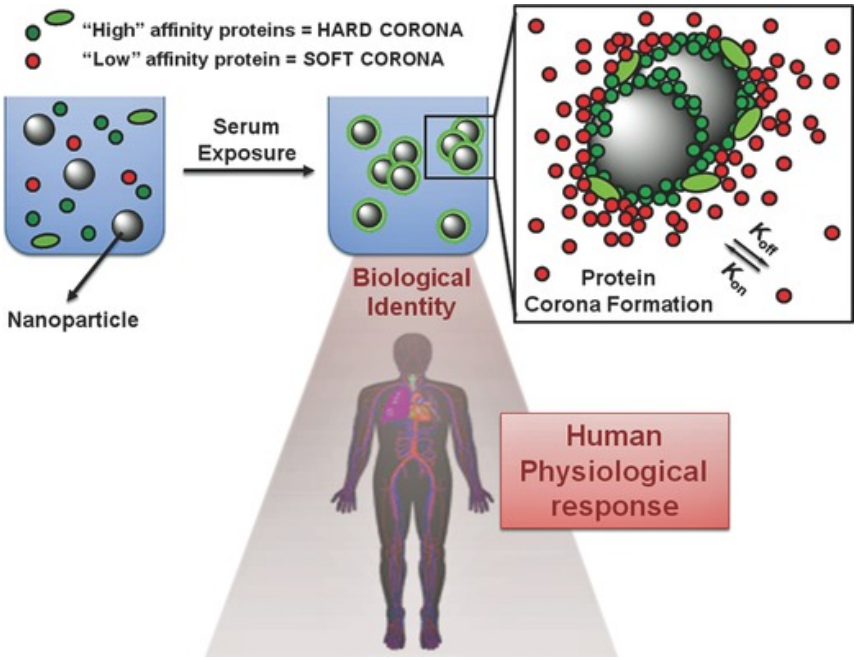


Figure 2.8: Protein corona formation on the surface of nanoparticles. Biomolecules with high affinity are tightly bound and form the hard cornoa (green) and biomolecules with lower affinity form are looslet and reversibly bound and form the soft corona (red).From [42].

nanoparticles with red blood cell membranes [50] and leukocytes [51] has also been proven to be an effective strategy [37].

For extravesation purposes, margination to the vascular wall is also an important factor. Association with the vessel wall can increase chances of passive (EPR effect) as well as in and active targeting where receptor-ligand binding is essential. Particle size and shape have a great effect on margination dynamic to the vascular walls [37]. Smaller and spherical nanoparticles tend to stay in the cell free layer of blood that is far from the endothelial layer where they can extravesate [52, 53, 37] while non spehrical and discoidal particles have shown better margination to the vessel walls [54, 52].

The high intratumoral pressure presents another substantial biological barrier for nanoparticles before they can accumulate at the tumor site. This high intratumoral pressure stems from poor lymphatic drainage in cancer tumors, dense extracellular matrix, interstitial fibrosis and compression from multiplying tumor cells all which result in elevated interstitial fluid pressures in tumors [37, 55].

If the theraputic goal is intracellular delivery of a theraputic agent, there are additional barriers that the nanoparticle must overcome. Cellular uptake also known as endocytosis can be divided into : Phagocytosis, macropinocytosis, caveolae mediated endocytosis and receptor-mediated (or clatherin mediated) endocytosis and clathrin-independent and caveolin-independent endocytosis (figure 2.9). Large particles are mainly engulfed by professional phagocytotic cells such as macrophages, neutrophils, or dendritic cells through phagocytosis or "cell eating" and go on to form an internal phagosome. Particles in the size range of 0.5âĂŞ5 um in diameter are taken up by non specific macropinocytosis which occur in almost all cell types. Smaller nanoparticles can be internalized through caveolar-mediated endocytosis (60nm), clathrin-mediated endocytosis (Up to 100-150 nm) in which cargo is deposited in small endocytic vesicles (usual diameter <100 nm) that fuse with early endosomes, and clathrin-independent and caveolin-independent endocytosis (90nm) [56, 57, 58, 59]. Nanoparticle uptake for most non specialized cells is through clathrin-mediated endocytosis [60]. In phagocytosis and clatherin mediated endocytosis, the phagosome or endosome formed after engulfing the particles will ultimately fuse with the acidic lysosome where most nanoparticles can be degraded , but could be detrimental for delivery of genetic material. On the other hand caveolae-mediated endocytosis results in the formation of caveolae that pinch off from the membrane and are fused with caveosomes that have neutral PH and have been shown to bypass lysosomes [37, 61, 62]. Coating the nanoparticles with ligands such as folic acid, albumin and cholesterol have been shown to facilitate uptake through caveolin-mediated endocytosis, whereas ligands for glycoreceptors promote clathrin-mediated endocytosis [37, 63]. Using or coating nanoparticles with cationic polymers, such as poly(l-lysine) (PLL), have been shown to facilitate endosomal escape through interaction of the cationic charge of the polymer with the outer negatively charged surface of the endosome, resulting in membrane flipping and destabilization (also known as the 'flip-flop' mechanism)[64, 37]

Upon successful internalization through cell, and achieving initial therapeutic effect, drug resistance is another major challenge of of conventional drugs, as well as drug delivery. Multidrug resistance (MDR), either intrinsic or acquired

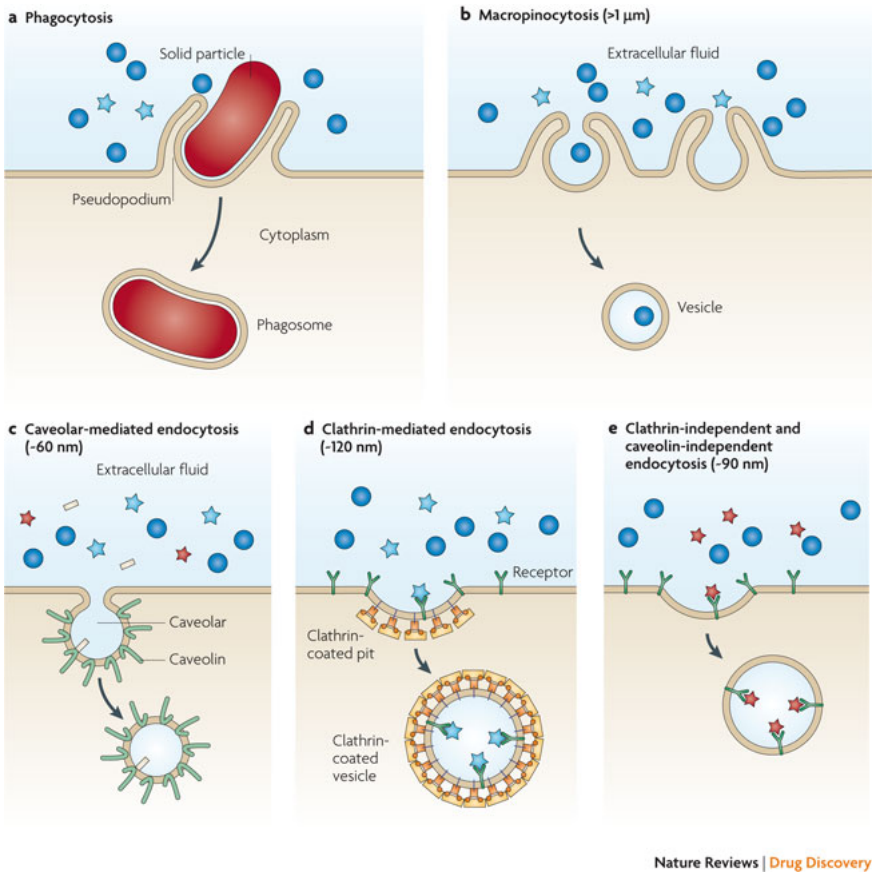


Figure 2.9: Large particles are internalized by phagocytosis (a). particles >1 µmcan be internalized via non specific macropinocytosis (b). Smaller nanoparticles can be internalized through caveolar-mediated endocytosis (60 nm , yellow rods) (c), clathrin-mediated endocytosis (120nm , blue starts) (d) and clathrin-independent and caveolin-independent endocytosis (90nm , red starts) (e) From [48]

from prolonged exposure to a certain therapy, results in the efflux of the drugs from cells, this will lower the intracellular concentration of the drug and result in an incomplete therapeutic impact [37]. However using nanoparticles can alleviate the severity of the drug resistance compared to conventional administration of the drugs[65, 66].

Figure 2.10 from [37] summerizes the biological barriers that was mentioned above.

An important factor to have in mind is that not all therapies need intracellular

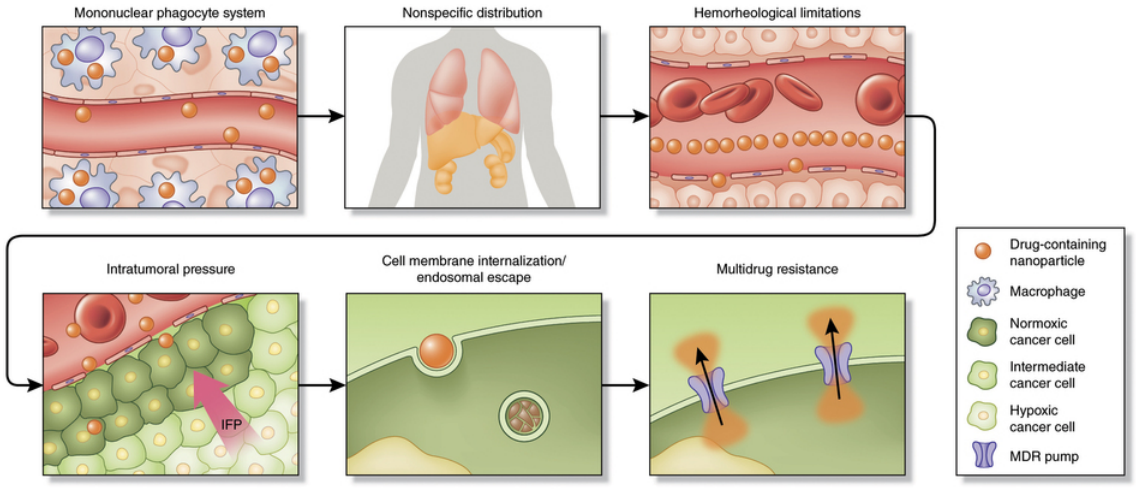


Figure 2.10: Biological Barriers for an intravenous injection of drug encapsulated nanoparticle starting with opsonization and RES uptake, unspecific distribution of the drugs in organs like spleen and kidneys,hemorheological/blood vessel flow limitations, increased intratumoral pressure, cellular internalization, escape from endosomal and lysosomal compartments and drug efflux pumps. From [37].

delivery, thus the biological barrier for each therapy, nanoparticle, and route of administration will be different. With that being said, keeping these biological barriers in mind is an important fact in designing an appropriate drug delivery platform for the intended therapeutic or diagnostic impact.

2.4.2 Nanoparticle Design Criteria

Size and Bio-elimination: Nanoparticle size plays an important role in the route by which it will be eliminated from the body. Systemically injected particles are bioeliminated as follows:Small particles (<5nm) are cleared from the body by the kidneys [55, 67, 48], particles that are in the nanometer range to 15 µm, accumulate mostly in the liver, the spleen, bone marrow adn the capillary of the lungs [48, 68, 37]. However nanoparticles in the range of 100-200 nm have been shown to be taken up by the tumor vasculator through the EPR effect and escape filtration by liver and spleen [37]. However this effect also depends on the permeability of the tumors, for instance poorly permeable tumors like pancreatic tumors are less permeable and only nanoparticles <50 nm are able to accumulate

in tumors [69, 37]. particles larger than 15 µm are removed from the circulation by mechanical filtration in capillaries and can be lethal depending on the dose [48, 68]. Figure 2.11 adapted from [37] shows a summary of the bioelimination of systemically injected particles based on size.

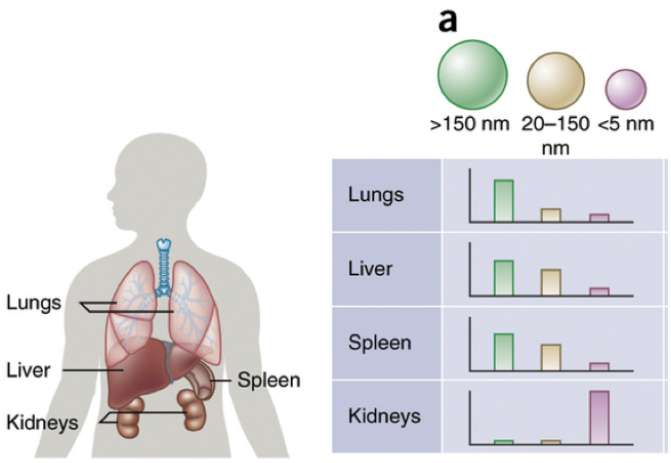


Figure 2.11: Bioelimination of systemically injected particles. Smaller (<5nm) particles are eliminated through the renal system, nanometer to -15um range particles accumulate in the liver, spleen and lungs. Adapted from [37]

Charge: Particles that are positively charged have been shown to be have high cellular uptake thus a rapid clearance from circulation (lower circulation half lives) [37]. Neutral and negatively charged particles on the other hand have shown to have longer circulation half lives [70]. Prolonged half lives can result in improved accumulation of the particles in the tumors.

Shape The majority of the nanoparticles that have been developed are in spherical shape. While it is now understood that just like nanoparticle size, the shape of the nanoparticle can also effect its *in vivo* distribution. Spherical nanoparticles tend to remain in the center of the flow, while rod like particles tend to tumble and drift towards the vessel walls due to variable forces and torques exerted on them (figure 2.12 from [71]). Thus rod like particles can marginate and bind to extravegate to the tumor through the endothelial vessel wall[71]. In addition to margination to the vessel walls, biodistribution studies have also demonstrated that spherical particles have increased uptake by macrophages compared to rod like particles [71]. For example, the liver uptake of 100-nm-long nanochains with

an aspect ratio of about 4 was significantly lower than 100nm in diameter spherical nanoparticles giving the nanochains increased circulation half life and thus increased extravasation to the tumor site [72].

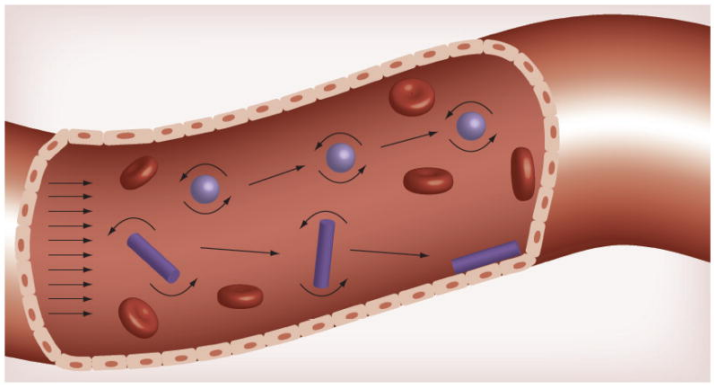


Figure 2.12: Nanoparticle shape effects margination to the vessel walls. Spherical particles tend to stay in the center while rod like particles tend to marginate to the vessel walls. From [71]

Stability: It is very important that the synthesized nanoparticle is stable in physiological conditions. Premature degradation of the nanoparticles can be detrimental to achieving the desired therapeutic effect as well as resulting in unintended accumulation in healthy organs [37]

Biocompatability and minimal toxcity: Needless to say, the material selected for the synthesis of the delivery system needs to be biocompatible with minimal toxicity. Maximum biocompatibility is achieved when the synthesized delivery platform interacts with the body without inducing unacceptable toxic, immunogenic, thrombogenic, and carcinogenic responses [73]. It is important to bare in mind that biocompatability for the most part is not an absolute term, factors such as desired therapeutic goal, target tissue, exposure half life of the delivery system can effect the biocompatability of the said delivery system [74, 73]. Use of FDA approved materials insures increased safety and can aid in commercialization aspect of the delivery platform.

Baring in mind the nanoparticle criteria and the biological barriers for drugs and drug delivery vehicles, nanoparticle design and modification should be done with out most attention to not mainly overcome these biological barriers, but by-

pass them when we can by incorporating novel and specific therapies such as amino acid depletion through enzyme encapsulated nanoparticles where the nanoparticles do not necessarily have to be delivered to the tumor, but depletion happens by the passage of blood through the nanoparticles that can be administered I.M or I.P [36].

2.5 Enzyme Delivery via Nanoparticles

2.5.1 Previous Work on Enzyme Delivery via Nanoparticles and their Shortcomings

Using enzymes as therapeutic agents comes with the advantage of their natural specificity for a given target, compared to general cytotoxic drugs, but due to the relative larger size of enzymes and their delicate nature compared to drugs, encapsulation of enzymes in nanoparticles have proven to be a challenging task in the past. Nevertheless, encapsulation of enzymes in nanoparticles have been widely studied in the past. For instance in single enzyme nanoparticles (SEN), each enzyme is surrounded with a porous composite organic/inorganic network of less than a few nanometers thick. The drawback of SENs is that it can only be applied to limited enzymes and in addition have weak retention at the target [75, 36]. Enzymes can also be encapsulated in protecting structures. These mico or nano strcutures could either release the enzyme such as mesoporous silica nanoparticles [76], polymeric nanoparticles [77, 78], liposome [79] or keep the enzyme associated with the platform such as enzyme immobilization platforms like mesa-porous silica, polymers and polysaccharides [80, 81, 82] , enzyme encapsulation platforms such as hollow silica, gold and polyelectrolytes nanoparticles [36, 83, 84, 85, 86].

One of the major pitfalls of methods that rely on enzyme release is premature and unspecific enzyme release *in vivo* [76]. In these methods although enzymes are associated with the particles, the mere fact that they are released makes them susceptible to immune clearance upon release and also nonspecific release at undesired sites. Polymeric particles for instance, mostly suffer from initial burst release of the cargo [87].These methods are also limited by toxicity,stability

issues, complicated synthesis processes and are limited by cargo size, charge and limited by cargo choice to enzymes that have the stability to withstand synthesis processes used to make the nanoparticles [84, 36].

