Biomimetic Nanocarriers for Enhanced Drug Diffusion in Prostate Tumors with a High Level Hyaluronan

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Abstract
Biomimetic Nanocarriers for Enhanced Drug Diffusion in Prostate Tumors with a High Level Hyaluronan
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Cancer therapies aim to eliminate malignant tumor tissues that grow uncontrollably within patients. However, due to many challenges unique cancer microenvironments pose; chemotherapeutic agents have low accumulation and diffusion through the tumor tissues. Pharmacokinetics and toxicity of these therapeutic agents can be altered with engineered biomimetic nanocarriers for targeted drug delivery applications to increase the efficacy and reduce the side effects of drugs.

The motivation of this project is to develop a biomimetic nanocarrier to increase drug accumulation and diffusion in tumors with high levels of hyaluronan (HA). We envision that red blood cell (RBC) membrane coated poly(lactic-co-glycolic acid) (PLGA) nanocarriers with extra cellular matrix degrading enzymes (ECMDEs) on surfaces are ideal candidates for this purpose because this platform combines the advantages of various biomimetic platforms. PLGA core supports the RBC vesicle and allows sustained drug release, while the RBC vesicle provides natural camouflage within the body extending circulation time. RBC membrane surface also allows the attachment of ECMDEs to degrade tumor ECM that poses a challenge for drug distribution.

For the first time RBC-PLGA nanocarriers with hyaluronidase (HAase) as the ECMDE on surfaces were successfully created. These RBC-PLGA-NPs with HAase on surfaces were shown to facilitate NP diffusion through a pericellular HA layer around prostate cancer cell line PC3, illustrating the potential of this nanocarriers for enhanced drug diffusion within tumors with high levels of HA.
1. Introduction

Cancer is one of the leading causes of death. Cancer cells can metastasize to other parts within the body through the circulatory and lymphatic systems and invade neighboring areas. Benign tumors, on the other hand, are not cancerous and they do not spread within the body. There are more than 200 cancer types known currently and cancer types are named after the organ or the type of tissue or cell which they originate [1].

Prostate cancer is the abnormal growth of the tissues of the prostate gland, which is located below the bladder and in front of rectum, in the male reproductive system (Figure 1-1). Prostate cancer is very uncommon for men under the age of 45; however it becomes more common with advancing age. Even though prostate cancer mortality rate has been declining in US due to more advanced treatment options and early stage diagnosis, it is still the most common cancer in men and it has the second highest leading cause of cancer related death in men, after lung cancer [2].

Figure 1-1 - Healthy prostate gland [3]
Cancer starts when a single cell or a group of cells undergo changes due to the damage to the genes or mutation. Therefore it is important to look at cancer at the tissue level to understand the threats and obstacles it creates. The unique tumor microenvironment surrounding cancer cells poses many challenges for effective treatment. Chemotherapy, radiotherapy and surgical approaches are the main treatment options available today; however these options have many drawbacks. While surgical approaches are very invasive, chemo and radiotherapies are very toxic. To achieve effective therapeutic outcomes, high doses are administered leading to many unwanted side effects. In the light of these challenges, nanocarriers with encapsulated drugs have been investigated as an alternative treatment mechanism.

Nanocarriers can be designed for different applications to have desired properties; therefore they have been investigated heavily for drug delivery applications. Depending on the material and the properties of the nanocarriers, the pharmacokinetics and toxicity of encapsulated drugs can be altered. As a result, higher efficiency with lower doses of chemotherapeutic agents can be achieved leading to reduced side effects. However, the aforementioned challenges tumor microenvironments pose, such as irregular and dense matrices surrounding tumor cells, can decrease the performance of nanocarriers by hindering their access to tumor. Furthermore, fast clearing of synthetic drug nanocarriers from the circulation due to being recognized as foreign in the body is also a common issue.

To overcome these obstacles red blood cell membrane-derived nanocarriers (RBC-NPs) with extracellular matrix degrading enzymes (ECMDEs) on surfaces were developed. Nanocarriers derived from RBC membranes obtained from the blood of patients are expected to be recognized as “self” by immune systems partly because of the immunosuppressive proteins on RBC membranes. Reduced clearance of nanocarriers will increase their tumor targeting, while the ECMDEs on surfaces will target the dense tumor stroma, allowing nanocarriers to penetrate and diffuse through the ECM to reach cancer cells. In the case of prostate cancer, the overexpression
and remodeling of type I collagen are associated with the development of the cancer, while the amount of hyaluronic acid (HA) in the prostate tumors is 3-8-fold of that in adjacent normal tissues [4]. Furthermore, the interaction between HA and cancer cells, in which HA matrix expands upon hydration allowing angiogenesis and tumor cell migration, is correlated to cancer metastasis. To enhance nanocarrier diffusion with tumors with a high level of hyaluronan, HA degrading enzyme hyaluronidase (HAase) is conjugated on the RBC membranes which are then used to coat PLGA nanoparticles.

To quantify the effective enzymatic activity of the HAase conjugated RBC-PLGA-NPs, an ELISA-like microtiter-based assay, which allows quick, simple yet sensitive evaluation of the enzyme activity, was employed [5]. HAase conjugated RBC-PLGA-NPs exhibited higher enzymatic activity compared to HAase conjugated on intact RBCs. To compare the effective activity of HAase conjugated RBC-PLGA-NPs to unmodified RBC-PLGA-NPs with free enzyme, confocal imaging and quantification of the NP uptake by PC3 cells was performed.

2. Background

2.1. Tumor Microenvironment

For the last couple of decades, tumors have been regarded as isolated masses of cancerous cells. In contrary to this belief, cancerous tissues have microenvironments with distinct properties, which contribute to tumor initiation, progression and metastasis as well as therapeutic resistance [6]. Similar to healthy tissues, tumor properties depend on the interactions between cancer cells and stromal cells within the tumor microenvironment. The stromal tissues of tumors also consist of fibroblasts, myofibroblasts, endothelial cells, immune cells and extra cellular matrix components.

Tumor microenvironments differ from healthy tissues for various reasons. Just like any other tissue, tumor tissues need a supply of oxygen and nourishment carried by blood for survival.
Therefore, the tumor stroma promotes angiogenesis to build new vasculature as the tumor grows. However, the vasculatures that grow within tumors are abnormal compared to healthy vessels due to rapid proliferation of tumor cells compared to endothelial cells. Endothelial cells are often discontinuous leading to large gaps between them. Depending on the location within the body, these gaps can vary from 100 to 780 nm in diameter [7]. What’s more, the walls of tumor vessels might have abnormalities such as lack of perivascular smooth muscle, discontinuous or absent basement membranes and integrated cancer cells [8]. As a result, tumor vessels tend to be leaky with irregular blood flow within the tumor tissues. This poor network of vessels cause some regions of tumor, which are distant (>100μm) from blood vessels, to have poor nutrient and oxygen transport leading to hypoxia and highly acidic regions (Figure 2-1) [8,9]. Many solid tumors are hypoxic including prostate cancer. The hypoxic cells in tumors are relatively resistant to cancer treatment options such as chemo and radiotherapies.

Unlike healthy tissues, tumor tissues lack lymphatic vessels and transport. Lymphatic system is responsible for the transportation of interstitial fluid and it plays an important role in the immune system. The lack of lymphatic drainage within tumor tissues leads to high interstitial fluid pressure. Combined with the abnormal blood vessels, high interstitial fluid pressure causes compressed vessels, preventing distribution of larger molecules within solid tumors. Figure 2-2 shows an overall model of a tumor tissue with the aforementioned issues.
Figure 2-1 - A) Diagrammatic representation of tumor microenvironment and how it is affected in relation to the nearest blood vessels. B) Graph depicting the decrease in oxygen and pH levels in relation to the nearest blood vessels [8].

Figure 2-2 – Tumor microenvironment: tumor growth gradient caused by the lack of adequate blood vessels. Abnormal vasculature and lack of lymphatic vessels leading to high pressure areas within the tumor tissue [10].
ECM composition of tumor tissues is different than healthy tissues and it can vary among different tumor types as well. It is found that tumor tissues are often tough and fibrotic due to increased activity within the ECM. This dense composition of ECM can slow down or completely inhibit movement of molecules within the tumor tissue. For example, it was found that one of the components of the stroma, fibroblasts, have an altered phenotype in prostate cancer, called myofibroblasts, which leads to increased activity in ECM linked to increased HA production [11]. Jenkins et al. found that myofibroblastic differentiation in tumor stroma leads to HA accumulation due to reduced hyaluronidase production and activity [12].