2.5.2 Enzyme Delivery via Nano-sharkcages

There is a great need to develop a nanoparticle platform that while maintaining enzymatic activity and integrity, overcomes the premature release of the cargo in unspecific sites, and also meet the criteria of bio-compatibility, low toxicity, bio availability and protection of the enzyme against immune clearance in a FDA approved, and cost effective manner with the possibility for passive and active targeting.

Enzymes have a natural specificity for their substrates and are not used up in reactions, by taking advantage of these natural quality of enzymes,as well as there relatively larger size compared to drugs and substrates, one could design a delivery platform that does not release the enzyme itself, but allows the substrate to reach the enzyme and be converted to product, and for the product to diffuse out. This system will operate like a nano-shark cage. The bars of the shark cage are big enough for small fish (substrate and products) to get in, but too small for the scuba diver (enzyme) to get out and for sharks (antibodies, large serum proteins like albumin) to reach the scuba diver (figure 2.13).

This dissertation will go over two different kind of nanoparticle platforms designed for such purpose. To give the nanoparticles the shark cage quality, they will be coated with a porous silica layer. In addition to the porous nature of silica, it is an excellent candidate due to its biodegradability, bio-compatibility, low toxicity, adjustable porosity, and thermal and mechanical stability, making it suitable for *in vivo* applications[1, 2, 3, 4, 5, 6].In comparison to other polymeric drug delivery vehicles, silica nanoparticles are more resistant to pH, heat, mechanical stress, and degradation by hydrolysis[6]. Chapter 3 of this dissertation will focus on Synthetic Hollow Enzyme Loaded Silica nanospheres (SHELS), its application in

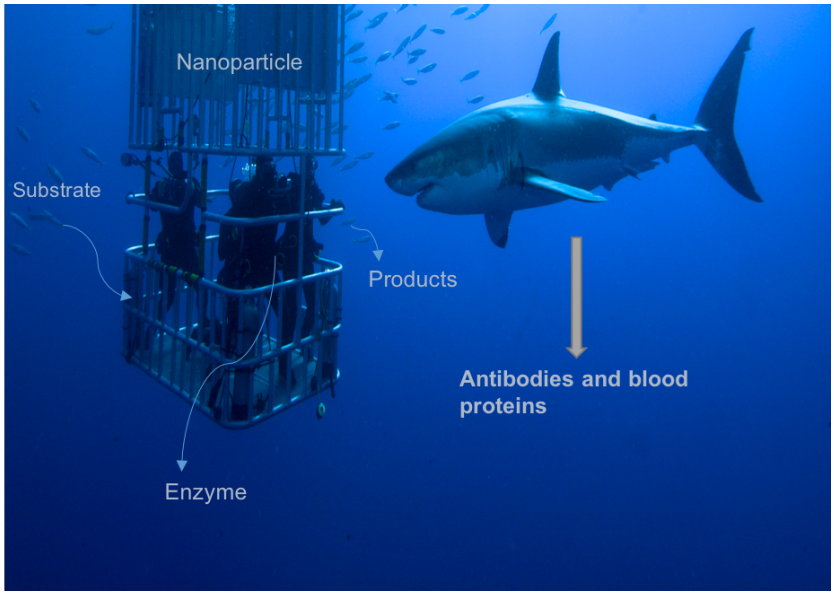


Figure 2.13: Enzyme delivery via nano- shark cages. The nanoparticle will act like shark cage, protecting the enzyme (scuba diver), and allowing small molecules like substrates and products (small fish) to diffuse in and out of the shark cage while larger molecules like antibodies and blood proteins (sharks) cannot diffuse in.

amino acid depletion for cancerous tumors. The third chapter will introduce a new nanoparticle: silica coated enzyme loaded PLGA nanoparticles (SiLGA) and its *in vitro* functional and structural characterization as well as its *in vivo* tissue residence time. Chapter 4 will explore SiLGA's application for enzyme prodrug therapy in caner and its preliminary results. Chapter 5 will focus on discussion and future directions of the nanoparticles.

Chapter 3

MethSHELS

3.1 Methionine Depletion Using SHELS

3.1.1 Background

Malignant tumors have high rate of growth and have increased need for nutrition and also amino acids compared to normal cells. Based on this "metabolic abnormality "of cancer cells, by depriving them of certain amino acids, one might be able to "starve" the cancer cells to death.

Amino acid depletion using enzymes of non-human origin has been extensively investigated and is a very well-known approach for cancer therapy [88, 89, 90, 91]

Therapeutic effect using this kind of therapy is mostly achieved from deep depletion which requires high bioavailability of the enzyme.

Since most of these enzymes are recombinant, meaning they have a foreign origin, they are rapidly cleared from the body by the immune system resulting in poor pharmacokinetics[92, 93]. This problem has been one of the major set backs of using foreign enzymes in enzyme therapy settings in the clinic.

Methionine is an essential amino acid, which means it can not be made in our bodies and needs to be ingested through diet. It has been known for many years that cancer cells have an abnormal methionine metabolism and increased methionine dependency compared to normal cells [94, 95, 96, 97].

In addition to these extensive literature resources, better understanding of the methionine pathway in the body can aid with further understanding increased methionine dependency in cancer cells. 3.1 slightly modified from [98] shows the methionine pathway in the body.

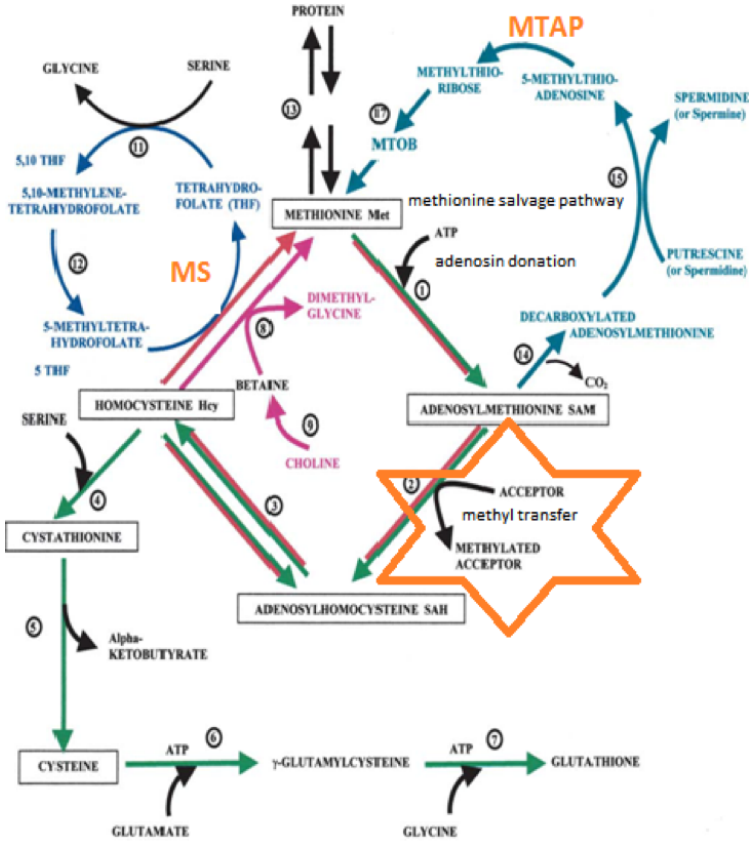


Figure 3.1: Methionine pathway in the body[98].

Since cancer cells are dividing with a faster rate increased DNA transmethylation is one of the main reasons attributed to methionine dependency in cancer cells [94]. In addition to that, methylthioadenosine phosphorylase (MTAP), a critical gene in the methionine salvage pathway which is located next to p16 (tumor suppressor gene) on chromosome 9p21,is often co-deleted in many cancer cells which makes them hyper sensitive to methionine depletion [94, 99, 100]. A summary of the correlation of methionine pathway with increased methionine dependency is shown in Figure 3.2[94, 99, 100].

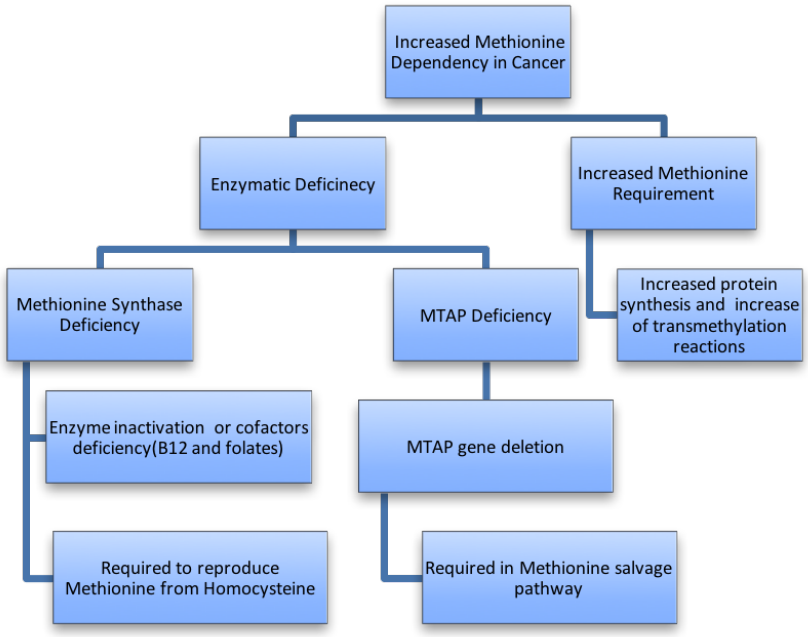


Figure 3.2: Correlation of methionine pathway with increased methionine dependency in cancer cells.

In previous studies,in a media where methionine was replaced by its immediate precursor homocysteine, normal cells were able to grow but cancer cells were not able to survive [101]. It was also shown that methionine depletion in cancer cells can cause ,the late S/G2 phase of their cell cycle to be halted and prevent their ability to divide [102]. This allows for methionine depletion to be used in synergy with other drugs that are effective in this manner like Doxorubicin [98].

One of the main advantages of methionine depletion is the vast majority of cancers that can be targeted for treatment. Methionine depletion has been shown to be effective in the treatment of many different types of *in vitro* cancer models like Walker-256 (rat breast carcinoma), L1210 (mouse lymphatic leukemia),RAG (mouse renal adenocarcinoma), TLX5 (mouse lymphoma), SK-N-MC (human neuroblastoma),CCRF-HSB-2 (human acute lymphoblastic leukemia) [97], as well as *in vivo* including neuroblastoma and breast cancer[103, 97, 104,

31, 105, 106].

Since methionine is an essential amino acid, one of the main ways of methionine restriction is through a methionine free diet. Although methionine restriction through diet can assist with methionine depletion *in vivo*, it is not sufficient to completely arrest tumor growth [107]. Moreover,prolonged methionine restriction can have harmful effects on nutritional status and the general health of the patients [98, 108] as well as being fatal in rats due to rapid weight loss and negative effects on their health[108].

An alternative way to achieve methionine depletion is by the means of enzymes. Methionine depletion with Methioninase from Pseudomonas putida has been extensively studies and tested *in vitro* and *in vivo* for its use in cancer as well as in many other diseases such as heart disease and Parkinson's disease [90, 91, 31, 105, 107, 109].

Methioninase has been cloned from Pseudomonas putida and expressed in Ecoli. It consists of 389-441 amino acids and forms a homotetramer of four subunits of 43 KDa each (172KDa total).

Each subunit contains one Pyridoxal -5'- Phosphate (PLP) as a cofactor[102, 110]. Figure 3.3 from [110] shows the three dimensional homotetramer structure of Methioninase from Pseudomonas putida (a) as well as the PLP binding site on a single monomer (b).

Methioninase catalyzes the conversion of methionine to α -ketobutyrate, methanethiol and ammoniac via α and γ elimination.

Pyridoxal -5'- Phosphate (PLP, also known as B6) is the cofactor of Methioninase. As mentioned before, cofactors are essential for the enzyme to function. In the resting state, the co-factor (PLP) is covalently bonded to the amino group of an active site lysine[102, 31, 110]. During the reaction PLP is transferred to the substrate (methionine) and is re- associated with the enzyme after the reaction[102, 110]. Figure 3.4 from [102] shows the mechanism of this reaction:

In a Pilot Phase 1 clinical trial, Methioninase was found to be safe in late stage breast cancer patients [111]. However, sufficient methionine depletion was

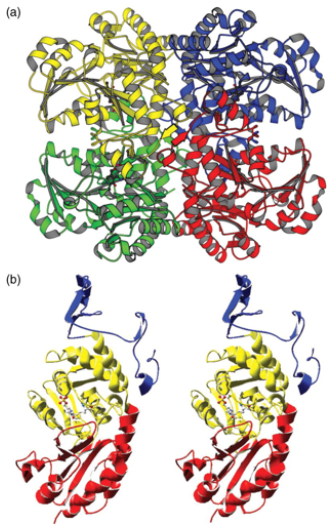


Figure 3.3: Three dimensional homotetramer structure of Methioninase from Pseudomonas putida (a) PLP binding site on a single monomer where the N-terminal domain is shown in blue, the PLP-binding domain in yellow and the C-terminal domain in red.PLP and PLP-binding Lys211 are shown in a ball and stick model (b)[110].

only seen for a short time due to the rapid clearance of the foreign enzyme by the immune system.

In another study, bare Methioninase triggered anaphylactic shock when it was administered to monkeys for repeated challenge doses in consecutive days [31, 106].

In an attempt to improve enzymatic circulation half life and immune reaction, the enzyme was later modified with Polyethylene Glycol (PEG). While PEGylation can help with increasing enzymatic half-life, it is a challenging and expensive task and has also been reported to reduce enzymatic activity [92, 31, 112].

Although PEGylating improved bio-availability, it was ultimately not able to sustain systemic methionine depletion for longer periods. It was later discovered that enzymatic activity is lost due to the rapid disassociation of PLP from the enzyme *in vivo*[113]. These findings were very surprising at the time since PLP is tightly bound to the enzyme *in vitro*[113].

Lys190 has been identified as the primary binding site for HSA [32, 114].

PLP was reported to be sequestered by blood proteins especially Albumin.

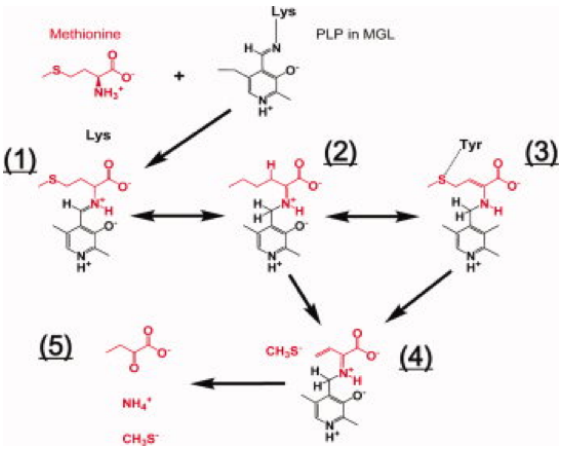


Figure 3.4: Methioninase catalysis reaction with methionine by α and γ elimination to produce α -ketobutyrate, methanethiol and ammoniac.(1) In the resting state, the co-factor (PLP) is covalently bonded to the amino group of an active site lysine, forming an internal aldimine. Once the substrate interacts with the enzyme's active site, a new Schiff base is made. (2) α- and β-hydrogens of the substrate are transferred to PLP (3) a Phenolic group of the adjacent tyrosine residue attacks the gamma-position of the substrate. (4) the thiol group is eliminated from the substrate (5) α -keto acid and ammonia are released from PLP as products [102].

This finding is in alignment with Albumin's high affinity to PLP and its role as the main carrier of more than %95 of circulating PLP in the body [32, 114, 115].

In an attempt to recover enzymatic activity PLP was supplied at super-physiological levels using mini-osmotic pumps *in vivo* and enzymatic activity was shown to be recovered [116]. This finding further supports that prevention of PLP loss is a very important factor for achieving adequate methionine depletion *in vivo*, since PEGylation might help with increasing the enzymatic half life but is ultimately not an ideal approach due to PLP disassociation *in vivo*.
Although these finding can aid with the activity recovery of the enzyme, clinical translation of such method is not feasible.

Since PLP is tightly bound to the enzyme *in vitro*, protecting the enzyme from PLP loss and blood protein access becomes very important in maintaining enzymatic activity that could result in achieving deep depletion of methionine *in vivo*.
As previously discussed, nanoparticles are a great vehicles for safe delivery of drugs

and enzymes *in vivo*.

Nanoparticles can participate in active and passive targeting. Due to the leaky vasculature of tumors, nanoparticles can be passively targeted using the enhanced permeation and retention effect [117, 118]. In addition to this, the surface of the nanoparticles can be functionalized with targeting ligands like vascular endothelial growth factor(VEGF) to target VEGF receptors in the vascular endothelium [119]. Moreover, since enzyme and substrate interactions are highly specific, enzyme therapy are more advantageous in terms of targeted therapies.

With nanoparticle enzyme delivery, the enzyme could be encapsulated, entrapped or immobilized on the nanoparticles [120, 121]. In some cases the enzyme can be released by the nanoparticles [122] or it can remain active inside the nanoparticles and allow for enzyme-substrate interaction [36].

However, enzyme delivery using nanoparticles has had limited success in the past. This can be attributed to the relatively large size of the enzymes in addition to their low stability due to their delicate nature.

In this chapter, by using a novel approach previously demonstrated by [36] referred to as synthetic hollow enzyme loaded nanosphere (SHELS) , we report encapsulation of Methioninase in SHELS (MethSHELS) for improved bio-availability as well as maintaining enzymatic activity. These Silica nanoparticles are able to act as a enzyme delivery vehicle for intramuscular (IM), intratumorural (IT), intra peritoneal(IP) or intravenous (IV) administrations.

Briefly, SHELS are made in 2 main steps. First, through a method called nanomasking [36], amine polystyrene spheres are used as templates and are combined with smaller carboxylate spheres that binds to their surface as nano-masks by electrostatic interaction. This process is followed by the deposition of a thin layer of silica on the nano-masked template. Here, the reaction only occurs on the template surface where it is prevented at the locations blocked by the nano-masks. Once silica is grown; the templates and masks are removed either by calcination or dissolution and silica shells with large pores are formed. In the second step Methioninase is loaded through these pores. Later the enzyme loaded nanoparticles are sealed by another layer of nano porous silica resulting in MethSHELS. This Nano porous silica layer allows small substrate such as methionine to freely

diffuse and react with Methioninase, however the large size of antibodies and blood proteins like albumin does not allow them access to the enzyme through the Nano porous silica. Moreover, using this novel method eliminates the need for direct enzyme modifications like PEGylation since the particles will be in a biocompatible nanoparticle. In our previous work we have shown that asparginase loaded SHELS are able to protect the enzyme from antibody neutralization [36]. Giving the nanoporous nature of these nanoparticles and the inability for large molecules to enter the particles and access the enzyme, we hypothesize that encapsulation will protect Methioninase from albumin (67 KDa) and antibody access therefore sustain enzymatic activity for longer periods of time compared to the unencapsulated enzyme.

3.2 SHELS

Figure 3.5 shows a scanning electron microscopy (SEM) image of Synthetic Hollow Mesoporous Nanospheres (SHMS and Synthetic Hollow Enzyme Loaded nanospheres (SHELS) that have been developed in our group [36]. As it can be seen, the meso-pores are relatively large in size (>5nm) which will be large enough for enzymes to enter the nanoparticle through diffusion. The enzyme loaded nanoparticles are later sealed with a nanoporous silica layer. As it can be seen, after the sealing process the holes are covered and the enzyme is encapsulated inside the SHELS.

In the subsequent sections enzymatic activity post encapsulation will be verified.

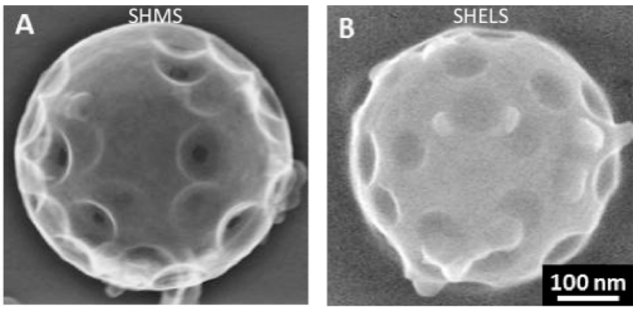


Figure 3.5: Scanning electron micrographs of A) Synthetic hollow mesoporous nanospheres (SHMS) and B) Synthetic Hollow Enzyme Loaded nanospheres SHELS[36].

3.3 Results and Discussions

3.3.1 Unencapsulated enzyme is not dose dependent on PLP *in vitro*

To asses the bare enzyme before the encapsulation process, unencapsulated enzyme was tested in different scenarios with its cofactor and albumin. Pyridoxal 5 phosphate (PLP) is the cofactor for Methioninase. This cofactor is essential for the enzyme to remain active (Holoenzyme) and in case the enzyme loses its cofactor it becomes inactive (Apoenzyme). To confirm that the enzyme is not dose dependent on PLP *in vitro*, we incubated the enzyme with different concentrations of PLP and carried out the assay as mentioned before. We were able to confirm that Methioninase is not dose dependent on the cofactor (PLP) *in vitro* Figure 3.6A. However in vivo, Human Serum Albumin (HSA) has been reported as the main cause of enzyme inactivation[17]. Albumin sequesters the cofactor due to its high affinity for PLP and consequently enzymatic activity is lost. To challenge the bare enzyme *in vitro*, we introduced different concentrations of human serum albumin to the assay. The bare enzyme was incubated with different concentrations of HSA and standard assay protocol was followed to measure enzymatic activity as mentioned before. As expected, introduction of HSA caused significant reduction in enzymatic activity Figure 3.6B.

By confirming that PLP is tightly bound to Methioninase *in vitro* and

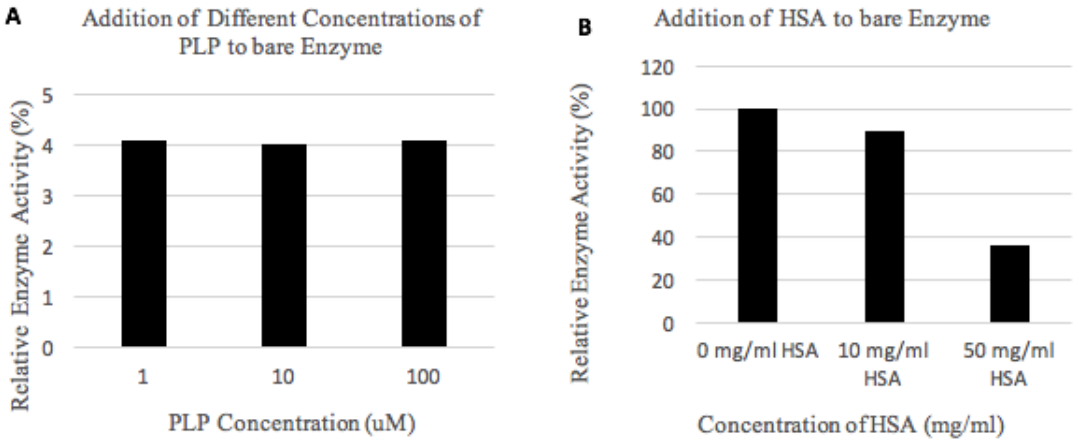


Figure 3.6: (A) Changing PLP concentration has no effect on the bare enzyme's activity, confirming that bare Methioninase is not dose dependent on PLP *in vitro*. (B) Although enzymatic activity was maintained at its full capacity while undisturbed, addition of Human Serum Albumin (HSA) had a significant effect on reducing enzymatic activity.

the role of HSA in reduction of enzymatic activity, we hypothesize that Methioninase could be protected from inactivation by encapsulation inside SHELS (MethSHELS). The porous silica coating will block the access of big molecules like antibodies and blood proteins like Albumin while allowing small molecules (like the substrate and products) to diffuse in and out.

3.3.2 MethSHELS

Methioninase was encapsulated in the SHELS (MethSHELS) based on our previous work [36] with minor modifications. To verify enzyme encapsulation and activity post encapsulation, MethSHELS and free enzyme were separately incubated with Proteinase-K (PK). Proteinase K is a serine protease that will cleave any free enzyme that is not encapsulated within the SHELS or any enzyme that is on the surface of the SHELS. The Activity measured post PK treatment will only come from the enzymes that are encapsulated inside the SHELS. The samples were incubated with and without PK at 37^0C overnight and assay was done on the

samples post incubation as described before. As it can be seen, the bare enzyme loses all its activity when exposed to PK Figure 3.7A while MethSHELS retain more than %60 of their activity upon exposure to PK Figure 3.7B.

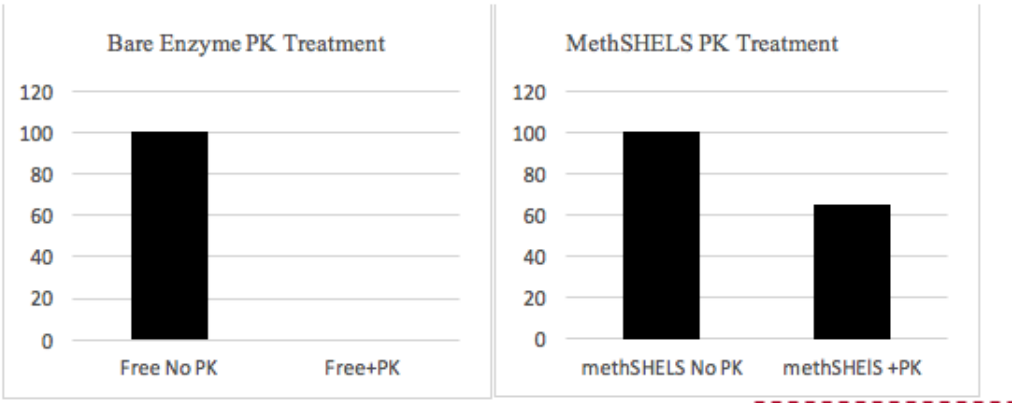


Figure 3.7: (A) Free enzyme was incubated with and without PK for overnight at 37^0C. Enzyme activity assay was done post incubation. While the bare enzyme with no PK maintains %100 of its activity, bare enzyme incubated with PK loses all its activity. (B) MethSHELS were incubated with and without PK for overnight at 37^0C. Activity assay was done post incubation. MethSHELS that were incubated with PK were able to retain more than %60 of their activity.

We believe that the partial loss of activity in MethSHELS could be attributed to the free and un-encapsulated enzyme that could be remaining in the solution from incomplete washing.

3.3.3 Effect of Albumin on MetSHELS *in vitro*

Human serum albumin (HSA) is the most abundant plasma protein and has a very high affinity for PLP [32]. More than %95 of the circulating PLP in the body is bound to human serum albumin [**32**]. Lys190 has been identified as the primary binding site for HSA [32, 114]. Previous studies has shown that cofactor (PLP) loss for Methioninase in vivo can be due to the high affinity of PLP to albumin [31]. During the catalytic reaction, PLP is sequestered by albumin and the enzyme loses its activity due to cofactor loss. Based on our hypothesis, encapsulation of Methioninase inside SHELS will prevent the access of blood proteins like albumin and neutralizing antibodies in the blood due to their inability to diffuse through the

pores of Silica because their larger size. However, substrate and products are able to diffuse in and out and react with the enzyme due to their smaller size. Thus the encapsulation can offer protection of the enzyme from larger molecules like albumin in vivo thus allowing the achievement of efficient methionine depletion. To test this hypothesis *in vitro*, MethSHELS were challenged by the introduction of HSA. MethSHELS were prepared as previously mentioned and incubated as follows: 1x10E11 MethSHELS was added to 30 mM methionine with or without 40mg/ml of HSA. The solutions were then incubated at 37^0C. At each time point a sample of the solution was taken out and the proteins in the solution were precipitated to isolate the products. We have used optical density (OD) at 320 nm as a measure of generated product. As shown in the Figure 3.8, while the free enzyme shows a decrease in activity over time and even lower activity in the presence of HSA, MethSHELS are able to maintain their activity throughout the experiment.

These results in addition to our Proteinase-K study with the MethSHELS supports our initial hypothesis stating encapsulation of enzyme within SHELS protects the enzyme resulting in maintaining enzymatic activity in the presence of albumin.

3.3.4 Toxicity in mice

For evaluating the toxicity of metSHELS, we used the standard preparation of 200 nm metSHELS described above. The maximum tolerable dose was found to be 3xE11 particles/injection/6 hours, which corresponds to around 250-300 U/kg enzymatic activity per injection.

3.3.5 In Vivo Analysis of MethSHELS

After the encouraging results from challenging MethSHELS with PK and HSA *in vitro*, we evaluated the activity of MethSHELS in vivo. For this purpose, we compared MethSHELS with an equivalent dose of bare Methioninase. A dose of 1.5 IU (international units) of bare Methioninase and 0.75UI MethSHELS was injected intramuscularly into the left flank of naive mice. Samples were collected

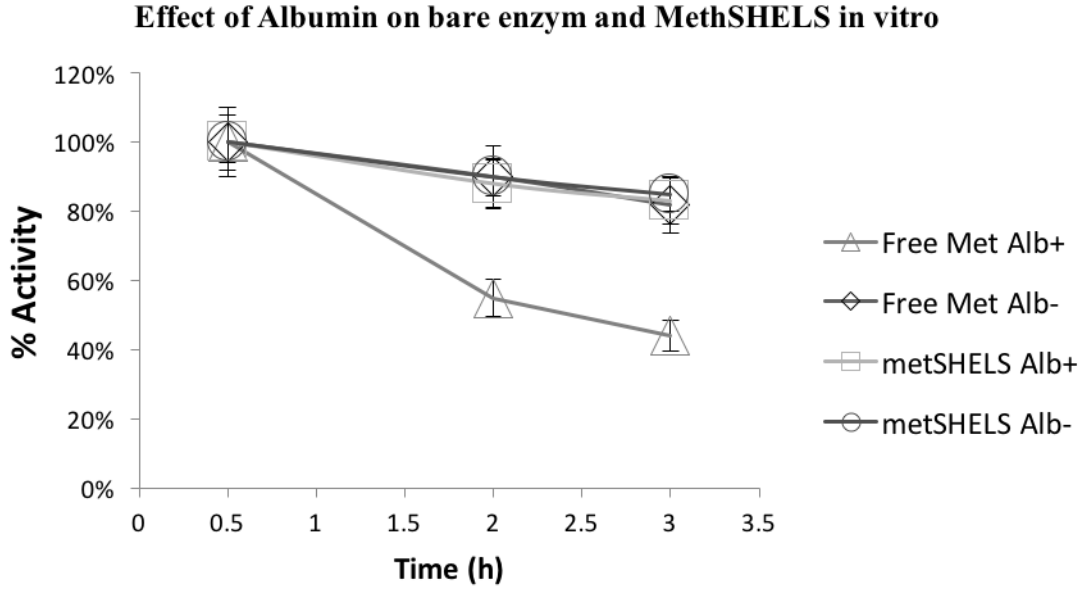


Figure 3.8: Effect of human serum albumin (HSA) on bare enzyme and MethSHELS *in vitro*. Bare Methioninase (Free Met) and Methioninase encapsulated in SHELS (MethSHELS) were incubated with or without HSA (Free Met Alb+, Free Met Alb-, MethSHELS Alb+ and MethSHELS Alb-). The bare enzyme loses a great portion of its activity when incubated with HSA while MethSHELS are able to maintain same level of activity with or without HSA.

at different time points and serum Methionine was measured over time (Figure **??**).

We observed a deeper and more durable Methionine depletion in mice that were injected with MethSHELS compared to bare Methioninase for up to 12 hours. This result is, to our knowledge, the first demonstration of sustained methionine depletion for 24 hours and suggests that encapsulation of Methioninase in SHELS provides protection against in activation *in vivo*.

3.4 Conclusion

In this study, we have successfully demonstrated encapsulation of Methioninase with high efficiency. We have challenged the encapsulated enzyme with Proteinase K and have shown MethSHELS maintain more than %60 of their activity.

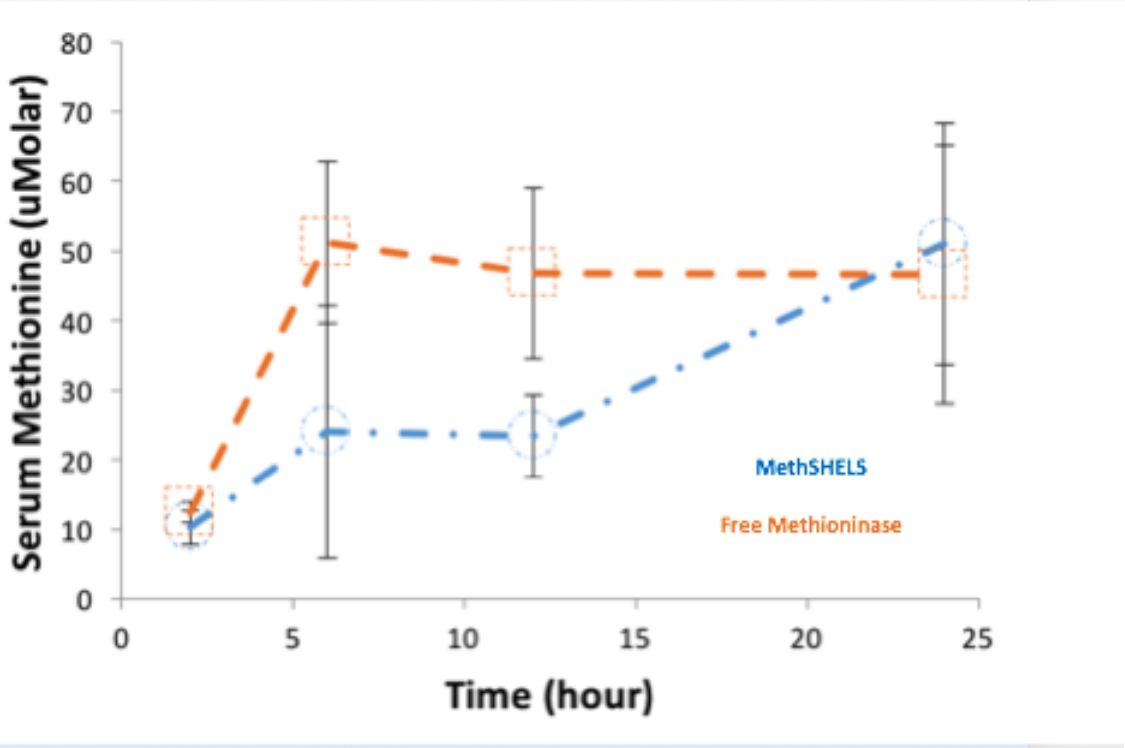


Figure 3.9: *In vivo* depletion of methionine by IM injection of MethSHELS and bare Methioninase in naive mice. A dose of 1.5 IU of bare Methioninase and 0.75 UI MethSHELS were injected intramuscularly into the left flank of naive mice. Serum Methionine was measured over time. Error bars represent the standard deviation of at least three replicate experiments.