These different properties of tumor microenvironment make it almost impossible to have “one fits all” type of treatment. This is the reason why various treatment options are available for each cancer type, and new methods are still being researched.

2.2. Prostate Cancer Therapy

Many treatment options exist for cancer in general with chemotherapy, radiotherapy and surgery being the most common ones. The type, the location, the stage and some other patient related factors determine what type of treatment could be used against cancer. However, these options have many drawbacks. Surgical approaches are invasive; they may cause nerve damage and loss of function. What’s more, not all cancers can be surgically removed. Chemo and radiotherapies are highly toxic with many side effects and they damage the healthy tissue as well as the cancerous tissues. Patients can grow resistance to chemotherapies, and increasing the dose of administered therapeutical agents is not always an option.

Although significant progress has been made regarding prostate cancer treatment over the past few decades, the currently available treatment methods, as shown in Table 2-1, remain very expensive, invasive and not effective enough.
Surgery, hormonal therapy, radiotherapy and chemotherapy are the most common treatment methods employed in the case of prostate cancer. Surgery is a very invasive procedure, and for prostate cancer cases, prostatectomy, the total removal of the prostate, is the primary approach. Sometimes radical prostatectomy, surgical removal of the entire prostate, the seminal vesicles and some of the surrounding tissue, can be performed if the case is considered high-risk. In metastatic cases orchidectomy, total surgical removal of testes, is also performed. These radical approaches to remove cancerous tissue from the body also reduce life quality with side effects such as nerve damage, loss of sexual function and urinary incontinence. However, in severe cases of metastatic
prostate cancer, in which the tumor spreads to bones and neighboring organs surgery is not even an option.

Hormonal therapy is another therapeutic method employed for the treatment of prostate cancer. Hormonal therapy can consist of androgen deprivation through anti-androgen or luteinizing hormone-releasing hormone (LHRH) agonists, and estrogen derivatives. Hormonal therapy has a lot of side effects such as hot flashes, mood changes, decreased libido, osteoporosis, weight gain, lethargy, cognitive decline and loss of muscle mass. Apart from the side effects, it has been suggested that continuous hormone therapy consisting of androgen deprivation can cause the prostate cancer to progress to an androgen-independent state [13]. Another thing to consider is that hormonal therapy is a very expensive treatment option in United States. The anti-androgen treatment costs more than $150000 per year alone [14].

Radiotherapy for prostate cancer is usually used for low-risk, localized cases. Radiotherapy can be administered through external-beam radiotherapy or internal radiotherapy (brachytherapy). Brachytherapy involves implanting radioactive seeds in or near the tumor, whereas external beam radiotherapy is involves application from an outside radiation source. Both methods are recommended for patients who are not suitable for surgery and have localized tumors. These methods can potentially cure patients by killing cancerous cells, however, in case of recurrence; surgery cannot be done on patients who underwent radiotherapy. Other disadvantages of radiotherapy include erectile dysfunction and significant damages to surrounding tissues leading to rectal and bladder injuries.

Chemotherapy is administered to patients with advanced prostate cancer. Taxotere (docetaxel), which was approved by the FDA in 2004 and Zytiga (abiratone acetate) are the commonly used chemotherapy agents for prostate cancer, as well as Jevtana (cabazitaxel) which is recently approved by the FDA. Alone or combined these drugs can extend the life expectancy of the
patients. However; similar to other treatment options, chemotherapy can cost up to $500,000 per patient, and it has many side effects [15]. Cost and side effects of chemotherapy can be lowered by increasing the efficacy of the drugs administered. Increasing the drug accumulation and diffusion in tumor could lower the number of doses administered leading to lower costs and lesser side effects due to highly toxic chemotherapy agents. Apart from increasing the efficacy, employing a mechanism that allows the drugs to be delivered specifically to the tumor can lower side effects by lowering the drug take up by other organs. Nanomedicine research aims to take on these challenges and solve the aforementioned problems.

Nanocarriers encapsulating anticancer drugs can alter the pharmacokinetics and toxicity of encapsulated drugs, leading to improved efficacy and reduced side-effects. Through specific targeting the accumulation of anticancer drugs in tumor tissues can be increased. Use of nanocarriers can improve the accumulation of therapeutic agents in tumor tissues due to the enhanced permeability and retention (EPR) effect. EPR effect is the result of newly formed leaky vasculature and poor lymphatic drainage present in tumor tissues. Nanoparticles within circulatory system can easily diffuse out of these leaky vasculatures into the tumor tissues, and can accumulate there due to poor lymphatic drainage. Improved accumulation and specific targeting can prevent administration of higher doses. However, while the unique tumor microenvironments can be targeted via nanocarriers, they also pose many barriers to drug delivery.

2.3. Barriers to Drug Delivery in Solid Tumors

Currently available options for cancer treatment can fail to treat cancer completely. This failure is usually associated with the resistance of the tumors to therapeutic agents that arise from genetic mutations, amplifications or epigenetic changes which influence the drug uptake [9]. For example, Sun et al. showed that mitoxantrone and docetaxel therapy, two common chemotherapy
agents used against prostate cancer, can induce the production of WNT16B proteins which promotes cancer cell proliferation and invasion in prostate cancer cells (Figure 2-3) [16].

![Figure 2-3](image)

**Figure 2-3** – (a) Immunohistochemistry assessment of prostate stromal WNT16B expression in prostatectomy tissue samples from men with prostate cancer who were either untreated (n = 30) or treated with chemotherapy (n = 50). Stromal WNT16B staining: 0, no expression; 1, faint or equivocal expression; 2, moderate expression; 3, intense reactivity. (c) Representative example of intense WNT16B expression in prostate stroma after in vivo exposure to mitoxantrone and docetaxel. The black arrows denote areas of the periglandular stroma with fibroblasts and smooth muscle. Grey arrows show the minimal WNT16B reactivity in the epithelium. Scale bars, 50 µm [16].

However, one of the most important reasons for treatment failure is the poor penetration and diffusion of drugs within the tumor tissue. It is important to reach all the tumor cells to deliver therapeutic agents in lethal concentrations. When anticancer drugs cannot penetrate and diffuse through the tumor tissue, the drugs cannot be delivered efficiently leading to resistance or regrowth of residual tumor cells. Increasing the dose of administered anticancer drugs doesn’t alleviate the problem; instead it leads to many side effects due to the toxicity of the drugs.

A successful drug delivery process requires the drugs to circulate within a vessel, diffuse out of the blood vessels into the tumor tissue, and penetrate the tissue to reach every cell. These steps
are influenced by the physicochemical properties of therapeutic agents such as size and diffusivity as well as the microenvironment of the tumor. While low molecular weight agents can circulate in the blood stream, diffuse out of the vasculature and acquire homogenous biodistribution in healthy tissues, solid tumors pose many barriers.

Diffusion is a passive form of transportation and it requires the presence of a gradient. Drug diffusion depends on the gradients of hydrostatic and osmotic pressures between the blood vessels and the interstitial space of the tumor [9]. Apart from the ability of the drug to diffuse and penetrate, half-life of the anticancer agents in the circulation are also important, since a short half life would lead to quick clearance of drugs before reaching cancer cells.

Tumor microenvironment plays a huge role in drug diffusion and extravasation and can affect how efficiently anticancer drugs are delivered. As mentioned in the previous chapter, tumor microenvironment has unique traits such as dense ECM, hypoxicity, acidity, leaky vasculature and lack of lymphatic drainage. All these different factors affect the sensitivity of the tumor tissues to drugs as well as the efficiency of drug delivery.

The acidity of the tumor microenvironment can alter the pharmacokinetics of anticancer drugs. The low pH in tumor microenvironment creates a gradient compared to the neutral to alkaline pH inside tumor cells. This gradient can lead to the protonation of the weakly basic drugs, which decreases the cellular uptake of weakly basic anticancer drugs such as doxorubicin and vinblastine, while weakly acidic drugs can experience an increased uptake [17]. Apart from passive transport, active transport of drugs can also be inhibited due to the acidic tumor microenvironment.