We believe that the limited loss of activity is due to the free and un-encapsulated enzyme that could be remaining in the solution from washing. We have also address one of the greatest challenges associated with Methioninase, which is enzyme in activation in the presence of blood proteins like albumin. Previous studies have shown the key role of human serum albumin (HSA) in sequestering PLP from Methioninase[17] during the catalytic reaction that has been attributed to albumin's naturally high affinity for PLP. We were able to successfully demonstrate our key hypothesis stating that encapsulation of Methioninase within nanoparticles improves the stability of the enzyme by protecting it from inactivation in the presence of human serum albumin. Moreover, we have shown a more durable and deeper depletion of IM injected encapsulated Methioninase (MethSHELS) compared to

bare (unencapsulated) Methioninase for up to 12 hours.

For future directions, we believe further optimization of the loading and dosing of SHELS should greatly improve the results. Despite promising results for IM injection of MethSHELS in mice, we believe that IV administration of MethSHELS is a more realistic approach for clinical applications. According to our calculations, after a single injection with SHELS the volume coverage is no more than a few cm^3, so in order to populate a muscle volume as large as 1000 cm3, one needs to make 300 injections to the patient which is not feasible, hence for future application, we would choose IV as the route of administration (figure 3.10)

IM Injection Volume		
Blood Flow Rate Through Muscle at Rest	4	mL / min / 100gr
Methionine Production Rate /humans - no diet	2	μM/hr
Methionine Therapeutic Depletion Level	5	μM
Blood Volume /average human body	5500	mL
SHELS injection Volume Required to Beat Production Rate, **no diet**	917	cm^3

Figure 3.10: Theoritical calculation for SHELS injection volume and muscle volume

In addition to this, while Methioninase has a validated mechanism of action and effects many different cancers, it is not commercially available, thus obtaining a high quality enzyme is very expensive and challenging. Moreover, in terms of the nanoparticles themselves, moving to a more simple synthesis process that achieves complete and efficient enzyme encapsulation and is not limited by enzyme size, as well as synthesizing the nanoparticles such that it is more inline with FDA approved material, will greatly ease the path to commercialization.

3.5 Methods

3.5.1 Nanoparticle Synthesis and Enzyme Loading

Nanoparticles (SHELS) were prepared based on [**35**] with minor modifications. Preparation of Synthetic Hollow Mesoporous Nanospheres (SHMS): 50 µl of 200nm Amine functionalized polystyrene beads (Polysciences, Inc) were used as a template and was mixed with 60 µl of 60nm Carboxyl-functionalized polystyrene latex particles (Life Technologies). The mixture was shaken overnight. Then 1000 µl of anhydrous Ethanol was added to the solution, followed by the addition of 1 µl of Tetramethyl orthosilicate (Aldrich-Sigma Ltd., St. Louis, Missouri) to initiate the silica growth. The mixture was shaken overnight. To stop the reaction and remove excess silica, the particles where centrifuged (10 min at 14000 rpm) and washed 3 times with deionized water. To remove the organic compounds, the nanoparticle solution was placed on a cover slide over a hot plate and calcined overnight at 450^0C. The calcined powder was transferred to a tube and re suspended in 50 µl water and dispersed by gentle sonication.

3.5.2 Preparation of Methioninase Loaded Synthetic Hollow Enzyme Loaded Nanospheres (Meth-SHELS)

Methioninase was obtained from(MtiBio) and dialyzed with 10mM of PLP (Pyridoxal 5-phosphate hydrate %98, Sigma-Aldrich) in 1X phosphate buffered saline (1XPBS) using a 10 KDa dialysis filter (Slide-A-Lyzer) MINI Dialysis Device, 10K MWCO, Thermofisher Scientific) at 4^0C. The solution was changed at 1 hour, 3 hours, and overnight. To prepare the particles for encapsulation, the SHMS solution was centrifuged and the supernatant was taken out. The dialyzed enzyme was added to SHMS pellet and incubated overnight while shaking at 4^0C. Later 50^0C µl of % 0.1 poly-L-lysine (Poly-L-lysine hydrobromide, mol wt>300,000, lyophilized powder, Îş-irradiated, BioXtra, Sigma-Aldrich) was added to the incubated solution. The solution was later diluted with 1000 µl of 1XPBS. Silicic acid was made by adding TMOS (Tetramethyl orthosilicate (Aldrich-Sigma Ltd., St. Louis, Missouri) to 1 mM HCl in 74:500 volume ratio and mixed for a few minutes.

25 µl of the silicic acid solution was added to the above SHMS solution immediately after dilution and shaken for 6 hours in order to generate MethSHELS. To wash the excess silicic acid, MethSHELS were centrifuged (10 min 14000 rpm) and washed 3 times with 1XPBS.

3.5.3 Proteinase K Treatment for loading efficiency analysis

Samples were exposed to Proteinase-K (Thermo Scientific Pierce Proteinase K) enzyme overnight at a concentration of 0.1 mg/ml in 1X phosphate buffered saline (PBS) solution at 37^0C.

3.5.4 Enzyme and Nanoparticle activity assay

The activity assay of Methioninase was done as follows: Solution preparation: A substrate solution of 30mM of L-methionine (DL-Methionine >%99, Sigma-Aldrich) was made in 1XPBS as substrate solution. 25 µl of MethSHELS was added to 975 µl of substrate solution. As controls, bare enzyme with matching activity or a background solution where nanoparticles or bare enzyme was replaced with 1XPBS was added to the substrate solution. Solutions were incubated at 37^0C for different time points. The reaction was stopped by adding 125 µl of a solution of %50 TCA(Trichloroacetic acid ACS reagent, >99.0%, Sigma-Aldrich). The resultant solution was centrifuged for 10 min at 14000rpm and 250 µl of the supernatant was added to 500 µl of 1M sodium acetate (3M Sodium Acetate, Corning-VWR) and 200 µl of %0.1 of 3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate %97, Sima-Aldrich). The solution was incubated at 50^0C for 30 minutes. After this the solution was removed from the incubator and set to reach room temperature and absorption (OD) was measured at wavelength of 320nm.Assay was carried as triplicates. All Absorbance intensities were measured on an Infinite00 Pro, TECAN, Switzerland.

3.5.5 *In vitro* Albumin Assay

A substrate solution containing HSA (Albumin from human serum lyophilized powder, >%97, Sigma-Aldrich) was prepared containing final concentration of 30mM L-Methionine in 1XPBS and different Albumin concentration. For experiments on bare enzyme 0.088mg of Methioninase was added to 1 ml of substrate solution with or without HSA. In controls, Methioninase was replaced by 1XPBS. For experiments on MethSHELS, 25 µl of MethSHELS was added to 1ml of HSA substrate solution. As a non HSA control; 25 µl of MethSHELS was added to a substrate solution containing only a solution of 30mM L-methionine in 1XPBS. The solutions were incubated at 37^0C and stopped at different time points. At each time point , 250 µl of sample was taken out and stopped with 32 µl TCA. The mixture were centrifuged for 10 min and 200 µl of supernatant was added to 200 µl of 3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate and 500l 1M Sodium Acetate. Mixture was incubated at 50^0C for 30 min and OD was measured at 320nm.The assay was carried in triplicates and bare enzyme and MethSHELS activity was matched, with bare enzyme activity being measured as %100 and MethSHELS activity measured accordingly. All Absorbance intensities were measured on an Infinite00 Pro, TECAN, Switzerland.

3.6 Acknowledgment

The content of Chapter 3 was obtained in collaboration with DevaCell INC.The scientific data on this chapter is ready to be submitted for publication.The authors of the publication are: Negin Mokhtari,Inanc Ortac, Jason Pianalto, Sadik Esener.The author of this dissertation is the first coauthor of the paper.

Chapter 4

Silica Coated Enzyme loaded PLGA (SiLGA) Nanoparticles- Preparation and Characterization

4.1 Background

With the goal of maintaining the nano-sharkcage effect by the porous silica coating, alternative enzyme encapsulation routes is investigated to address the limitations that was discussed in the previous chapter. Moving to a more simple synthesis process that is not limited by enzyme size, as well as using more FDA approved material can result in more efficient encapsulation and ease the path to commercialization. With that goal in mind, Silica coated enzyme loaded PLGA (SiLGA) nanoparticles, their successful synthesis, and structural and functional characterization is investigated in this chapter.

4.1.1 PLGA

One of the most popular and widely used drug delivery vehicles to date is poly(lactic-co-glycolic acid), or PLGA. PLGA has been used in sutures since the 1970's [123]

PLGA owes its popularity to many of its unique features including: bio-compatibility,

biodegradability, potential for surface modification for targeting different cancers and organs, and great chemical control over synthesis parameters like size and drug release[124]. Moreover, PLGA is approved by both the Food and Drug Administration (FDA) and the European Medicine Agency (EMA).

PLGA delivery vehicles exist in several forms, though predominantly as particles and implants. A summary of some of the current therapies from [125] are shown in 4.1.

Product name	Dosage Form	Distributor	Active	Duration, months
Decapeptyl®	Microparticle	Ferring	Triptorelin acetate	1
Decapeptyl® SR	Microparticle	Ipsen-Beaufour	Triptorelin acetate	1, 3
Zoladex®	Implant	AstraZeneca	Goserelin acetate	1, 3
LupronDepot®	Microparticle	Takeda Pharma NA	Leuprolide acetate	1, 3, 4, 6
Sandostatin LAR® Depot	Microparticle	Novartis	Octreotide acetate	1
Somatuline® LA	Microparticle	Ipsen-Beaufour	Lanreotide acetate	0.5
Profact® Depot	Implant	Sanofi-Aventis	Buserelin acetate	2, 3
Suprecur® MP	Microparticle	Sanofi-Aventis	Buserelin acetate	1
Eligard®	Liquid	Sanofi-Aventis	Leuprolide acetate	1, 3
Luprogel®	Liquid	MediGene AG	Leuprolide acetate	1
Trelstar™ Depot	Microparticle	Watson	Triptorelin pamoate	1
Trelstar™ LA	Microparticle	Watson	Triptorelin pamoate	3
Arestin®	Microparticle	OraPharma	Minocycline HCl	0.5
Atridox®	Liquid	CollaGenex Ph.	Doxycycline hyclate	0.25
Risperdal® Consta™	Microparticle	Johnson & Johnson	Risperidone	0.5
SMARTShot B12	Microparticle	Stockguard Labs	Vitamin B12	4, 8
Vivitrol®	Microparticle	Alkermes	Naltrexone	1
Revalor®-XS	Implant	Intervet	Trenbolone/estradiol	6
Ozurdex™	Implant	Allergan	Dexamethasone	1
Propel™	Implant/device	Intersect ENT	Mometasone furoate	1
Bydureon™	Microparticle	Amylin	Exenatide	2
Longrange™	Liquid	Merial Limited	Eprinomectin	5
Lutrate Depot®	Microparticle	G P Pharm	Leuprolide acetate	1

Figure 4.1: Example of current therapies using PLGA[125]

Poly(lactic-co-glycolic acid) is a co-polymer of two monomers; lactic acid and glycolic acid.

In the presence of water, the ester linkage between lactic and glycolic acids is hydrolysed and PLGA is decomposed to its building monomers (figure4.2[124]).

Since lactic and glycolic acids are endogenous, it is easily metabolized via the Kreb's cycle. This feature gives PLGA minimal toxicity.

The degradation rate of PLGA depends on the molar ratio of is building

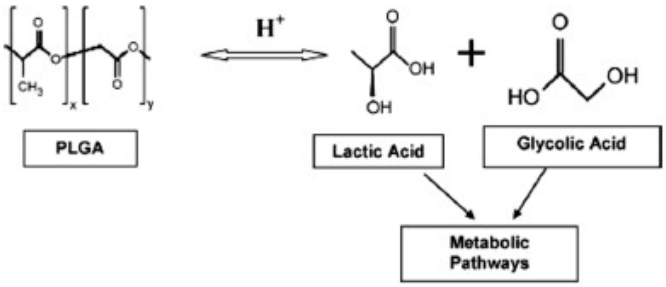


Figure 4.2: PLGA structure[124]

monomers. This ratio can also effect the crystallinity, hydrophilicity, and glass transition temperature. The glass transition temperatures of PLGA co-polymers are above the physiological temperature of 37^0C (around 40-60C) and decreases with reducing molecular weight and lactic acid content; thus, PLGA possess brittle characteristics in physiological conditions[126].

Higher lactic-to-glycolic acids ratio will result in a more hydrophobic composition due to higher hydrophobicity of lactic acid [127]. As such, higher glycolic acid ratios yield in lower degradation time. An exception to this is PLGA composed of a 50:50 monomer ratio that has a faster degradation rate [128].

Depending on the resulting PLGA nano/micro particle or implant, PLGA degrades through bulk and surface diffusion as well as through bulk and surface erosion (figure from [129]). Moreover, the degradation of the polymer to its acidic monomers results in auto-catalysis of the degradation process itself.

The initial burst release of the cargo is related to cargo type, cargo concentration, and the monomer ratio of the PLGA in use. In addition, cargo release is also effected by the solubility of the cargo itself, as well as penetration of water into the polymer matrix[128].

The type of cargo loaded in PLGA particles is another factor contributing to the degradation rate of PLGA. The presence of different kinds of drugs may change the degradation mechanism from bulk erosion to surface degradation, as well as affect the rate of matrix degradation[130].

Cargo-to-polymer ratio is also an important factor in determining the release profile of the resulting PLGA particle, with higher drug or enzyme cargo

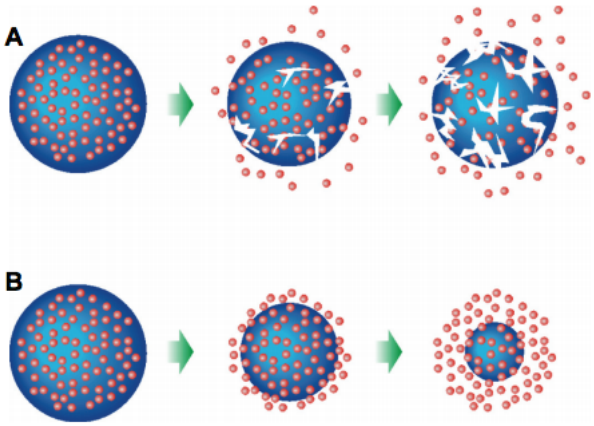


Figure 4.3: Degradation mechanisms of biodegradable polymeric nanoparticles: A) bulk erosion, B) surface erosion from [129].

content resulting in a larger initial burst release [128].

Figure from [131] shows an example of different release profiles of BSA encapsulated PLGA nanoparticles. Depending on the formulation, different modes of BSA release has been reported. Initial burst release as well as biphasic model combining a moderate initial burst and a subsequent sustained release, or triphasic model with a lag of release between both initial and sustained release phases, or incomplete release, can all be observed. Continuous release can be seen when the diffusion of cargo is faster than the particle degradation. The biphasic release can be seen as an initial burst at or near the particle surface followed by a second phase in which BSA is progressively released by diffusion. A third triphasic release profile can also be seen when a lag release period occurs after initial burst and until polymer degradation starts. Finally, it is possible to obtain an incomplete protein release as a result of protein-polymer interaction or protein instability.

Regardless of the mode of release, however, initial burst release is a common phenomena among PLGA delivery vehicles, especially with protein cargo.

4.1.2 Encapsulation of Proteins in PLGA

As it has been discussed before, unencapsulated foreign enzymes have a low half-life *in vivo*. Their oral delivery for instance is limited by their susceptibility to degradation by gastrointestinal enzymes, while other routes of systemic admin-

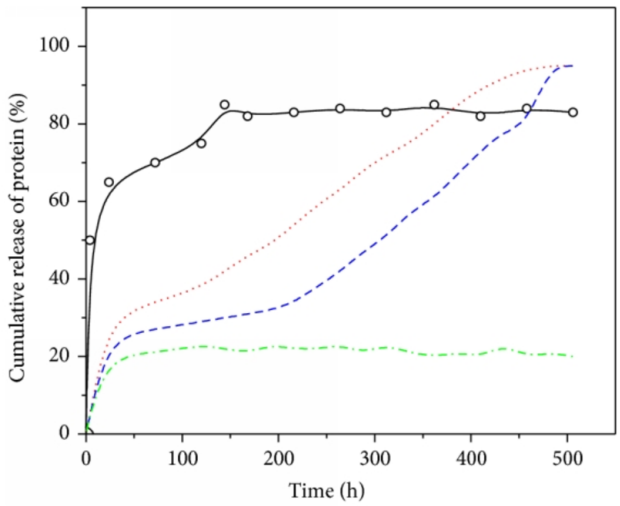


Figure 4.4: Release profiles. (circle) BSA release from PLGA nanoparticles with high initial burst release; (red dots line) biphasic model combining a moderate initial burst and a subsequent sustained release; (blue dash line) triphasic model with a lag of release between both initial and sustained release phases; (dash-dot green line) incomplete release.[131]

istration is susceptible to rapid clearance by the immune system. Encapsulating these foreign enzymes in polymeric nanoparticles, especially in PLGA nanoparticles, has shown promising results in the past[122, 77, 30, 132].

Due to the hydrophilic nature of most proteins, the most common method of protein encapsulation in PLGA particles is through double emulsion solvent evaporation, or W/O/W. However, successful encapsulation of proteins even through this method is met with challenges like protein stability, protein-polymer interaction, and protein aggregation in the water phase[124, 133]. Achieving smaller particle size also requires subjecting the particle solution to higher sonication powers, which can result in loss of enzymatic activity. Due to these challenges, modified methods need to be applied to successfully encapsulate enzymes in PLGA particles.

In an attempt to protect neurons from oxidative stress, [122] reported successful encapsulation and release of Catalase from PLGA nanoparticles. Catalase is an enzyme that converts hydrogen peroxide to water and oxygen, preventing oxidative stress induced by the presence of reactive oxygen species like hydrogen peroxide.