The abnormal leaky vasculature and poor lymphatic drainage of tumors can cause high interstitial pressures. This increased pressure affects the pressure gradient between the blood vessels and the tumor interstitial tissues. When the interstitial pressure is high, drug molecules cannot diffuse out
of blood vessels. Also since tumors have layers of tissues that not in the vicinity of blood vessels, drug molecules need to be able to penetrate and diffuse further distances to reach all the cells. As shown in Figure 2-4, anticancer drugs may not be able to penetrate into the hypoxic regions further away from blood vessels in tumor tissues. Leakiness is not the only abnormal thing about tumor vessels. Blood vessels in tumors usually have branching patterns with dead ends and excessive loops which lead to disorganized blood flow. In general, blood flow in vessels within tumors is lower compared to healthy tissues which create a barrier to drug delivery [7].

Figure 2-4 – Fluorescent microscopy of a murine breast cancer demonstrates the perivascular distribution of an anticancer drug (doxorubicin) in vivo. Drug has little to no diffusion in hypoxic regions. Blood vessels are shown in red, drug in blue and hypoxic tumor cells in green [18].

Dense ECM of tumor microenvironments negatively affects drug penetration and diffusion. Tredan et al. indicates that “tumors that have a well-organized and richly interconnected collagen network display lower penetration by high molecular weight agents than those with a poorly organized collagen network” [9]. Lower drug penetration in tumor with dense ECM is due to the
ECM acting as a physical barrier. Large molecules such as antibodies cannot diffuse through the ECM due to their poor diffusion coefficients as well as due to binding to epitopes in the ECM [18]. Aaltomaa et al. found that all prostate cancer specimens express HA in their stroma and 78% of the tumors show strong stromal expression [19]. This accumulation of ECM components such as HA can cause uneven distribution of administered chemotherapy agents, even completely preventing the penetration of large drug molecules.

### 2.3.1. ECM Component: Hyaluronic Acid

Hyaluronic acid (HA) also called hyaluronan or hyaluronate, is an anionic high-molecular mass polysaccharide found in the extracellular matrix. Since it has repeating disaccharide units and an amino sugar as seen in Figure 2-5, it is categorized as a nonsulfated glycosaminoglycan (GAG) or a mucopolysaccharide. HA is a high molecular weight GAG; one HA molecule can have several thousands of repeating disaccharide units.

![Figure 2-5 - Chemical structure of hyaluronic acid](image)

Unlike other GAGs which are produced in the Golgi apparatus in cells, HA is produced in the plasma membrane of fibroblasts and other cells such as synovial cells, and it lacks covalently linked peptides as well as epimerized uronic acid residues [21]. Within the body HA is mostly found in the extracellular and pericellular matrix but it can also be present in the cells as well.
The synthesis of HA is carried out by the enzymes hyaluronan synthases which are integral membrane proteins, that are permanently attached to a biological membrane. Mammalians have 3 different types of hyaluronan synthases: HAS1, HAS2 and HAS3 located at the cytoplasmic side of the plasma membrane, which synthesize HA by adding glucuronic acid and N-acetylglucosamine to a co-synthesized polysaccharide. Growth factors such as EGF, PDGF, TGFβ, IGF-I are involved with the activation of the synthesis process [22].

HA plays an important role within humans. It is most commonly found in soft-connective tissues providing resilience since it can hold a lot of water as mentioned before, as well as increasing the viscosity of synovial fluid and providing lubrication. However it is not just a “goo” molecule as it was described until late 1970s as explained by Toole [23]. HA has many different functions within the body. HA found in the skin promotes wound healing through facilitating the inflammation process. Zhao et al. stated that HA optimizes the matrix composition during the wound healing process, and even though the early decomposition products of HA promotes vascularization, it doesn’t lead to hypervascularization [24]. Apart from these, experiments of Camenisch et al. suggested that HA has an important role in facilitating cell behavior such as migration, proliferation and differentiation, therefore HA is an important part of the development of many vital organs such as heart and brain in an embryo [25].

In cancer

During the development of an embryo, many HA filled spaces as well as HA coated cells are present. This HA coatings around the cells are called pericellular HA, and they disappear as the embryo grows as shown in Figure 2-6. Pericellular HA protects the cells from cytotoxic lymphocytes and viruses, and provides a highly hydrated medium which assembles other matrix components that aid the cells growth and differentiation.
As mentioned, pericellular HA disappears in a growing embryo; however pericellular HA can be present in many tumor tissues such as prostate cancer, breast cancer, bladder cancer and pancreatic cancer. The pericellular matrix can harbor molecules such as HA, collagen, chondroitin sulphate and aggrecan. This increased production of HA and formation of HA matrix around tumors are attributed to the over expression of HAS2 and HAS3, which in return results in enhanced tumorigenicity of the cells [27]. Increased production of HA in various different cancer cell cultures was characterized by Jiang et al. [28]. Among the various breast cancer and prostate cancer cell linings they cultured, 4T1 breast cancer and PC3 prostate cancer cultures showed the highest HA expression in the culture media; 473.83 ng/mL and 294.45 ng/mL respectively.

The dense HA matrix around the cancer cells presents many challenges for the treatment of the cancer. According to Koyama et al, increased HA production induces epithelial to mesenchymal transition (EMT), which is responsible for cancer cells to become metastatic [29]. Apart from
that, it is shown in various cases that HA may contribute to multi-drug resistance with its receptor CD44 by increasing the expression of factors responsible for the removal of drugs from the cell [27]. Another mechanism HA impairs drug effectiveness is by causing high interstitial pressure in tumor tissues which leads to compressed vessels. When the blood vessels in tumor tissues are compressed, the permeability of the vasculature is decreased which means the administered drugs cannot leave these compressed blood vessels due to limited diffusion (Figure 2-7).

Figure 2-7 - HA matrix affects the efficiency of drug delivery. HA degrading enzymes can alleviate the issues caused by the HA matrix [30].

To overcome these barriers, various not so successful treatment strategies have been utilized such as administering increased doses of the therapeutic agents which comes with many harmful side-effects. Another strategy has been the utilization of nanomedicine for drug delivery by
administering the therapeutic agents within nanocarriers to increase the accumulation and penetration of the drug within the tumor tissues.

2.4. Nanomedicine and Drug Delivery

Nanotechnology has been the key to many innovations. With the advancements in nanotechnology over the years, many inter-disciplinary fields that utilize nanomaterials were born. Nanomedicine is one of these inter-disciplinary fields and it brings together nanotechnology and related research including but not limited to biology, chemistry, physics, and material science for medical applications. One of the areas that nanomedicine focuses on is drug delivery. Drug delivery combines the efforts of various interdisciplinary fields to utilize the desirable properties of new materials with the biochemical properties of therapeutic agents.

Drug discovery and FDA approval process is a very long and expensive process, therefore the focus have been mainly on improving the pharmacokinetic and pharmacodynamic properties of current drugs. However, completely novel nano-platforms for drug delivery have also emerged recently [31]. Today, drug delivery research aims to combine drugs or molecules of interest with biomaterial carriers such as polymers, liposomes, polysaccharides, cells or cell ghosts to deliver them to specific cells in the body to increase the bioavailability and reduce toxicity. Specific targeting can increase the effectiveness of drugs as well as eliminating the need to administer high doses, which can mean fewer side effects. Specific targeting also reduces costs and human suffering through this highly selective mechanism [32].

Nanoparticles have been a desirable platform for drug delivery because their structure, surface properties and drug release rate can be tailored for broad applications. Nanosize of the particles provide a very large surface to mass ratio which allows the particles to bind or carry a greater amount of other molecules such as targeting ligands. Besides large loading capacity and specific targeting, nanocarriers can also protect the loaded drugs from degradation and provide sustained
release. Another important advantage of the nanosize is that it can allow access into cells directly as well as allowing benefiting from the EPR effect in cancer therapy. However, as discussed earlier, factors such as increased interstitial pressure and low circulation half-life can hinder the diffusion and accumulation of nanoparticles in tumor tissues.

2.4.1. Current Strategies for Increasing Nanoparticle Diffusion in Tumors

Therapeutic agent diffusion and retention in tumors are important factors that determine the efficiency of drug delivery systems; therefore they have been subjects of interest. Researchers have been studying various approaches to increase nanoparticle diffusion and retention such as strategies aiming to alter the properties of nanocarriers, strategies aiming to utilize electronic device assisted methods and strategies aiming to alter the solid tumor microenvironment.

Pharmacokinetics of nanocarriers is one of the important aspects that can affect drug diffusion in tumors. For adequate drug delivery, the nanocarriers need to have long circulation half-life. Long circulation half-life means enough time for the nanoparticles to diffuse out of vessels and accumulate in tumors. Circulation half-life is affected by kidney, liver and reticuloendothelial system (RES) activities which aim to filter the blood from foreign substances. Since administered therapeutic agents are foreign objects in the blood stream, they need to be stealthy to evade the filtering mechanisms. Filtration in the kidneys occurs at the glomerular basement membrane which has pores about 6 nm in diameter [10]. Therefore, therapeutic agents above the size of 6 nm diameter are predicted to be poorly removed by the kidney. However larger molecules can be removed from the circulation by RES and liver recognition. What’s more, surface charge and hyrophilicity of nanocarriers can affect their circulation half life as well. Hydrophobic and highly charged molecules are recognized by the liver and RES as foreign, therefore they are cleared from the circulation quickly. Kobayashi et al. indicated that limiting therapeutic agent size to 300 nm
in diameter, maintaining a surface charge close to neutral and providing a hydrophilic surface would be useful design parameters for therapeutic agents [10].

Use of targeting ligands, such as antibodies and peptides for drug delivery purposes has been widely investigated. Tumor and cell-targeting peptides have been used in conjunction with other therapeutic methods to increase the efficiency of anticancer treatments [33,34]. Sugahara et al. investigated the delivery of nanoparticles and other compounds via tumor targeting peptide iRGD which mediates penetration into tissues and cells through a multistep binding and penetration mechanism as shown in Figure 2-8 [35]. The researchers achieved 12-fold higher tumor homing with the use of iRGD compared to control peptides; however, the molecular mechanism that leads to rapid tumor tissue penetration has not been elucidated.

Figure 2-8 – The iRGD peptide accumulates at the surface of cells that express αV integrin on the surface. RGD mediates the integrin binding which leads to the cleavage of the peptide. The exposed elements of the peptide mediates binding to neuropilin-1, resulting in tissue penetration [35].
Kong et al. developed magnetically vectored nanocarriers as shown in Figure 2-9 with a drug release mechanism that can be switched on and off through the application of external magnetic field [36]. Intratumor tissue penetration of these particles can also be increased with the application of external magnetic field. The in vivo studies showed that the entrapment of SiMNCs in tumor cells was 200 times greater with the application of magnetic field compared to samples with no magnetic field application.

![Figure 2-9 - Synthesis of hollow silicone magnetic nanocarriers (SiMNCs) [36]](image)

Apart from utilizing external magnetic fields, researchers also investigated the use of ultrasound to enhance nanoparticle penetration. Grainger et al. utilized ultrasound exposure to increase the penetration of polystyrene nanoparticles into MCF-7 breast cancer spheroids [37]. Ultrasound application increased the penetration and accumulation of nanoparticles 6-20 fold higher compared to samples that are not exposed to ultrasound by generating a fluid flow that increased the convective transport of nanoparticles into the tumor. Similar to Grainger et al., Wei et al. utilized ultrasound exposure to improve the therapeutic effects of anticancer drugs [38]. However, instead of generating a fluid flow, Wei et al. utilized the thermogenic quality of ultrasound. The study showed that the application of ultrasound increased the tissue temperature which increased the blood flow, vascular permeability hence the drug content in tumor tissues.
Solid tumor microenvironment creates many barriers to drug delivery as discussed earlier. One of the barriers researchers have been focusing on is the high interstitial pressure within tumors. To alleviate this issue different strategies have been employed such as targeting vascular endothelial growth factor (VEGF) to decrease vessel permeability, induction of apoptosis by pretreatment with drugs to reduce tumor cell density, using prostaglandin E1 to decrease stromal cell contraction, agonizing bradykinin to increase pore size of tumor vasculature and the total vascular surface area and lastly, targeting platelet-derived growth factor beta (PDGFβ) to decrease stromal cell contraction and interactions between stromal cells and ECM [9]. These strategies aim to lower interstitial pressure which might help by increasing therapeutic agent diffusion out of the vasculature; however these methods don’t work every type of cancer and some of the mechanisms such as targeting VEGF actually lower the vessel permeability which might hinder nanoparticle and drug diffusion. Apart from aforementioned strategies, an “artificial lymphatic system” (ALS) model which can be used in conjunction with other therapeutic methods was proposed by DiResta et al [39]. This model includes a mechanical drainage system consisting of a vacuum source and drain. Results of the in vivo studies showed that the ALS device lowered the interstitial fluid pressure significantly; however this approach is very invasive, it can only be used for accessible tumors and without a portable vacuum source, immobilization is required during the use of ALS [39].

Another strategy for increasing nanoparticle diffusion and accumulation is to improve blood flow within tumors by inhibiting neoangiogenesis to prevent immature vasculature and modulating the vessel muscular tone to “normalize” the blood vessels. Tong et al. indicated that by pruning immature vessels and improving the integrity of blood vessels drug delivery could be enhanced due to improved blood flow within the tumor [40]. However it was also mentioned that this approach can hinder the leakage of macromolecules out of the vessels and cause increased levels of hypoxia due to the decreased number and leakiness of vessels [10]. In contrast to this strategy
Curnis et al. aimed to increase tumor blood permeability and drug penetration by damaging tumor endothelium [41]. However, damaging tumor endothelium can lead to increased interstitial pressure which hinders drug delivery.

Modification of tumor ECM is another strategy used to alter the tumor microenvironment to increase drug diffusion and retention. Neeves et al. investigated three different methods for the manipulation of brain ECM [42]. First method is co-administration of nanoparticles with hyperosmolar solution of mannitol, the second method is pre-infusion of an isotonic buffer solution followed by administration of nanoparticles to dilate the ECM and the third method is the administration of hyaluronidase to degrade the ECM and increase nanoparticle penetration overall. The researchers used microfluidic devices to administer the samples intercranially, since the nanoparticles or the administered therapeutic agents wouldn’t be able to pass the blood-brain barrier. These methods resulted in significant increase in nanoparticle distribution in the brain: first method resulted in 51%, second method 123% and the third method 64% increase in nanoparticle distribution.

Eikenes et al. showed that treatment of tumors with ECMDE collagenase enhances the interstitial diffusion accumulation of macromolecules [43]. What’s more, in 1992, Beckenlehner et al. pretreated a breast cancer xenograft model with hyaluronidase prior to the administration of doxorubicin and found out that susceptibility to doxorubicin was highly increased [44]. Besides, intratumor drug concentration was also increased due to the pretreatment of hyaluronidase. In 1996, Muckenschabel et al. conducted a melanoma study and concluded that administration of hyaluronidase shortly before chemotherapy increased the melphalan concentration drastically (16- to 32-fold) in nude mice [45]. Several preclinical studies have also been conducted and showed that hyaluronidase pretreatment can enhance efficiency and efficacy of anticancer drugs as shown in Table 2-2.
Table 2-2. Clinical studies investigating the coadministration of hyaluronidase with chemotherapy [46]

<table>
<thead>
<tr>
<th>Study</th>
<th>Trial type</th>
<th>Tumor type</th>
<th>Chemotherapy</th>
<th>Number of patients</th>
<th>Endpoint</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klocker et al.</td>
<td>Phase II</td>
<td>Adv. SCC-HN</td>
<td>Cisplatin/vindesine</td>
<td>48</td>
<td>Response</td>
<td>CR in 84%, 47% survival &gt;5 y</td>
</tr>
<tr>
<td>Baumgartner et al.</td>
<td>Phase III</td>
<td>Bladder cancer</td>
<td>Mitomycin C</td>
<td>56</td>
<td>Recurrence</td>
<td>27% vs. 59% recurrence in HYAL-treated vs. untreated</td>
</tr>
<tr>
<td>Pillwein et al.</td>
<td>Phase II</td>
<td>Malignant brain</td>
<td>Carboplatin/etoposide</td>
<td>40</td>
<td>Survival</td>
<td>3-y survival, 84% vs. 50% in HYAL-treated vs. untreated</td>
</tr>
<tr>
<td>Smith et al.</td>
<td>Phase I</td>
<td>Kaposi’s sarcoma</td>
<td>Vinblastine</td>
<td>6</td>
<td>Toxicity/recession</td>
<td>0% vs. 50% recurrence in HYAL-treated lesions, no added toxicity</td>
</tr>
<tr>
<td>Baumgartner et al.</td>
<td>Phase I</td>
<td>Gastrointestinal and others</td>
<td>Adriamycin and others</td>
<td>12</td>
<td>Toxicity/recession</td>
<td>PR/MR in 5 of 12 resistant, no added toxicity</td>
</tr>
</tbody>
</table>

Abbreviations: Adv. SCC-HN, advanced squamous cell carcinoma of the head and neck; CR, complete response; HYAL, hyaluronidase; MR, minimal response; PR, partial response.