In this method Catalase is encapsulated in PLGA nanoparticles using double emulsion solvent evaporation method. Catalase is co-encapsulated with Rat Serum Albumin (RSA) to stabilize the enzyme in the water phase as well as protect enzyme-polymer interactions. Dimethyl L-tartrate (DMT) is used in the oil phase to facilitate enzyme release from the nanoparticles. Addition of the extra material and other experimental methods, resulted in successful synthesis of Catalase encapsulated PLGA nanoparticles with an average size of 280 nm, a polydispersity of 0.034, and an average zeta potential of -20.mV. These nanoparticles released Catalase activity for a month *in vitro*.

Although successful encapsulation of Catalase in PLGA nanoparticles was shown, the release mechanism of PLGA nanoparticles remains the same. Due to the nature of the polymers, the cargo will be burst-released *in vivo*.

4.1.3 Shortcomings of PLGA

Even with successful encapsulation of cargo, PLGA faces substantial challenges specifically as an enzyme delivery vehicle for *in vivo* applications.

Protecting the foreign enzyme's activity *in vivo* is essential for the enzyme to reach its therapeutic effect.

One of the most prominent shortcomings of PLGA is the initial burst release of the payload once PLGA is introduced *in vivo*. The polymeric structure of PLGA causes a burst release profile followed by a period of slow release. This initial burst release, specially for foreign enzymes, is a great disadvantage. Mostly this initial burst release is an inefficient use of drug or enzyme from both therapeutic and financial points-of-view [87]. Since enzymes are not used in the chemical reaction that they catalyze, they retain their therapeutic effect while encapsulated. In fact, protecting the enzyme from release will substantially decrease the immune response against foreign enzymes. This is protection becomes crucial for enzymes that have cofactors (like in the case of Methioninase and its cofactor PLP, metioned in Chapter2); if such enzyme is released, it will suffer the same fate as its free counterpart: inactivation due to immune response and cofactor loss.

Moreover the premature release of cargo from nanoparticles causes problems

when delivery is intended for sight-specific drug or enzyme release [6].

The most important issue remains; the enzyme is going to be released into the body, facing all of the issues that have been previously discussed. In the following sections, enzyme-loaded PLGA nanoparticles will be coated with a Silica layer that, in addition to preserving all of the advantages that PLGA has to offer, addresses the shortcomings of these nanoparticles and prevents the enzyme from leaking from the nanoparticles.

4.2 Silica Coated Enzyme Loaded PLGA (SiLGA)

One of the biggest challenges of enzyme and drug delivery with PLGA is the initial burst release of the cargo. With the initial burst release, in case for cargo like drugs, the drug might not be able to reach the target tissue or cells, leading to a loss of efficacy. Enzymatic cargo will suffer the same fate, even after the enzyme-loaded nanoparticle reaches its phase of slow release (assuming it is releasing to the point of having a therapeutic effect), the enzyme will be exposed to the antibodies and blood proteins that the nanoparticle was intended to protect it from in the first place.

Coating the surface with a layer that will prevent the enzyme from leaking combats the burst release of the enzyme *in vivo* and protect the enzyme from degradation.

The selected material should be porous enough for the enzyme's substrate and the resulting product to enter and exit the nanoparticles, but small enough that enzymes are unable to escape the nanoparticles.

For this purpose, Silica was selected as the coating material for enzyme-loaded PLGA nanoparticles.Silica is an excellent candidate due to its biodegradability, bio-compatibility,low toxicity, adjustable porosity, and thermal and mechanical stability, making it suitable for *in vivo* applications[1, 2, 3, 4, 5, 6]. In comparison to polymeric drug delivery vehicles, Silica nanoparticles are more resistant to pH, heat, mechanical stress, and degradation by hydrolysis[6].

PLGA nanoparticles encapsulating enzyme, drug, virus, nucleic acids can

be coated with silica, a porous inorganic material. Applications of these loaded, silica-coated PLGA (or, "SiLGA") nanoparticles are well-known in biotechnology, nanomedicine, and enzyme/drug delivery.

In brief, a payload like an enzyme or drug is encapsulated inside poly(lactic -co-glycolic acid) (PLGA) nanoparticles. The PLGA nanoparticles, which encapsulate their payload, are then coated with a porous silica layer.

One simple analogy describing how this system works is to think of a SiLGA nanoparticle like a shark cage. The scuba diver (here, the drug or enzyme) inside the cage is too big to escape; however, small fish (here, prodrugs or substrate molecules) can enter the cage and interact with the scuba diver. Anything big like a shark (here, the antibodies and blood proteins) cannot get into the cage.

Since the payload is located inside the nanoparticle, the porous silica layer hides it from the immune system. The silica coating also protects the payload itself from *in vivo* inactivation, like co-factor loss, which often limits the use of certain enzymes *in vivo*. Once the nanoparticle goes through endocytosis, the very acidic conditions of the endosome degrade the particle.

4.2.1 Synthesis of Silica-Coated, Enzyme-Loaded PLGA (SiLGA)

The first step of synthesis utilizes PLGA nanoparticles as templates - encapsulating the payload - onto which a porous silica layer is deposited.

In the first step of the synthesis, the enzyme is encapsulated inside PLGA using a double emulsion solvent evaporation based on [122] with minor modifications.

In summary,first, an appropriate ratio of enzyme to Rat Serum Albumin (RSA) is dissolved in water, yielding the initial aqueous phase (W1). RSA protects the enzyme during the encapsulation process.

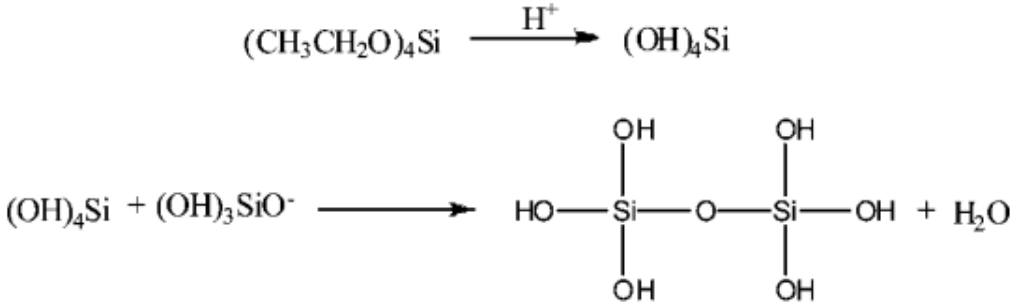
The W1 mixture is then added to the oil phase (O1), containing PLGA (poly(lactic-co-glycolic Acid) and DMT (dimethyl L-tartrate) in of dichloromethane (DCM), while vortex mixing; immediately afterwards, probe sonication on ice

generates the water-oil emulsion. Here, DCM acts as an organic solvent, and DMT serves as a plasticizer. The mixture is then added to a solution of %5 PVA (polyvinyl alcohol) in water, which serves as the second aqueous phase (W2). PVA acts as an emulsifier. This mixture is vortex mixed and probe sonicated on ice to create the water-oil-water double emulsion. The organic solvent (DCM) is evaporated overnight at room temperature, allowing the PLGA nanoparticles to harden.

The nanoparticle solution is then vacuum-dried for one hour, centrifuged, and washed three times with water to remove excess PVA.

The (W/OW) nanoparticles are then coated with Silica using Silicic Acid (a ratio of 75:500µl tetramethyoxysilane to hydrochloric acid) in 1X PBS overnight.

Tetramethoxysilane (TMOS) is hydrolyzed in aqueous solution to give silicic acid. The initial chemistry of the coating process is shown below [134].



A summary of the process is shown in 4.5

The silica shell has pores allowing the small molecules to pass through. However, the payload cannot exit the holes because it is sealed within the particle. There are many variations of this fabrication approach for different applications. For instance, PLGA size, lactic-to-glycolic-acid ratio, payload, silica coating time, and probe sonication duration and power can all potentially be modified.

4.3 Structural Characterization of SiLGA

The nanoparticles size and charge were characterized using scanning electron microscopy (SEM) and dynamic light scattering (DLS). In order to visually

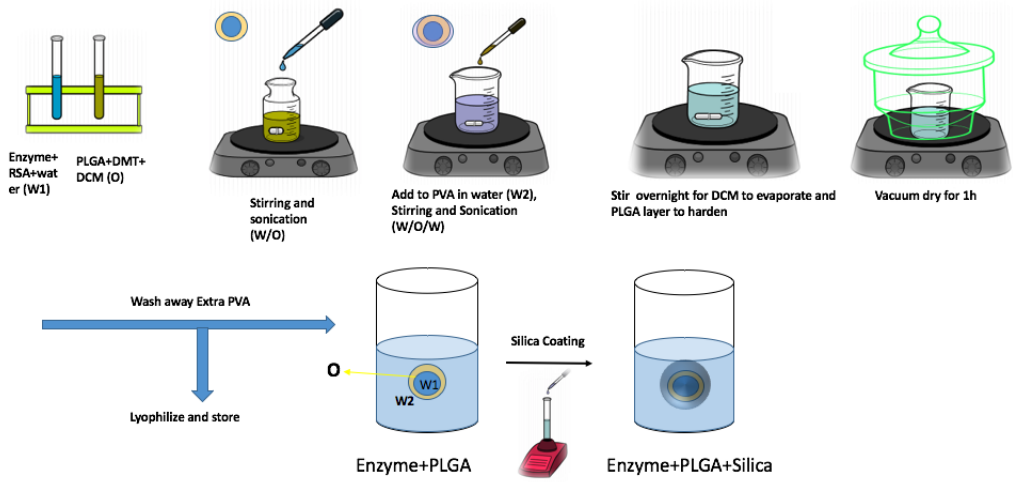


Figure 4.5: Step by step synthesis of SiLGA.

confirm that PLGA is coated with Silica, samples were imaged using SEM before and after coating (figure 4.6). As it can bee seen, there is a clear visual indication of Silica coating on the PLGA nanoparticles before [fig 4.6A and fig 4.6B] and after [fig 4.6C and fig 4.6D] the coating process.
Our SEM results indicate a size range of 100 nm - 200 nm for the PLGA nanoparticles and 100nm - 260 nm for SiLGA nanoparticles (figure 4.7).

For further confirmation of Silica coating, size and surface charge of the nanoparticles were compared before and after the coating process using DLS. PLGA nanoparticles show a size range of 201.9 nm with a polydispersity index (PDI) of 0.093, while SiLGA nanoparticles show a size range of 386.7 nm with a PDI of 0.239.

As it can be seen in 4.8,the DLS data confirms a substantial difference between the size and charge of the nanoparticles before and after Silica coating. This should be taken as an additional sign of successful coating. A range of low PDI values indicates that both PLGA and SiLGA nanoparticles are well-dispersed in the solution.

We believe that the discrepancy in size between SEM measurements and DLS data is due to hydrodynamic radius surrounding the charged particle surfaces and the incomplete removal of the emulsifiers used during synthesis.

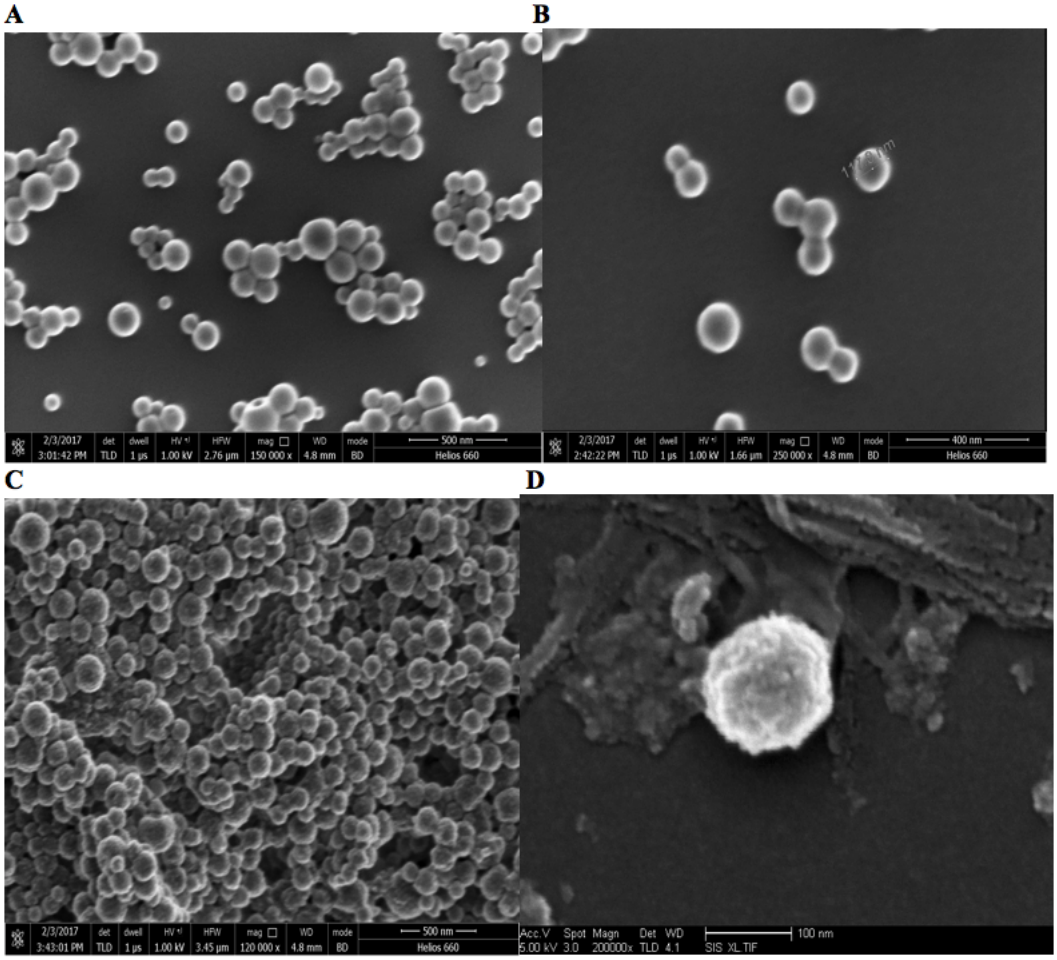


Figure 4.6: SEM images of nanoparticles. A and B) PLGA before coating with Silica. C and D) PLGA after coating with Silica (SiLGA)

This data, along with the SEM images, clearly shows the correct pattern of change in the characteristic of PLGA nanoparticles before and after Silica coating.

4.4 Functional Characterization of SiLGA

After successfully characterizing the structural changes due to coating PLGA with Silica (SiLGA), enzyme activity assays were used to verify enzyme loading and activity post-encapsulation and coating.

Penicillinase (BLA) was chosen as a mock enzyme to test activity, as well

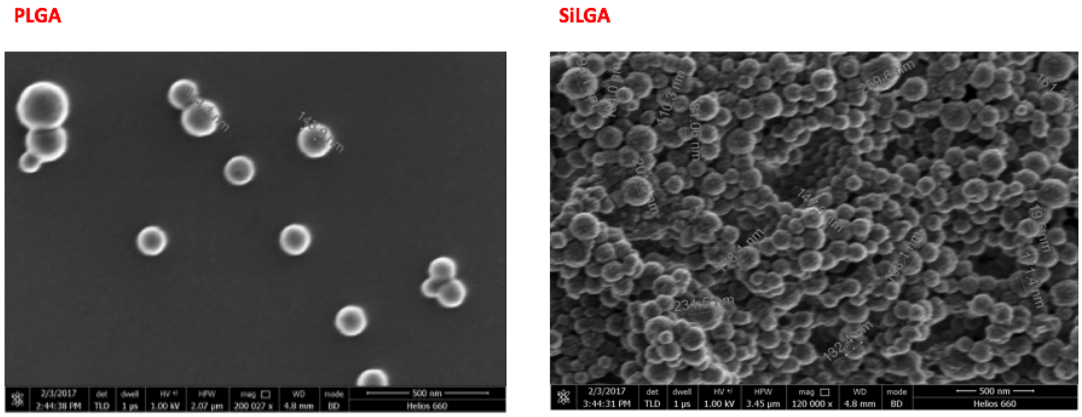


Figure 4.7: PLGA and SiLGA nanoparticles size via SEM

as SiLGA's protection efficiency against protease enzymes, *in vitro*.

Penicillinase from Bacillus cereus is a member of the family of beta-lactamases, which catalyze the hydrolysis of the beta-lactam ring [135].

Unencapsulated (BLA), BLA encapsulated in PLGA (BLA-PLGA), BLA encapsulated in SiLGA (BLA-SiLGA) were separately incubated with Proteinase -K (PK). Proteinase K is a serine protease that will cleave any free enzyme not encapsulated within the nanoparticles or on the particle surfaces.

Remaining activity post PK treatment indicates activity from enzymes protected from PK exposure from within the nanoparticle's interior.

The samples were incubated with PK at a final concentration of 0.1 mg/ml of PK at 37^0C in a shaking incubator (300 rpm) overnight. In controls (samples without PK), PK was replaced with 1XPBS. Using nitrocefin as a substrate, an enzyme activity assay was performed on the samples after incubation at 37^0C. Nitrocefin is a chromogenic Îš-lactamase substrate that changes color from yellow (λmax = 390 nm at pH 7.0) to red (λmax = 486 nm at pH 7.0) as the amide bond in the Beta-lactam ring is hydrolyzed by BLA.

Each reaction was 100 µl in total volume and contained 250 ug/ml nitrocefin as substrate; absorbance was measured at 486 nm.

The results can be seen in 4.9 As expected, it can be seen, the unencapsulated BLA loses all its activity when exposed to PK. BLA-PLGA retains only %4

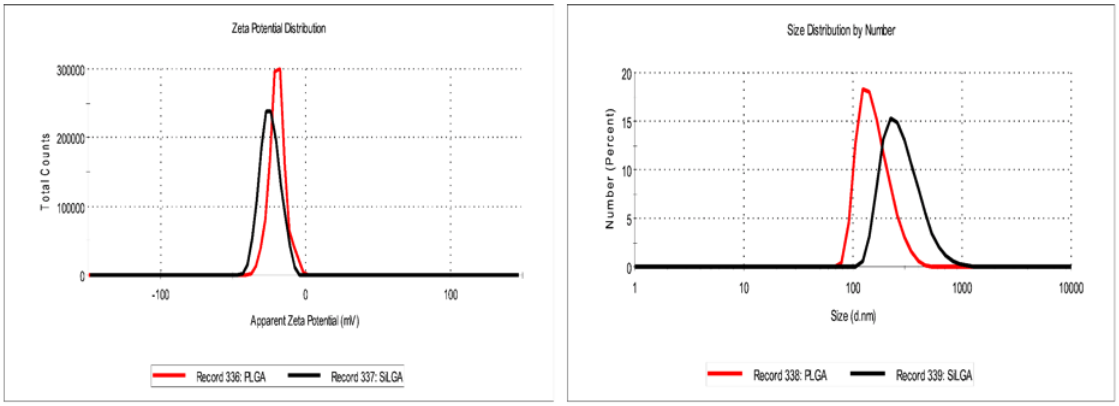


Figure 4.8: Size and Zeta Potential measurement of PLGA and SiLGA via DLS.

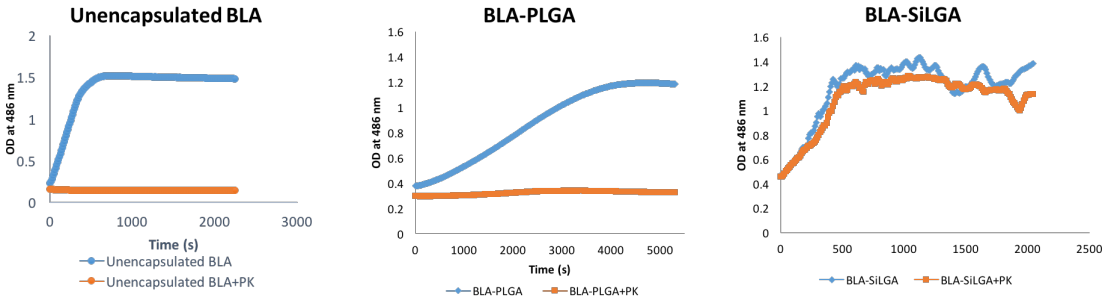


Figure 4.9: BLA activity at 37^0C as A) Unencapsulated BLA incubated with or without PK B) BLA encapsulated in PLGA (BLA-PLGA) incubated with or without PK. C) BLA encapsulated in SiLGA (BLA-SiLGA) incubated with or without PK.

of its activity of its activity while BLA-SiLGA retains more than %83 of its activity before and after incubation with PK. We believe that activity loss in BLA-PLGA is from the burst release of BLA followed by polymer degradation and subsequent PK access to the enzyme. while such loss is not seen with the SiLGA nanoparticles due to the protection offered by the Silica coating

This substantial protection of enzymatic activity by SiLGA against PK *in vitro* demonstrates the potential effectiveness of SiLGA against proteolysis of the cargo (enzyme) *in vivo*.

4.5 *In vivo* Tissue Residence time of SiLGA

To asses the residence time of SiLGA in tissue, Alexa fluor 680 conjugated to Bovine Serum Albumin (BSA) was loaded into SiLGA nanoparticles. Athymic, nude mice were purchased from the Jackson Laboratories (Stock : 002019). Mice were housed in high-efficiency particulate air (HEPA) cages in a specific-pathogen free (SPF) facility at OHSU. Mice were fed a diet of PicoLab Mouse Diet 20 (LabDiet, 5058) ad libitum and started one week prior to imaging. 10 week old male nude mice were given a single 100µl intramuscular injection of either BSA loaded SiLGA in the left hind leg muscle or free BSA in the right hind leg muscle. Mice were imaged for fluorescence (excitation wavelength = 680nm, emission wavelength = 710nm, exposure = 0.2sec) after correcting for background fluorescence using the IVIS Lumina XRMS Series III (PerkinElmer). All experiments performed were approved by the Institutional Animal Care and Use Committee (IACUC) at OHSU. As it can be seen in figure 4.10 while the unencapsulated BSA is almost gone in 2 days, the BSA-SiLGA nanoparticles are localized in the tissue for at least 60 days.

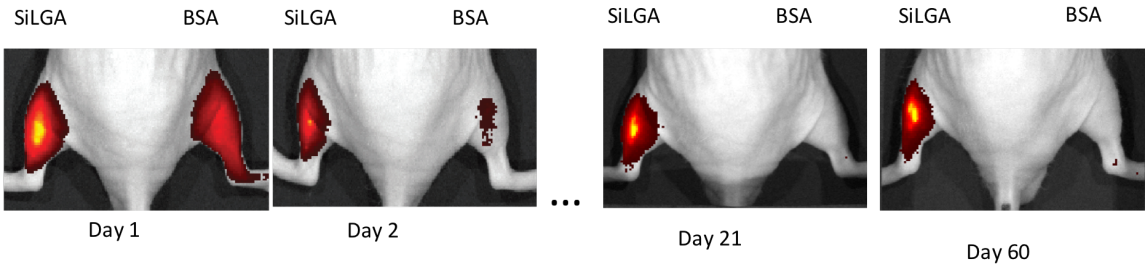


Figure 4.10: *In vivo* tissue residence time of BSA encapsulated in SiLGA compared to unencapsulated BSA

Residence time is important for many applications including applications in amino acid depletion and enzyme pro-drug therapy using SiLGA nanoparticles. The promising results of this experiment clearly indicates SiLGA's stability, minimal toxicity and biocompatibility, as well as its superior tissue residence time compared to free BSA.

4.6 Discussion

To conclude this chapter, we have successfully encapsulated enzymes and proteins in Silica-coated PLGA nanoparticles (SiLGA) as demonstrated by structural and functional characterization both *in vitro* and *in vivo*.

Our method allows for the entrapment of proteins and enzymes without modifying the enzyme or protein itself. We have shown that adding a porous silica coating does not reduce enzymatic activity. The porous silica layer of the SiLGA nanoparticles is too small for the enzyme to escape and for big molecules, like antibodies and proteolytic enzymes, to reach the enzyme; however, these pores are sufficiently large to allow substrates to reach the enzyme and for the reaction product to diffuse out. The structural characterization confirmed differences in size and charge between PLGA nanoparticles and SiLGA nanoparticles. PLGA nanoparticles show a size range of 100-200 nm with a polydispersity index of 0.093, while SiLGA nanoparticles show a size range of 100-260 nm with a PDI of 0.239.The particles were visually confirmed using SEM.

In the functional characterization, Penicilinase (BLA) was loaded into PLGA nanoparticles (BLA-PLGA) and then coated with Silica (BLA-SiLGA). We were able to confirm retention of enzymatic activity throughout the synthesis process.

To confirm superior protection capabilities of SiLGA and successful silica coating, particles were incubated with a serine protease (Proteinase K) at 37^0C for 24 hours where unencapsulated BLA and BLA-PLGA lost almost all of their activity while BLA-SiLGA retained %83 of its original activity. These strong *in vitro* results, in addition to our structural characterization results, give us confidence that silica coating has been successful and that SiLGA nanoparticles can offer significant protection against proteolytic enzymes *in vivo*.

To confirm tissue residence time of SiLGA nanoparticles *in vivo*, our results confirms that unencapsulated Alexa fluor-BSA vanishes within one day while Alexa fluor-BSA encapsulated in SiLGA persists for at least 60 days.

Small particle size combined with inert, FDA-approved materials used in synthesis will ease the way for clinical translation of these drug delivery vehicles.

Furthermore, different polymers may be used in this method (e.g. PLA,PMMA, etc) and different coatings can be applied to the polymer surfaces (e.g. calcium phosphate, titanium oxide, etc).

The next chapter will address some preliminary applications of SiLGA using different enzymes.

4.7 Methods

4.7.1 SiLGA Synthesis

The PLGA nanoparticles are made by double emulsion solvent evaporation method by [122] with minor modification. First an appropriate ratio of enzyme to Rat Serum Albumin (RSA) (for example: 22mg:8mg or 8mg:22mg) are made in 300 µl of water. This makes the initial water phase (W1). RSA protects the enzyme during the encapsulation process.

The oil phase (O1) consists of 81 mg of PLGA (Poly lactic-co-Glycolic Acid) and 3mg DMT (Dimethyl L-tartrate) in 3mL of dichloromethane (DCM). Here DCM is used as the organic solvent and DMT as a plasticizer.

The second water phase (W2) contains a solution of %5 PVA. To prepare the %5 PVA, first 100 mL of water was heated up to 80C on a hotplate. Then 5 g of PVA was slowly added while mixing water. Then, the solution was let to reach room temperature and vacuum filtered. PVA acts as an emulsifier.

The W1 mixture (enzyme and RSA in water) was added drop-wise to the oil phase (O1:PLGA and DMT in DCM) while vortex mixing for one minute. Afterwards, this mixture was probe sonicated on ice for 2 minutes with a total energy output of approximately 1400 Joules.

The mixture was then added drop-wise to 18ml of %5 PVA (polyvinyl alcohol) in water, vortex mixed for one minute, and probe sonicated on ice for 2 minutes with a final energy output of approximately 2800 Joules; this process yielded the second water phase (W2).This makes the W1/O/W2 solution.

The solution was then left stirring overnight at room temperature to re-

move the organic solvent. This was followed by one hour of stirring in a vacuum desiccator to completely remove the organic solvent while also allowing the PLGA nanoparticles to harden.

To wash away excess PVA and any unencapsulated enzyme, the nanoparticles were centrifuged and recovered three times using ultra centrifugation (32000 rpm for 30 minutes at 4C). After each wash, the nanoparticles were resuspended in water and probe sonicated on ice as mentioned before.

The nanoparticles were then centrifuged at 1500 rpm for 10 minutes, and the supernatant was recovered. After this step, the synthesis of enzyme encapsulated PLGA nanoparticles is complete and particles are ready to be coated with Silica.

In empty nanoparticles, enzyme is replaced with RSA.

In order to coat the nanoparticles with silica, 50 µl of PLGA nanoparticles are added to 1ml of 1XPBS while mixing. A solution of silicic acid is made by adding 74µl of Tetramethoxysilane (TMOS) to 500µl 1mM HCl. The solution is mixed for a minute. TMOS is hydrolyzed in aqueous solution to give silicic acid. Then 15 µl of silicic acid is added drop wise to the particle solution.The resulting solution is left mixing at 4^0C over night.

To remove the excess silica, the solution is centrifuged at 4000 rpm for 15 minutes in 4^0C. The nanoparticles are resuspended in 1XPBS at each step. The final solution is probe sonicated on ice for 30 seconds (%20 amplitude, 2 seconds ON / 1 second OFF).

The resulting nanoparticles from this step are enzyme-loaded, Silica-coated PLGA nanoparticles (enzyme-SiLGA).

Samples are characterized using SEM and DLS as well as enzyme activity assays for each corresponding enzymes.

4.7.2 Characterization

Absorbance and fluorescence measurements for activity assays were taken with the TECAN Spark(R) 20M Te-cool(TM). The Helios Nanolab(TM) 660 scanning electron microscope produced micrographs of particles. Particle size, Zeta potential, and polydispersity index were measured using the Malvern Zetasizer Nano

ZSP dynamic light scattering instrument.

4.7.3 Beta Lactamase Activity Assay

For the colorimetric determination of Beta Lactamse activity, 100 µl of enzyme solutions were transferred onto a 96-well plate. To obtain the working solution One milligram nitrocefin was dissolved in 100 µl DMSO, and 1.9 mL 1X PBS was added.
25 µl of the working solution was added to each well and absorbance at 492 nm was measured at 37^0C on a Tecan Spark 10M.

4.7.4 Amplex Red Assay

The Amplex Red Assay Kit (A22180) was used from Invitrogen. Amplex Red reagent reacts with a 1:1 stoichometry with any unreacted H2O2 in the presence of horseradish peroxidase (HRP) to produce the highly fluorescent oxidation product, resorufin.Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively.

4.7.5 Materials

The following materials were purchased from Sigma Aldrich and used as received: amorphous PLGA ("Resomer(R) RG 504 H"; acid terminated; lactide(38 kDA):glycolide(54 kDa); monomer ratio of 1:1); lyophilized powder form of penicillinase ("Penicillinase from Bacillus cereus"; 1,500 to 3,000 units per milligram of protein); lyophilized powder form of rat serum albumin (>99 percent purity); dimethyl-L-tartarate; dichloromethane (anhydrous; 40 to 150 ppm amylene stabilizer; >99.5 percent purity); polyvinyl alcohol (87 to 90 percent hydrolized; average molecular weight: 30 to 70 kDa). The presterilized Stericup and Steritop Vacuum Driven Disposable Filtration System was utilized to filter the %5 PVA aqueous solution. The Q500 Ultrasonic Processor from QSonica (output: 500 W, 20 kHz) was used to create the water-oil-water double emulsion.

4.8 Acknowledgment

Chapter 4 its methods are patented (Nanoscale delivery device and uses thereof,15/449,830). Collaborator for the patent are: Sadik Esener, Negin Mokhtari,Yasan Yeh, Mukanth Vaidyanathan, Ajay Sapre, Bartu Ahiska. The author of thisdissertation is a co-inventor of the patent. Chapter 4 and its scientific methods are ready to be submitted for publication.The author of the publication are: Negin Mokhtari, Kyle Gustafson, Jared Fischer,Mukanth Vaidyanathan, Yasan Yeh, Sadik Esener.The author of this dissertation is the first author of the paper

Chapter 5

SiLGA Application

5.1 Applications of SiLGA

SiLGA's exceptional localization in tissue, as well as its minimal toxicity, holds great promise for exploring its in vivo applications for therapeutic and diagnostics purposes. SiLGA's application in enzyme prodrug therapy as well as its characterization for antioxidant therapy has been investigated in this chapter. It is important to bare in mind that SiLGA is a new delivery platform, thus its application is not just limited to cancer therapy or diagnosis. SiLGA nanoparticles can have many different application in other diseases. For instance it can be used as an enzyme replacement therapy platform for patients with Phenylketonuria (PKU) a metabolic genetic disease where phenylalanine, an essential amino acid, is accumulated in the body and can result in mental retardation. The enzyme, Phenylalanine ammonia lyase can be used to degrade phenylalanine and has been shown promising results in the clinic [136]. SiLGA can be loaded with antioxidant enzymes like catalase and superoxide dismutase for antioxidant therapy and have been shown to protect neurons against oxidative stress [122], this can be utilized in diseases such as cerebral ischemia and multiple sclerosis [137, 122]. Catalase loaded nanoparticles could also be used as a diagnostic tool for *in vivo* detection of acute kidney injury (AKI) [138].

5.1.1 Enzyme Prodrug Therapy

The treatment of solid or metastatic tumors could benefit from enzyme-prodrug therapies using foreign enzymes that convert a non-cytotoxic prodrug into its toxic forms at the tumor site by a highly specific localized enzymatic reaction [11, 139, 140, 18, 141, 142]. Enzyme-prodrug therapy involves the systemic administration of an inactivated non toxic form of a drug (prodrug), which can be converted in the the active form of the drug by an exogenous enzyme. The chosen exogenous enzyme should either be of foreign origin or have none or very low endogenous expression. The prodrug needs to be non toxic in its inactivated form and not be a substrate to endogenous enzymes that would lead to its unspecific activation in non target tissues [139].

As the limitation of administration of free enzymes have been described in previous chapters, in enzyme prodrug therapy those issues will be even more devastating resulting in non specific activation of the prodrug throughout the body. Thus one of the best ways to circumvent these issues, is to encapsulate the enzyme in nanoparticles, where the exogenous enzyme intended for the enzyme prodrug therapy will be encapsulated in a nanoparticle and localized within the target, followed by systemic administration of the prodrug that is the specific substrate for the encapsulated enzyme. This will result in local activation of the drug in the tumor. [139]. With the promising *in vivo* data obtained with SiLGA, we hypothesize SiLGA will be an excellent candidate as an enzyme delivery vehicle for applications in enzyme prodrug therapy (Figure 5.1).

5.1.2 Enzyme Prodrug Therapy Using HRP loaded SiLGA and IAA

Indoleacetic acid (IAA), is a plant hormone found in most plants. In humans IAA is a breakdown product of tryptophan metabolism and is often produced by the action of bacteria in the mammalian gut. Some endogenous production of IAA in mammalian tissues has also been seen [143]. IAA has been detected in the central nervous system, urine and blood plasma of humans [144, 145, 146, 147, 148]. Thus IAA is well tolerated in humans [144, 149]. However while IAA

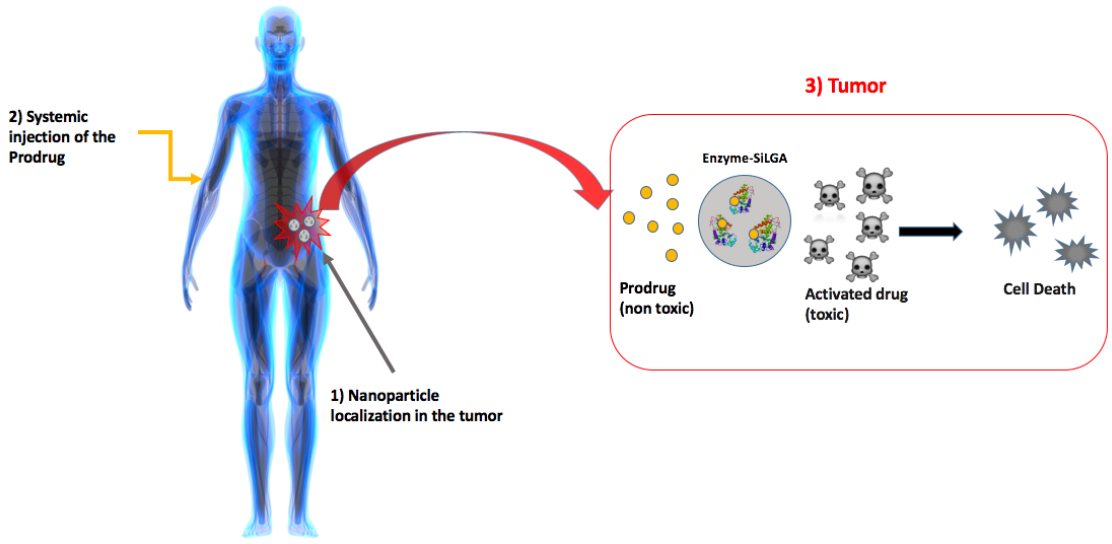


Figure 5.1: Enzyme prodrug therapy via nanoparticles. First enzyme loaded nanoparticles will be injected and localized, then the prodrug (inactivated and non toxic form of the drug) will be systemically injected, and activated to its cytotoxic form at the tumor site by the enzymes loaded in the nanoparticles.

is not oxidized by mammalian peroxidases [144], it can be converted to reactive radicals by horseradish peroxidase (HRP) (figure 5.2 [150].)and be cytotoxic to cells [151, 152]. HRP is found in the roots of horseradish and is a very robust heme-containing peroxidase and can oxidize a wide variety of substrates including IAA in the presence of hydrogen peroxide. IAA is only toxic after oxidative decarboxylation; the effect of IAA/HRP is thought to be due in part to the formation of methylene-oxindole, which may conjugate with DNA bases and protein thiols [153]. The IAA/HRP-mediated apoptotic pathway induces caspase-8 and caspase-9 activation, which results in caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage [151].