In 1998, Smith et al. demonstrated the assistance of hyaluronidase in anticancer therapies through a series of pilot clinical trials for treating cutaneous lesions of Kaposi’s sarcoma [47]. Pillwein et al. found that event-free and overall survival rates were improved when 40 pediatric brain cancer patients were pretreated with bovine hyaluronidase [48]. A similar reduction of recurrence was observed in bladder cancer patients from 32% to 7% in a study of mitomycin C conducted by Baumgartner et al [49]. Klocker et al. added hyaluronidase to chemotherapy plus radiation therapy for squamous cell carcinoma of the head and neck and reported an overall survival of 47% of 80 patients at 5 years [50].

Apart from using hyaluronidase as a pre-treatment, researchers also have been investigating the targeting of HA by inhibiting the synthesis of it. For example corticosteroids are shown to inhibit the synthesis of HA by interacting with HAS [46]. However, according to Whatcott et al., clinical research of adding corticosteroids to anticancer therapies during the treatment of patients has been limited [46]. Apart from corticosteroids another HAS inhibitor, 4-methylumbelliferone, has been developed and it is currently being investigated in clinical trials [46].
While HAS inhibitors aim to decrease the HA production, HA degrading enzymes can disintegrate the existing HA matrixes. Research shows that the use of HA degrading enzymes can increase the efficacy of administered drugs.

### 2.4.2. Hyaluronidase

Hyaluronidases (HAase) are a family of enzymes that degrade HA. HAase is an endoglycosidase which means it is an enzyme that cleaves the polysaccharide chains to separate the oligosaccharides from glycoproteins or glycolipids. Unlike exoglycosidases which remove monomers at the terminal residue one by one, endoglycosidases can be used to release long chains of polysaccharides from their conjugated molecules at once. To be specific, HAase “hydrolyzes HA by splitting the β 1,4-glucosaminidic bond between C1 of the glucosamine moiety and C4 of the glucuronic acid [51]” as shown in Figure 2-10.

![Figure 2-10 - HAase hydrolizing HA [51]](image)

In humans, 6 genes responsible for HAase synthesis have been identified: HYAL1, HYAL2, HYAL3, HYAL4, HYALP1 and PH20 [4]. These different genes are responsible for different HAases found in the various parts of the body, for example, HYAL1 gene encodes the HAase
found in the human serum, whereas the PH20 gene encodes the HAase found in the testicles. HYAL1 and HYAL2 catalyze the hydrolysis of the highest amount of HA in mammals [46].

HAase can be found in the tumor tissues and the stroma; however the native HAase found in these tissues are not enough to degrade the thick HA matrix and let administered drugs into the tissue. Therefore researchers have been investigating the additional administration of HAase to increase the efficiency of current drugs and facilitate the entry of these molecules into the tumor tissue. Provenzano et al. [52] targeted the pericellular matrix of pancreatic cancer with HAase, which is a cancer type that can express high levels of HA, to decrease the interstitial pressure, to increase the permeation of blood vessels and diffusion of drug molecules. The researchers administered a pegylated version of the enzyme called PEGPH20 intravenously along with Gemcitabine, a chemotherapeutic agent for pancreatic cancer, and managed to decrease the interstitial pressure in the cancerous region which lead to higher efficacy of the administered drug as shown in Figure 2-11.

Figure 2-11 - Administration of HAase increased the efficacy of the therapeutic agent. (A) High resolution images of the tumor site (t). Scale bar, 1 mm. (B) Quantitative analysis of tumor volume, G: drug alone, GP: drug with HAase [52].
Similar to this study, Beckenlehner et al. [53] combined the use of HAase and anticancer drug Adriamycin, also known as Doxurubicin, and found that the combination therapy can increase the susceptibility of breast cancer cells to the anticancer drugs.

Overall, HAase is shown to decrease the drug resistance in spheroid models of cancer by increasing the drug penetration as well as facilitating cell death [46]. Use of HAase along with other drug delivery techniques can increase the efficacy of the existing drugs and drug delivery systems.

2.5. Biomimetic Drug Delivery Platforms

2.5.1. Liposomes

Investigation of the use of nano-platforms for medical applications dates back to 1965, which is when Bangham et al. described the first example of lipid vesicles, also known as liposomes [54]. Liposomes are small vesicles, usually 0.05-5.0 μm in diameter, which are made artificially from certain lipids [55]. As shown in Figure 2-12, they have bilayer lipid membranes thus they should not be confused with micelles which has a hydrophobic core of lipid tails.

![Figure 2-12 - Structural difference between micelles and liposomes [56]](image-url)
Liposomes have been studied to encapsulate drugs within the aqueous volume inside, within the phospholipid bilayer or within the bilayer interface [31]. Liposomes have been of interest due to their biocompatible and biodegradable properties; however they also have many limitations. Some of the major problems with liposomes are the low drug entrapment, stability and particle size control issues. Also they cannot be produced in large batches and the consistency of the product between each batch differs.

After liposomes, the first long-circulating biodegradable polymeric nanoparticles were introduced in 1994 by Gref et al [57]. Gref and colleagues utilized two biocompatible polymers, poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL), and their co-polymers. One of the advantages of using polymers is that through controlling the chemical composition and the molecular weight of the polymer, the degradation time of the resulting particles can be altered for different applications. However, these particles can be cleared from the body very quickly by macrophages due to opsonization, the process of a foreign body to be marked for ingestion by phagocytes. To avoid opsonization Gref and colleagues covalently attached polyethylene glycol (PEG), which provides a steric barrier to avoid opsonization when it is attached to a liposome [58]. As a result, the synthesized nanocarriers were shown to have increased blood circulation times. Unlike liposomes, Gref and colleagues showed that polymeric nanocarriers can be freeze dried and easily redispersed without additives in aqueous solutions which allows the production of these nanocarriers in big batches.

### 2.5.2. PLGA Nanoparticles: Fabrication and Properties

PLGA is a biodegradable polymer due to its chemical structure. As shown in Figure 2-13, when PLGA is hydrolyzed it releases two metabolite monomers: Glycolic acid and Lactic acid, meaning these monomers can be processed within the body.
PLGA is synthesized by ring-opening co-polymerization of glycolic and lactic acid. Changing the ratio of these two monomers determines the resulting form of PLGA obtained from the synthesis. The degradation time of PLGA is dependent on this ratio, since it degrades faster with increasing content of glycolic acid. PLGA degrades in the presence of water but it is not water soluble. It can be dissolved by organic solvents such as acetone and ethyl acetate.

Among all the possible polymers, PLGA is one of the most commonly used polymers for nanoparticle applications. This is due to its many attractive properties summarized by Danhier et al. such as “(i) biodegradability and biocompatibility, (ii) FDA and European Medicine Agency approval in drug delivery systems for parenteral administration, (iii) well described formulations and methods of production adapted to various types of drugs e.g. hydrophilic or hydrophobic small molecules or macromolecules, (iv) protection of drug from degradation, (v) possibility of sustained release, (vi) possibility to modify surface properties to provide stealthness and/or better interaction with biological materials and (vii) possibility to target nanoparticles to specific organs or cells [60]”.