Neither IAA nor HRP by themselves show cytotoxicity to cells [154, 155]. The robust nature of HRP and the low toxicity of IAA makes the combination of IAA and HRP an excellent candidate for targeted enzyme prodrug therapy [152, 154, 17, 144, 156, 157]. HRP has previously been conjugated to PEG [158, 159]increasing the molecular weight of the protein by approximately 25 kDa. However

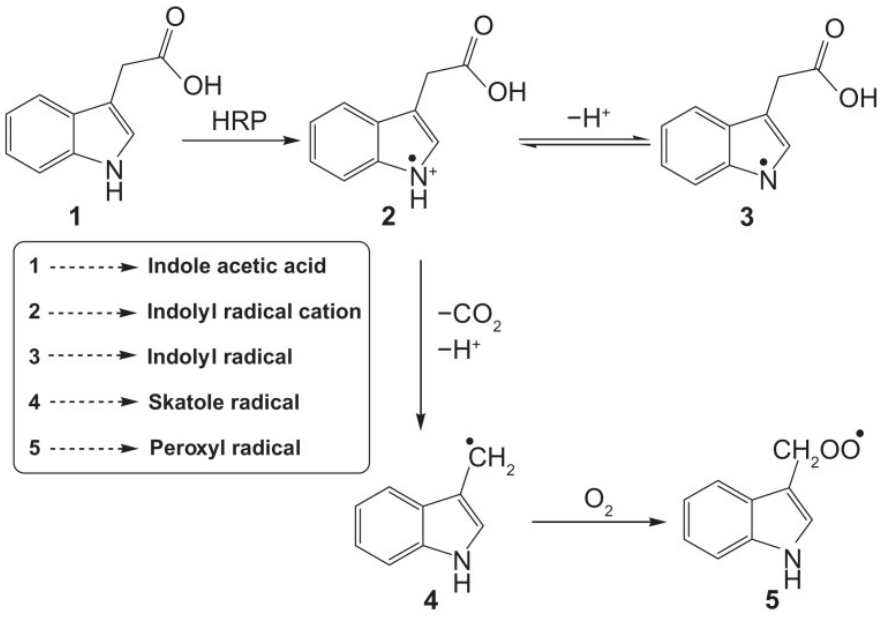


Figure 5.2: Free radicals formed by oxidation of indole-3-acetic acid by horseradish peroxidase (HRP) [150].

with this conjugation %50 of the enzymatic activity was lost [157]. Moreover Administration of HRP-PEG to tumour-bearing animals indicates a problem of liver uptake. [157, 160].

There is a clear need for a better and non toxic delivery platform for HRP without compromising enzymatic efficiency. This can be achieved by encapsulation of HRP in nanoparticles and accumulation of the said nanoparticles in the tumor followed by systemic administration of IAA that would lead to the localized activation of the prodrug at the tumour, preventing normal tissue damage.

5.1.3 HRP loaded SiLGA Activity and Dosing

HRP encapsulated SiLGA (HRP-SiLGA) was synthesized as mentioned before. Horse Radish Peroxidase (HRP) was encapsulated in PLGA and SiLGA. Empty PLGA and SiLGA particles were made as controls. In controls, enzyme was replaced with rat serum albumin (RSA).

To evaluate whether the encapsulation process had effected the functionality of the enzyme, unencapsulated and encapsulated HRP activity was evaluated using the Amplex Red assay kit.

As it can be seen in 5.3 HRP encapsulated in PLGA (HRP-PLGA) and HRP encapsulated in SiLGA (HRP-SiLGA) have maintained enzymatic activity which confirms the preservation of enzymatic activity after each step of encapsulation.

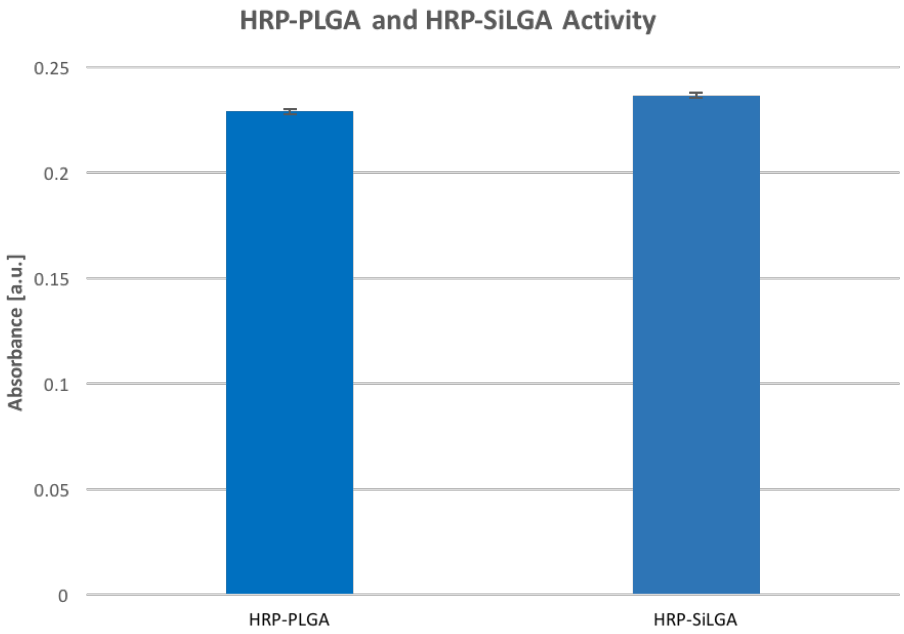


Figure 5.3: HRP activity compared

As expected, the activity varies as different concentrations of HRP-SiLGA are used in a dose dependent manner as shown in 5.4

5.1.4 HRP SiLGA Cytotoxcity

Two different concentrations of HRP-SilGA,5.6E9 (HRP-SiLGA-a) and 2.8E11 (HRP-SiLGA-b) , were incubated with HeLa cells for 5 hours. In the positive control cells, HRP-SiLGA was replaced with 1XPBS. In the negative control, cells were incubated with triton X and imaged using a confocal microscope. As it can

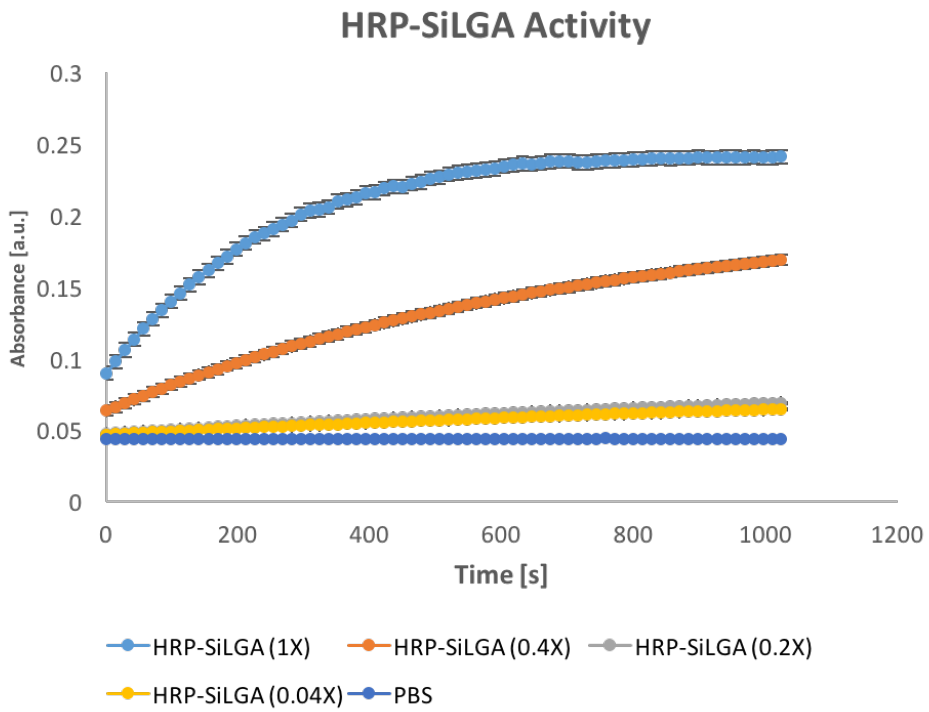


Figure 5.4: HRP-SiLGA Dose

be seen in 5.5, the cells incubated both doses HRP-SiLGA and control cells have similar characteristics to the control, indicating that HRP-SiLGA has minimal toxicity in HeLa cells, even in higher doses.

5.1.5 HRP-SiLGA and IAA Cell Viability via MTT

As a preliminary investigation for the application of HRP-SiLGA in enzyme prodrug therapy using IAA, MTT cell viability assay was performed in HeLa cells in the presence of IAA. HeLa cells were incubated with 0.8 mM IAA alone and 0.8mM IAA in combination with 5.6E9 HRP-SiLGA nanoparticles for 24 hours. After 24 hours MTT viability assay was performed. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) is a small molecule that is positively charged and undergoes NADPH-mediated conversion in live cells to form Formazan, changing its color from blue to purple in live cells that can be detected using a spectrophotometer. As it can be seen in 5.6, while 0.8mM of IAA shows no toxicity to the HeLa cells, the combination of IAA incubated with 5.6E9 HRP-SiLGA nanoparti-

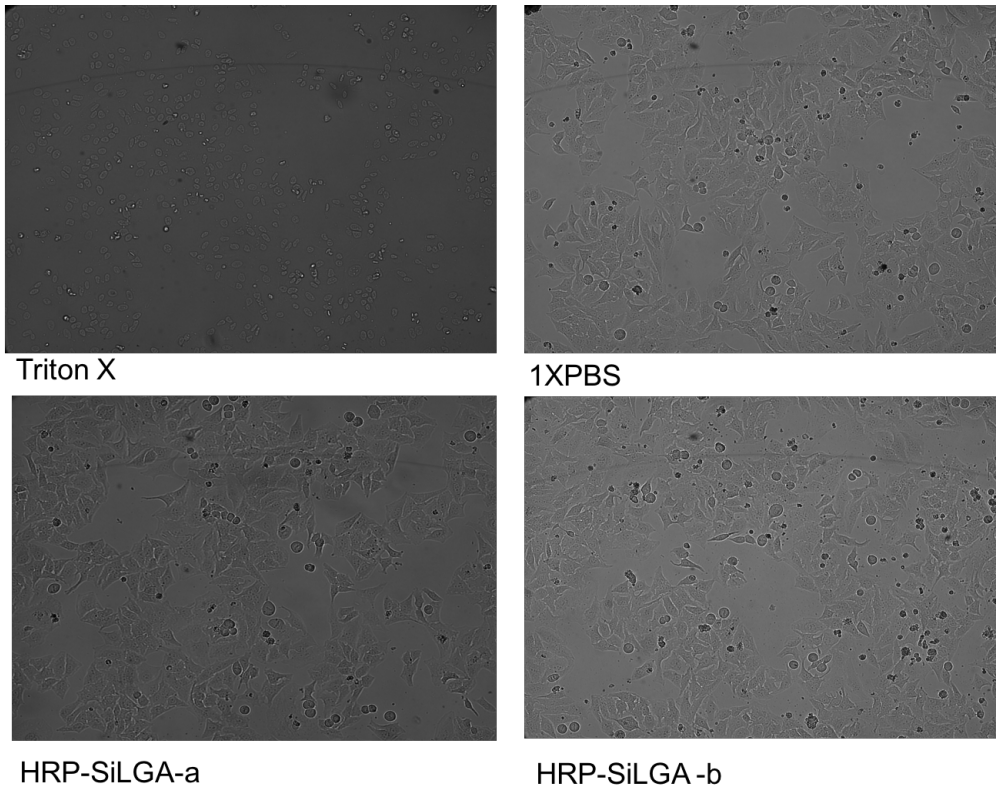


Figure 5.5: HRP-SiLGA's toxicity to HeLa cells. HRP-SilGA,5.6E9 (HRP-SiLGA-a) and 2.8E11 (HRP-SiLGA-b) , were incubated with HeLa cells for 5 hours. In the positive control cells, HRP-SiLGA was replaced with 1XPBS. In the negative control, cells were incubated with triton X. Cells were imaged using a confocal microscope.

cles were able to kill more than %94 of the cells.

5.1.6 Conclusion and Future Direction

Our preliminary experiment with enzyme prodrug therapy using HRP and IAA has shown promising results. While we have previously shown SiLGA high tissue residence time and minimal toxicity in mice, our results suggest minimal toxicity when SiLGA is incubated with HeLA cells. Our results also confirms that while the dose of IAA was not toxic to cell, its combination with IAA was able to kill more than %94 of the HeLA cells. These are of course preliminary results and more dosing studies and time studies are needed for HRP-SiLGA, as well as IAA in different cell lines. The effects of HRP-SiLGA and IAA can be further

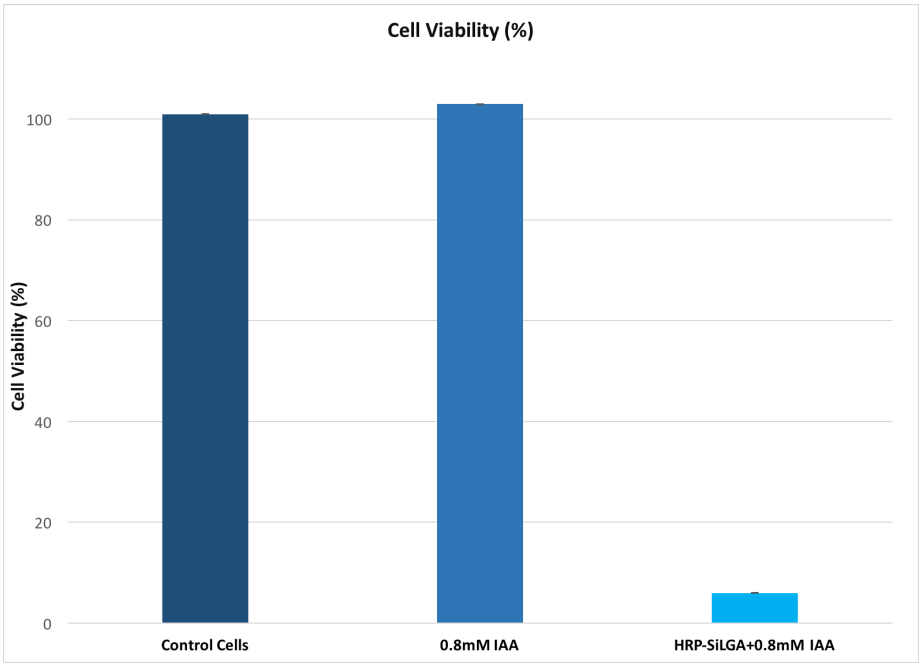


Figure 5.6: Cell viability analysis using MTT. HeLa cells were incubated with 0.8 mM IAA alone and 0.8mM IAA in combination with HRP-SiLGA nanoparticles for 24 hours. MTT viability assay was performed. Samples were done in triplicates, error bars as shown.

investigated by tumor shrinking studies *in vivo*.

5.2 Catalase and Antioxidant Therapy

In our bodies, Reactive Oxygen Species (ROS) such as superoxide anion, OH (hydroxyl radical) and H2O2 (hydrogen peroxide) are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis [161] These free radicals have one or more unpaired electrons, making them highly unstable. During normal conditions in our bodies there is a balance between the formation of reactive oxygen species and their clearance. However under certain conditions, this balance can be disrupted and lead to oxidative stress. Oxidative stress can be seen in in or as a results of many different conditions such as ischemia and reperfusion in stroke and heart attacks, cancer, multiple sclerosis, Parkinson's disease, etc [137, 122, 162, 163, 161].

To battle ROS, cells make a variety of antioxidant enzymes to convert reactive oxygen species to unharmful substances. Two important players are SuperOxide Dismutase (SOD), which converts super-oxide radicals into hydrogen peroxide, and catalase (CAT), which converts hydrogen peroxide into water and oxygen gas. Recent studies have utilized delivery platforms for antioxidant therapy via catalase [122, 164, 165].