Fabrication of PLGA nanoparticles can be done in various techniques depending on the desired properties and the final application. Emulsification-solvent evaporation technique is the most
common technique for PLGA preparation [60] which allows the encapsulation of desired compounds. This technique consists of dissolving the PLGA and the compound of interest such as hydrophobic drugs in an organic solvent, and then adding water and a surfactant like Span-80. After all the components are added together, the solution is sonicated or homogenized to induce nanosized droplets. These nanoparticles can be extracted by evaporating the solvent or centrifuging the solution. Adding an initial step of dissolving components in water before the addition of oil (surfactant) and water allows hydrophilic drugs to be encapsulated as well.

Another method is the nanoprecipitation method, also known as the solvent displacement method. In this method, the polymer and the compound of interest is dissolved in an organic solvent and then added dropwise into the water. The organic solvent is then evaporated by continuous stirring. When the organic solvent evaporates, the PLGA particles can either be used in this aqueous solution or they can be obtained by centrifuging. Through utilizing the aforementioned methods, compounds can be encapsulated within the PLGA nanoparticles as shown in Figure 2-14, which depicts a particle formed with emulsion method. However, other techniques like spray-drying won’t allow encapsulation of compounds. In that case, compounds can be adsorbed onto the nanoparticles after their fabrication [60].

![Figure 2-14 - The structure of PLGA nanoparticles encapsulating compounds, obtained with emulsion method [61]](image-url)
PLGA alone is known to be cleared from the bloodstream very quickly due to opsonization since PLGA is a hydrophobic material. To hide the hydrophobicity of PLGA, it can be coated with a material that can create a hydrophilic layer such as PEG as mentioned earlier. Apart from coating the particles in PEG, other materials can be used to modify the surface of the PLGA particles. For example, attaching ligands to the surface of the nanoparticles can make the particles target specific cells. Also, surface charges of the nanoparticles can be altered through surface coatings as well. Surface charges are known to affect the interaction of the nanoparticles with cells, since negatively charged cells are attracted to positively charged nanoparticles. This makes it easier for the particles to be internalized by the cells. When internalized, positively charged particles accumulate around the nucleus, avoiding the lysosome. PLGA nano-particles have negative surface charges without any modification [61].

Apart from the aforementioned surface modification techniques, a new approach has been investigated in the recent years. This approach is coating PLGA nanoparticles with erythrocyte, simply known as RBC, membranes. This novel technique aims to combine the advantages of two separate platforms such as the long circulation lifetime of the RBCs and the controlled drug retention and release of PLGA nanoparticles [62].

2.5.3. Erythrocytes As Nanocarriers: Their Advantages and Limitations

Many different platforms have been considered for drug delivery purposes. What separates RBCs from these platforms, are their unique features. First of all, RBCs are completely biodegradable as well as biocompatible. They can be broken down in the body without generating any toxic residues. Secondly, there is virtually an endless source for red blood cells and they can be obtained in massive quantities, one drop of human blood (approximately 1 μL) contains about 5 million RBCs [63]. Thirdly, they can be easily modified to encapsulate drugs or be coupled to other molecules of interest. Encapsulation of drugs into RBCs protects drugs from degradation while the RBCs remain immunologically and biochemically intact [42]. Apart from
these, RBCs can circulate in the vascular system up to three months which is more than other carrier platforms [63]. What is more, since RBCs have their own enzymatic activities, they can convert some prodrugs such as Dexamethasone 21-P (Dex 21-P) shown in Figure 2-15 into diffusible, active drugs [32].

![Figure 2-15 - Encapsulation of Dex (21-P) into RBC and release of Dexamethasone [32]](image)

The aforementioned properties of RBCs make it possible to use them in various applications for vascular drug delivery. They can be used for specifically targeting macrophages to deliver drugs, since macrophages are responsible for eliminating RBC from the vascular system by phagocytosis. They can be conjugated to enzymes and function as circulating bioreactors within the vascular system [32]. For example conjugating thrombolytic or fibrinolytic enzymes to RBC makes it possible to dissolve unwanted blood clots within the vascular system. Covalent and non-covalent crosslinkers as well as recombinant fusion proteins allow drugs to be coupled to the surface of RBCs.

Conjugation is not the only way to deliver a desired enzyme or a drug with RBCs. RBCs can be loaded with compounds by fusing artificial lipid vesicles loaded with drug molecules. This allows the drug molecules to enter the RBCs. Another method of compound loading involves lysing the RBCs to harvest the membrane and encapsulate compounds within the membrane obtaining RBC
ghosts. RBC ghosts are devoid of the inner structures of natural RBCs but they maintain the membrane structure.

While loading molecules of interest into RBCs is one way of administering drugs, RBCs can also be used to enhance the efficacy of the already existing drugs. RBCs can increase the efficacy of drugs by enhancing the drug’s circulation half-life by maintaining sustained release over a longer period of time. This would avoid administration of higher doses of drugs than needed, since they won’t be cleared from the body too rapidly.

2.5.4. RBC Coated PLGA Nanoparticles

As discussed in the previous sections, both synthetic biomaterials and RBC derived nanoparticles have different advantages and disadvantages. Synthetic biomaterials allow fabrication of particles with various sizes and shapes to control the pharmacokinetics for different applications; they allow controlled release of drugs and specific targeting of areas of interest while reducing the negative side-effects. However, even though the synthetic biomaterials used for drug delivery have been studied and developed to have advanced functionality and variety, they fall short of reaching the level of complexity exhibited by biological molecules produced within the body. Synthetic biomaterials often face the challenge of being cleared from the blood stream before achieving successful drug delivery. Modification of biomaterial surfaces with PEG is shown to improve circulation half-life but as shown by Parveen et al. after 72 hrs nanoparticles are cleared from the vascular system [62 - 64]. Therefore, this improvement doesn’t compare to innate biological entities such as RBCs which can circulate in the system up to three months.

On the other hand RBCs are very delicate and modifications such as drug loading or conjugation can affect RBCs adversely by damaging the cytoskeleton reducing their plasticity and resistance to osmotic and mechanical damage [63]. As a result, opsonization occurs and phagocytes eliminate these ‘damaged’ RBCs from the circulation which lowers drug bioavailability and circulation half life.
Coating PLGA nanoparticles (PLGA-NPs) with RBCs, as shown in Figure 2-16, combines the advantages of the two distinct drug delivery platforms while eliminating their disadvantages. Instead of trying to functionalize the synthetic polymer nanoparticle surfaces to act like innate biological entities, coating them with RBC membranes allows the PLGA-NPs to be camouflaged to achieve a long circulation time without extensive surface modification steps. Apart from this, the bilayer lipid membrane of RBCs provides a medium for embedding transmembrane proteins without chemically attaching them to the surface of PLGA-NPs which could alter the functionalities of the proteins [66]. While the RBC membrane helps camouflage the PLGA-NPs, PLGA-NPs provide support to the RBC vesicles covering it. Without the hard polymeric shell inside, RBCs are prone to osmotic and mechanical damage upon modification as mentioned previously. PLGA core can also allow sustained release through controlled degradation. As mentioned in earlier chapters, by changing the monomer ratios, the degradation rate of PLGA can be altered.
3. Problem Statement and Objective

As described above, currently available treatment options for prostate cancer such as chemotherapy and radiotherapy are highly toxic and surgical approaches are very invasive. Furthermore, these treatments are not efficient against hypoxic tumors. Nanocarriers have been investigated heavily for therapeutic agent delivery in order to alter the pharmacokinetics and toxicity of drugs. Use of biotic materials such as, liposomes and erythrocytes as drug carriers have been proposed previously but these drug delivery platforms have issues regarding low drug entrapment and stability of particles. Apart from biotic platforms, use of polymeric carriers such as PLGA, have also been proposed but since they are prone to opsonization, surface modification or coating is necessary. Therefore the objective of this thesis is to fabricate biomimetic composite nanoparticles with extra cellular matrix degrading enzymes on surfaces to achieve high tumor-targeting capability and superior diffusion within tumors compared to particles without ECMDEs. The efficient diffusion of nanocarriers will enhance the bioavailability of encapsulated drugs to hypoxic tumor cells and be promising to improve the overall survival rate of patients.

To develop these biomimetic nanocarriers, three main criteria are required: (1) a composite nanocarrier which can be loaded with drugs, (2) can be marked as self within the body, (3) and can overcome the dense extra cellular matrix surrounding hypoxic tumors. In this thesis these criteria were addressed by developing RBC membrane-derived nanocarriers with a PLGA core and surface modified HAase. First of all, PLGA was chosen as the polymeric carrier because of its desirable qualities such as biocompatibility and biodegradability, FDA approval for drug delivery systems, and ability to encapsulate and protect hydrophobic or hydrophilic drugs. Secondly, red blood cell membranes are used to coat the PLGA nanoparticles to allow the particles to be recognized as self within the body. Finally, HAase is attached onto the surface of the nanoparticles to increase the diffusion and penetration of particles within hypoxic tumors.
4. Materials and Methods

4.1. Materials

Poly(D,L-lactide-co-glycolide) with PLA:PGA ratio of 50:50 with a molecular weight (Mw) of 47.5 kDa was purchased from DURECT Corporation. The inherent viscosity of the purchased PLGA is 0.66 dL/g. PC-3 cell lining derived from human metastatic site (bone) was purchased from ATCC. FITC-BSA was purchased from Sigma-Aldrich. HRP-Streptavidin, Traut’s Reagent and Short PEG linker (SM-(PEG)2) was purchased from Thermo Scientific, whereas the long PEG linker (Maleimide-PEG-NHS) was purchased from NANOCS. Additional salts, solvents and buffers were purchased from various vendors. Table 4-1 shows the chemicals used in this thesis and corresponding chemical structures as well as vendors the materials are obtained from.

Table 4-1. Materials list

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<tr>
<th>Material Name</th>
<th>Chemical Structure</th>
<th>Vendor</th>
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</thead>
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<td>NaCl</td>
<td>Fisher Scientific</td>
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<td>MgCl</td>
<td>Fisher Scientific</td>
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<td>Acros Organics</td>
</tr>
</tbody>
</table>
4.2. Methods

4.2.1. PLGA Nanoparticle Preparation

As a part of this thesis two different methods for PLGA NP fabrication were employed. First method is the solvent displacement method [67]. PLGA stock solution was prepared by dissolving PLGA in acetone at concentrations ranging from 1-2 mg/mL depending on the desired final particle size [68]. This stock solution was added into deionized (DI) water in a dropwise manner on a stir plate at 1:2.4 volume ratios. The mixture was let stir at room temperature for 2 hours. After 2 hours, the remaining acetone was removed by dialysis against DI water overnight. The PLGA NP was filtered through 450 nm and 200 nm syringe filters to obtain a narrow size distribution. The sizes of the NPs were confirmed using Dynamic Light Scattering (DLS). NPs with the average sizes ranging from 65-80 nm were obtained depending on the initial PLGA concentration used. NP size could be increased by increased PLGA concentration in stock solution.

The second method that was employed is a modified version of the nanoprecipitation method. PLGA stock solution was prepared in a similar fashion, 8 mg/mL for 70 nm NPs and 15 mg/mL for 115 nm NPs. The stock solution was added into DI water in a single swift motion at a volume ratio of 1:4. The solution was placed in a vacuum chamber overnight. The PLGA NP was filtered through 450 nm and 200 nm syringe filters to obtain a narrow size distribution. The particle size was confirmed using DLS.

For in vitro imaging purposes PLGA with, lipophilic carbocyanine dye, DiD was also prepared by additionally adding 0.05% (wt%) of DiD in acetone together with PLGA. (1mg/ml DiD stock solution in acetone and 0.5uL of this stock solution/mg PLGA) After DiD and PLGA was added to acetone the second method was followed.
4.2.2. RBC Membrane Modification

Blood is obtained from mice using submandibular bleeding method also called the facial vein method. RBCs were washed three times in PBS (10 ml PBS each time, spinning down at 600g for 5 minutes) before any modification. As a part of this thesis RBCs were modified with two different proteins; initially with bovine serum albumin with fluorescein isothiocyanate (FITC-BSA) to optimize the conjugation method for RBC modification and secondly with HAase as the ECMDE.

4.2.2.1. Conjugation of FITC-BSA to RBC Membranes

Short PEG linker molecules (MW = 425.39) were first dissolved in DMSO at a dilution ratio over 2000:1 and added into PBS at final concentration of 100 μM which is 0.0425 mg/mL of linker. RBCs were added at a concentration of 0.01 mL worth of whole blood/mL of PBS. This reaction took place at room temperature while shaking the cells. After 20 minutes, the cells were washed two times with PBS (10 mL of PBS each time, spinning down at 600 g for 5 minutes) and then resuspended in PBS at a concentration of 0.04 mL worth of whole blood/mL before adding the thiolated FITC-BSA. FITC-BSA thiolation was carried out by mixing 2-iminothiolane (Traut’s reagent) with FITC-BSA at a molar ratio of 5:1 and let shake at room temperature in the dark for an hour. After the FITC-BSA and Traut’s Reagent mixture was filtered through a desalting column, it was added to the RBCs modified with the PEG linker at various concentrations to investigate the optimum concentration before incubating at room temperature in the dark for 20 minutes. The controls for this study were: linker modified RBCs incubated with not thiolated FITC-BSA, unmodified RBCs incubated with thiolated FITC-BSA and unmodified RBCs incubated with not thiolated FITC-BSA.
4.2.2.2. Conjugation of HAase to RBC Membranes

After the conjugation method is optimized with FITC-BSA studies, HAase is used to modify RBCs. PEG linker with MW = 3400 (long), was used to conjugate HAase to RBCs. RBCs were washed three times in PBS (10 mL of PBS each time, spinning down at 600 g for 5 minutes). Linker molecules were first dissolved in DMSO and added into PBS at a final concentration of 100 μM (0.17mg/mL) and RBCs were added at a concentration of 0.01 mL worth of whole blood/mL of PBS. This reaction took place at room temperature while shaking the cells. After 1 hour, the cells were washed two times with PBS and then resuspended in PBS at a concentration of 0.04 mL worth of whole blood/mL before adding the thiolated HAase at a concentration of 25 μg/mL. This reaction took another hour at room temperature followed with three times of washing with PBS before isolation of membranes.

Thiolation of the enzyme HAase was through Traut’s reaction by adding 8-fold of Traut’s reagent into 1mg/mL HAase solution in PBS and let react for 1 hour at room temperature. This was done by preparing 1 mg/mL stock solution of Traut’s reagent in PBS and addition of 12 μL of this stock solution into 1 mL of 1mg/mL HAase solution. After the reaction, the thiolated HAase was purified by filtering through a desalting column.

4.2.3. RBC Membrane Coated PLGA-NP Fabrication

To obtain RBC membrane coated PLGA-NP, RBC (modified or unmodified) membranes are isolated. To isolate the membranes, 950 μL of 0.2 mM EDTA in DI water was added to centrifuged cells to burst the RBCs. 50 μL of 20 times concentrated (20X) PBS was added after the burst to maintain the salt concentration. The membrane was centrifuged at 18000 g for 7 minutes. After three times of washing with EDTA and 20X PBS, the membrane was resuspended in DI water at a concentration of 50 μL worth of whole blood/mL. This membrane solution was
sonicated for 10 minutes and then was extruded through 400 nm extrusion membrane. After extrusion, RBC membrane was mixed with PLGA or PLGA-DiD NP solution at a volume ratio of 3:1 resulting in final concentrations for membranes 37.5 μL worth of whole blood/mL and PLGA of 0.25 mg/mL. The mixture was then filtered through 200 nm syringe filter if the PLGA was prepared with the modified solvent displacement method, and through 100 nm extrusion membrane if the PLGA was prepared with the solvent displacement method, for 11 times back and forth before collecting the sample. NP without the dye DiD was prepared for the activity assay and NP with DiD incorporated inside PLGA was prepared for the study of NP uptake by PC3 cells.