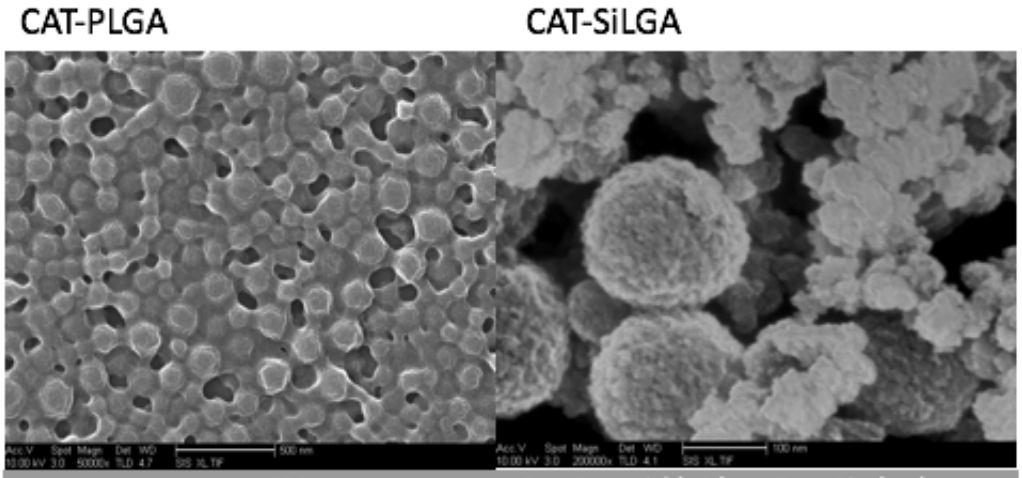
As mentioned before,in an attempt to protect neurons from oxidative stress, [122] reported successful encapsulation and release of Catalase from PLGA nanoparticles. Catalase is encapsulated in PLGA nanoparticles using double emulsion solvent evaporation method. These nanoparticles released Catalase activity for a month *in vitro*. Although successful encapsulation of Catalase in PLGA nanoparticles was shown, the release mechanism of PLGA nanoparticles remains the same. Due to the nature of the polymers, the cargo will be burst-released *in vivo*.

5.2.1 Catalase Loaded SiLGA Synthesis and Activity

In the first step of synthesis, the PLGA nanoparticles are used as a stepping stone to encapsulation of catalase using double emulsion solvent evaporation method [122] (CAT-PLGA) and afterwards the whole nanoparticle is sealed with a porous silica layer (CAT-SiLGA). This delivery platform,allows for catalaseto be protected inside the nanoparticles from immune clearance, while small molecules like hydrogen peroxide can diffuse inside the nanoparticle from the pores of silica and get converted to unharmful water and oxygen.

Zeta potential charge and poly dispersity index (PDI) was measured using Dynamic Light Scattering (DLS). PLGA nanoparticles and Silica coated PLGA nanoparticles (SiLGA) were imaged using Scanning Electron Microscopy (SEM) (figure 5.7).

After successful syhtnesis and characterization of CAT-SiLGA, enzymatic activity was measured using the amplex red assay kit. CAT-SiLGA's activity was measured to be 125 U/ml per 2.8E11 particles. As it can be seen in 5.8, the silica



	PDI	Zeta Potential (mV)	Z avg Size(nm)
PLGA	0.174	-19	239.3
Silica Coated PLGA	0.271	-25.6	318.7

Figure 5.7: SEM images of nanoparticles. Catalase loaded PLGA (CAT-PLGA) before coating with Silica (left) and Catalase loaded PLGA after coating with Silica (SiLGA) (right). Bottomn table shows hydrodynamic size, zeta potential charge and polydispersity index as measured by dynamic light scattering

coating process does not effect catalase activity.

5.2.2 Conclusion and Future Direction

CAT-SiLGA nanoparticles have been successfully synthesized and characterized with high activity. CAT-SiLGA can be utilized in antioxidant therapies against ischemia and reperfusion injury, cancer, diabetes, Parkinson's or any disease or condition that can benefit from antioxidant therapy. CAT-SiLGA can also be used as a diagnostic tool using ultrasound for detection of regional oxidative stress [138].

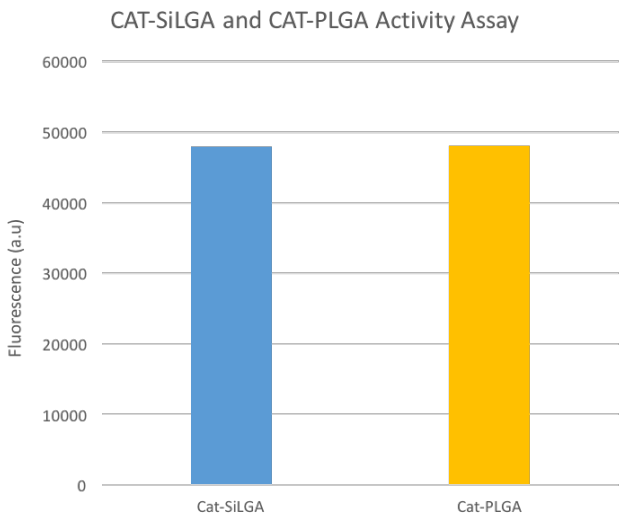


Figure 5.8: CAT-SiLGA and CAT-PLGA activity showing preservation of enzymatic activity throughout the sealing process.

5.3 Methods

5.3.1 Catalase and HRP Loaded SiLGA Synthesis

The PLGA nanoparticles are made by double emulsion solvent evaporation method by [122] with minor modification. First an appropriate ratio of enzyme to Rat Serum Albumin (RSA) (for example: 22mg:8mg or 8mg:22mg) are made in 300 µl of water. This makes the initial water phase (W1). RSA protects the enzyme during the encapsulation process.

The oil phase (O1) consists of 81 mg of PLGA (Poly lactic-co-Glycolic Acid) and 3mg DMT (Dimethyl L-tartrate) in 3mL of dichloromethane (DCM). Here DCM is used as the organic solvent and DMT as a plasticizer.

The second water phase (W2) contains a solution of %5 PVA. To prepare the %5 PVA, first 100 mL of water was heated up to 80C on a hotplate. Then 5 g of PVA was slowly added while mixing water. Then, the solution was let to reach room temperature and vacuum filtered. PVA acts as an emulsifier

The W1 mixture (enzyme and RSA in water) was added drop-wise to the oil phase (O1:PLGA and DMT in DCM) while vortex mixing for one minute. Afterwards, this mixture was probe sonicated on ice for 2 minutes with a total

energy output of approximately 1400 Joules.

The mixture was then added drop-wise to 18ml of %5 PVA (polyvinyl alcohol) in water, vortex mixed for one minute, and probe sonicated on ice for 2 minutes with a final energy output of approximately 2800 Joules; this process yielded the second water phase (W2). This makes the W1/O/W2 solution.

The solution was then left stirring overnight at room temperature to remove the organic solvent. This was followed by one hour of stirring in a vacuum desiccator to completely remove the organic solvent while also allowing the PLGA nanoparticles to harden.

To wash away excess PVA and any unencapsulated enzyme, the nanoparticles were centrifuged and recovered three times using ultra centrifugation (32000 rpm for 30 minutes at 4C). After each wash, the nanoparticles were resuspended in water and probe sonicated on ice as mentioned before.

The nanoparticles were then centrifuged at 1500 rpm for 10 minutes, and the supernatant was recovered. After this step, the synthesis of enzyme encapsulated PLGA nanoparticles is complete and particles are ready to be coated with Silica.

In empty nanoparticles, enzyme is replaced with RSA.

In order to coat the nanoparticles with silica, 50 µl of PLGA nanoparticles are added to 1ml of 1XPBS while mixing. A solution of silicic acid is made by adding 74µl of Tetramethoxysilane (TMOS) to 500µl 1mM HCl. The solution is mixed for a minute. TMOS is hydrolyzed in aqueous solution to give silicic acid. Then 15 µl of silicic acid is added drop wise to the particle solution.The resulting solution is left mixing at 4^0C over night.

To remove the excess silica, the solution is centrifuged at 4000 rpm for 15 minutes in 4^0C. The nanoparticles are resuspended in 1XPBS at each step. The final solution is probe sonicated on ice for 30 seconds (%20 amplitude, 2 seconds ON / 1 second OFF). The resulting nanoparticles from this step are enzyme-loaded, Silica-coated PLGA nanoparticles (enzyme-SiLGA).

Samples are characterized using SEM and DLS as well as enzyme activity assays for each corresponding enzymes

5.3.2 Amplex Red Assay

The Amplex Red Assay Kit (A22180) was used from Invitrogen. Amplex Red reagent reacts with a 1:1 stoichometry with any unreacted H2O2 in the presence of horseradish peroxidase (HRP), or HRP-SiLGA to produce the highly fluorescent oxidation product, resorufin. Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively.

5.3.3 Cell Culture

HeLa (ATCC CCL-2) cells was used to test toxicity of HRP-SiLGA . The cells were sub cultured at least 4 times prior to the study. 5000 cells/ well were plated and incubated 24 hours. The treatments were only studied upon 60-70% con fluency each well. The cells were incubated with 14E10 (high concentration of HRP-SiLGA) and 56E8 (low concentration of HRP-SiLGA). In the control HRP-SiLGA was replaced with PBS. Cells were imaged using a microscope.

5.3.4 Cell Viability via MTT Assay

HeLa (ATCC CCL-2) cells were used to test toxicity and these cells were sub cultured at least 4 times prior to the study.5000 cells/ well were plated and incubated 24 hours prior to any treatment. The treatments were only studied upon 60-70% con fluency each well. The cells were incubated with 0.8mM IAA and 1.4E11 HRP-SiLGA+0.8 mM IAA for 24 hours. In the controls IAA or HRP-SiLGA was replaced with PBS. Before the addition of the MTT reagent, the media was removed and replaced with the media containing 0.2mM MTT. The cells were incubated at 37^0C incubator for 3 hours before the addition of 50µl of DMSO to determine the absorbance at 540nm. All samples were done in triplicates.

5.3.5 Characterization

Absorbance and fluorescence measurements for activity assays were taken with the TECAN Spark(R) 20M Te-cool(TM). The Helios Nanolab(TM) 660 scanning electron microscope produced micrographs of particles. Particle size, Zeta potential, and polydispersity index were measured using the Malvern Zetasizer Nano ZSP dynamic light scattering instrument.

5.4 Material

The following materials were purchased from Sigma Aldrich and used as received: amorphous PLGA ("Resomer(R) RG 504 H"; acid terminated; lactide(38 kDA):glycolide(54 kDa); monomer ratio of 1:1); MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Thermo Fisher Scientific M6494; Pierce Horseradish Peroxidase from Thermo Fisher Scientific; Catalase from bovine liver, lyophilized powder, 2,000-5,000 units/mg protein from Sigma-Aldrich. lyophilized powder form of rat serum albumin (>99 percent purity); dimethyl-L-tartarate; dichloromethane (anhydrous; 40 to 150 ppm amylene stabilizer; >99.5 percent purity); polyvinyl alcohol (87 to 90 percent hydrolized; average molecular weight: 30 to 70 kDa). Molecular Probes Amplex Red Catalase Assay Kit from Fisher.The presterilized Stericup and Steritop Vacuum Driven Disposable Filtration System was utilized to filter the %5 PVA aqueous solution. The Q500 Ultrasonic Processor from QSonica (output: 500 W, 20 kHz) was used to create the water-oil-water double emulsion.

5.5 Acknowledgment

Chapter 5 and its methods are patented (Nanoscale delivery device and uses thereof,15/449,830). Collaborator for the patent are: Sadik Esener,Negin Mokhtari,Yasan Yeh, Mukanth Vaidyanathan, Ajay Sapre, Bartu Ahiska. The author of thisdissertation is a co-inventor of the Chapter 5 and its scientific methods are ready to be submitted for publication.The author of the publication are: Negin

Mokhtari, Mukanth Vaidyanathan, Kyle Gustafson, Jared Fischer, Sadik Esener. The author of this dissertation is the first author of the paper.

Chapter 6

Conclusion and Future Potentials

In this dissertation, two types of silica nanoparticles are discussed as efficient and robust enzyme delivery platforms. The porous silica coating on the surface of these nanoparticles allow them to operate like a nano-shark cages: the encapsulated enzyme (the scuba diver) is too large to diffuse out from the nanoparticle interior, antibodies (the sharks) are too big to reach the enzyme by diffusing into the nanoparticle interior, and small molecules like substrates or reaction products (the small fish) can freely diffuse in and out of the nanoparticles. The nano-shark cage-like effect of these nanoparticles demonstrate great advantage over conventional enzyme therapies, where the recombinant enzyme is rapidly cleared from the body due to its foreign nature and current enzyme delivery technologies (e.g. direct modification of the enzyme with PEG) which prompts some loss of enzymatic activity and only partially protects the enzyme from immune clearance.

The first nanoparticle system discussed are Synthetic Hollow Enzyme-Loaded Silica nanospheres (SHELS). SHELS were utilized for the depletion of the amino acid methionine using the enzyme, methioninase, via methioninase-loaded SHELS (MethSHELS). The experimental results for methSHELS showed successful encapsulation of Methioninase. MethSHELS maintained more than %60 of their activity after exposure to protease enzymes (proteinase K). One of the greatest challenges associated with Methioninase is cofactor loss, which leads to enzyme inactivation in the presence albumin, the most abundant blood protein. Our experimental results showed that methioninase encapsulation in SHELS improves the enzymatic stabil-

ity by preventing inactivation in the presence of human serum albumin. Moreover, a more durable and deeper methionine depletion was seen by MethSHELS compared to bare (unencapsulated) Methioninase for up to 12 hours when injected intramuscularly. Future directions include :acquiring higher quality Methioninase to be loaded into the SHELS as well as further optimization of loading and dosing of MethSHELS, specifically for *in vivo* studies.

Even though SHELS show promise as a delivery platform, they entail a complicated synthesis process. Due to size limitations of the holes, enzyme loading, especially for bigger or slightly aggregated enzymes, will be challenging. This issue increases in magnitude as the particle size decreases; we anticipate pore size to limit 100 nm SHELS spheres notably more than 200 nm SHELS spheres. Even so, the two-step synthesis of SHMS and SHELS both involve silica and utilizing an FDA-approved material already common in the clinic has clear benefits.

With that in mind, a new delivery platform was developed using FDA-approved poly(lactic-co-glycolic acid) (PLGA). PLGA was used as a scaffold to encapsulate different enzymes and was coated with a porous silica layer to overcome the greatest challenge of PLGA nanoparticles: the burst release of their cargo. For this purpose, silica-coated enzyme-loaded PLGA nanoparticles (SiLGA) were developed and their structural, in-vitro functional, and in-vivo tissue residence time were characterized. Structural characterization results confirmed differences in size and charge between PLGA nanoparticles and SiLGA nanoparticles.PLGA nanoparticles had an average size range of 100-200 nm with a polydispersity index of 0.093, while SiLGA nanoparticles showed a size range of 100-260 nm with a PDI of 0.239. The particles were also visually characterized using SEM to confirm successful silica coating. For functional characterization, Penicilinase (BLA) was loaded into PLGA nanoparticles (BLA-PLGA) and then coated with Silica (BLA-SiLGA). BLA-SiLGA maintained of enzymatic activity throughout the synthesis process, exhibiting more than %83 of the BLA activity upon exposure to the protease enzyme, proteinase K. This shows a clear advantage over the %64 retention of activity previously achieved by SHELS and the %4 retention of activity by PLGA alone. Strong *in vitro* results further confirm successful silica coating. The superior protection capabilities of enzyme-loaded SiLGA nanoparticles gives us more con-

fidence that SiLGA nanoparticles offer significant and similar protection against proteolytic enzymes *in vivo*. To explore *in vivo* behavior, Alexafluor680-conjugated BSA was encapsulated in SiLGA (BSA-SiLGA) and injected intramuscularly into mice. While unencapsulated BSA cleared within a day, the BSA-SiLGA was non-toxic to mice and show an in-tissue-residence time at least 60 days when they are injected intramuscularly. Repeated dosing, quantification of immune response, and alternative routes of *in vivo* administration require further study.

SiLGA's application was investigated in enzyme prodrug therapy using Horseradish Peroxidase (HRP)-loaded SiLGA (HRP-SiLGA). Indoleacetic acid (IAA), a non-toxic plant hormone used as a non-active prodrug, can be systemically injected and activated at the tumor site by localized HRP-SiLGA to produce free radicals toxic to cancer cells. For this purpose, HRP-SiLGA was developed and characterized *in vitro*. While we have previously shown high tissue retention time of SiLGA nanoparticles with minimal toxicity in mice, our preliminary results also show that HRP-SiLGA has minimal toxicity to HeLa cells. Moreover, our preliminary MTT results showed high cytotoxicty to HeLA cells (%>94) when HRP-SiLGA was combined with IAA, but minimal cytotoxicity for IAA alone. These preliminary results are quite promising. Further optimization of HRP-SiLGA, as well as more detailed dosing studies, could greatly improve these results. The effects of HRP-SiLGA and IAA will be further investigated by tumor-shrinking studies *in vivo*. Catalase-loaded SiLGA (CAT-SiLGA) was also developed as a potential antioxidant therapeutic and diagnostic. CAT-SiLGA was successfully synthesized and characterized, demonstrating high activity. These promising results makes CAT-SiLGA a promising delivery platform for application in antioxidant therapies against ischemia and reperfusion injury, cancer, diabetes, Parkinson's, and any disease or condition that can benefit from antioxidant therapy. Used in-conjunction with ultrasound, CAT-SiLGA can also serve as a diagnostic system for detecting regions of oxidative stress.

SiLGA nanoparticles are a novel nanoscale delivery platform and can be employed in therapeutic and diagnostic applications in different diseases.The inert nature of their constituent, FDA-approved materials as well as their small size and mechanism of action, makes SiLGA practical and effective for enzyme deliv-

ery in many diseases and conditions including cancer (for amino acid depletion therapy and enzyme prodrug therapy), antioxidant therapy for oxidative stress for example in ischemia and reperfusion injury, Multiple Sclerosis,Diabetes,etc, as well as enzyme replacement therapy for genetic diseases. SiLGA nanoparticles could potentially be used for imaging by encapsulating a fluorescent payload or as bio-sensors. Additionally, the silica surfaces of these particles can be modified to enhance circulation and targeting *in vivo* without modifying the payload itself.

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