4.2.4. Prostate Cancer Cell Lining (PC3) Studies

4.2.4.1. Cell Culture

PC3 cells are cultured in 75 cm² flasks. When the cells became confluent or needed to be transferred to new flasks, the culture medium was first removed in the sterile hood. The flask was washed with 10 mL of PBS to rinse the cell layer. After PBS was removed 3.5 mL Trypsin solution (0.25% (w/v) Trypsin with 0.53 mM EDTA) was added followed by incubation for 7 minutes at 37°C. The flask was removed from the incubator and cell detachment was confirmed using light microscope before adding 13 mL of F-12K cell medium in the sterile hood. This solution was transferred into two 15 mL conical tubes, equally separated, and spun down at 1000 rpm for 5 minutes. The supernatant was removed in the sterile hood, and the cells were resuspended in 6 mL of media. This solution was separated into two by adding 3 mL into one flask. 12 mL of F-12K medium was added onto the cell solution in each flask. The flasks were placed in the incubator at 37°C.
4.2.4.2. Nanoparticle Uptake Studies

Nanoparticle uptake studies were done to study the effectiveness of HAase-RBC-PLGA-NPs as delivery vehicles within PC3 cancer lining. Controls for this study were unmodified RBC-PLGA-NP and unmodified RBC-PLGA-NP with free enzyme fluorescently labeled with DiD. The amount of PLGA in both samples was equalized via fluorescent intensity of DiD.

PC3 cells were cultured on glass slides in 12-well plate for 2 days before the treatment and reached 90% confluence. NP solution with concentration of 0.25 mg worth of PLGA/mL was diluted in cell culture media by 5 times before adding 1 mL/well and incubated at 37 °C for 2 hours. The cells were washed 3 times with PBS and then fixed by using 4% formaldehyde in PBS and incubated for 20 minutes at room temperature. After incubation the samples were washed and mounting media with DAPI (Vector Laboratories Inc.) was added before they were observed under confocal microscope using 405 nm and 633 nm lasers for the DAPI and DiD respectively.

4.3. Characterization and Imaging Techniques

4.3.1. Dynamic Light Scattering and Zeta Potential

Dynamic light scattering (DLS) is a physical characterization technique that can be used for measuring the size and distribution of particles and molecules in suspension within the range of below a nanometer to several microns. DLS (Zetasizer Nano ZS90) was used to characterize the fabricated PLGA and RBC nanoparticles before and after co-extrusion to monitor the size of the resulting particles.

The DLS samples consisted of particles dispersed within solution, PLGA particles in deionized water and RBC particles in PBS. Disposable macro-cuvettes were used as sample holders. The DLS instrument was automatically calibrated before each measurement according to the cuvettes, solvents and room temperature.
“Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion or attraction between particles, and is one of the fundamental parameters known to affect stability [69].”

Determining the zeta potential of particles is important for determining the stability of the particles within the solution. A zeta potential value between -25 mV to +25 mV typically means the particles are not stable and they can aggregate or precipitate out of the solution due to Van der Waals interactions. Values lower than -25 mV or higher than +25 mV means particles are relatively stable within the solution.

Zetasizer (Zetasizer Nano ZS90) was used to obtain zeta potential information of the same samples prepared for DLS measurements. Disposable capillary cells were used as sample holders. The instrument automatically calibrated according to the aforementioned variables before each measurement.

4.3.2. Transmission Electron Microscopy

Transmission electron microscopy (TEM, JEOL 2100JEM) was used to characterize the PLGA nanoparticles, with and without RBC coating, after fabrication. The TEM samples were prepared by first adding one drop of prepared PLGA nanoparticle sample solution onto the glow-discharged carbon coated copper grid. The grid is let sit for half an hour before the remaining excess solution is absorbed with a filter paper. The staining solution, uranyl acetate, is added onto the copper grid with the sample. An acceleration voltage of 200 keV was used for imaging in bright field mode.

4.3.3. Optical Microscopy

Various optical microscopes such as light microscope (Leica DMI 4000B) fluorescence microscope (Leica DMI 4000B) and confocal microscope (Olympus IX81) have been utilized to characterize the samples at various stages.
Light microscope, which uses bright-field illumination for reflection and absorption, was mainly used for confirming the viability and the confluence of PC3 cells. PC3 samples were imaged within the flask or plates they were cultured. Phase contrast mode of the light microscope was used to image RBC samples. RBC samples were prepared by adding a drop of blood (cell concentration at 8*10^5 cells/mL) in a 12 well plate.

In addition to the capabilities of a bright field microscope, a fluorescence microscope also uses fluorescence and phosphorescence of samples for imaging. A fluorescence microscope was used to confirm chemical conjugation technique used to modify RBCs as well as confirming the viability of the cells after modification. Samples were prepared by adding a drop of modified RBCs in PBS (cell concentration at 8*10^5 cells/mL) in a 12 well plate.

**4.3.3.1. Confocal Microscopy**

Confocal microscope (Olympus IX81 with Fluoview FV1000) was used to study the chemical conjugation of proteins to RBCs and study the PC3 cells after treatment with NPs. Samples were prepared by first fixing by using 4% formaldehyde in PBS and incubating for 20 minutes at room temperature before washing. After the wash, mounting media with DAPI was added onto glass slides with samples. Kalman filter was used to capture images.

**4.3.4. HAase Enzymatic Assay**

Enzyme assays were done in order to measure the enzymatic activity of the HAase in various conditions. The activity of HAase on cells and NPs was detected with an ELISA-like microtiter-based assay.

**4.3.4.1. Preparation of Biotinylated HA (bHA)**

HA was dissolved in 0.1 M MES hydrate (4-Morpholineethanesulfonic acid) with a pH of 5.0 at a final concentration of 1 mg/mL by stirring overnight at 4°C. Sulfo-NHS was added to the
hyaluronate MES solution to a final concentration of 0.184 mg/ml. Biotin hydrazide was dissolved in DMSO as a stock solution of 100 mM and then added to the HA solution to a final concentration of 1 mM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was prepared as a 100 mM stock solution in DI water and then added to the HA-biotin solution at a final concentration of 30 mM. This solution was left stirring overnight at 4°C. After the addition of 4 M guanidine-HCl, unlinked biotin and EDC were removed by dialysis against DI water overnight. Prepared batches of bHA are stored at -20°C.

4.3.4.2. Immobilization of bHA onto Plates

Sulfo-NHS was diluted to 0.23 mg/ml in DI water and then mixed with the bHA solution with a volume ratio of 4:1. The resulting solution was pipetted into 96-well Covalink-NH plates at 50 μl per well. EDC was dissolved in DI water at a concentration of 0.123 mg/ml and pipetted into the Covalink plates at 50 μl per well. The plates were incubated for 2 hours at room temperature. After covalent immobilization of bHA on the microtiter plates, the coupling solution was removed washing three times with PBS containing 2 M NaCl and 50 mM MgSO4.

4.3.4.3. Assay for HAase Activity

The Covalink plates with immobilized bHA were equilibrated with 100 μL/well PBS. A set of standards was generated by diluting 1 mg/mL HAase solution with original activity of 116000 U/mg to a series of solutions with HAase activity increasing from 1 U/mL to 1000 U/mL. The samples are diluted five times in PBS before assaying 100 μL/well in triplicate. After 45 minutes of incubation in 37°C, the reaction was terminated by adding 200 μL 6M Guanidine-HCl per well followed by three washes of 300 mL/well with PBS, 2 M NaCl, 50 mM MgSO4, 0.05% Tween 20 (buffer B). A horseradish peroxidase (HRP) conjugated streptavidin solution was prepared by diluting stock solution with the concentration of 1.25 mg/mL in PBS containing 0.1% Tween 20 at a ratio of 1:10000. The solution was added 100 μL/well and incubated for 30 minutes at room
temperature. The plate was then washed five times with buffer B. An ophenylenediamine (OPD) substrate was added at 100 mL/well by dissolving one 5 mg tablet of OPD in 5 mL of 0.1 M citrate-PO4 buffer with a pH of 5, and 3.75 μL of 30% H2O2. The plate was incubated in the dark for 5 minutes before adding 50 μL/well of 4M H2SO4 solution. The absorbance was read at 492nm using TECAN (Infinite M200). The activity for each sample was determined graphically by comparing with the standards.

5. Results and Discussion

5.1. Optimizing PLGA-NP Fabrication Method for Desired Size and Concentration

5.1.1. PLGA-NP Fabrication with Solvent Displacement Method

Optimizing the fabrication method for PLGA-NPs to obtain desired nanoparticle sizes at desired concentrations was the first major step for this thesis. The size of the fabricated NPs is an important parameter which affects the biodistribution of the nanocarriers within the body. The size of the final nanocarriers can affect various aspects such as the accumulation at tumor sites via the EPR effect, the diffusion through dense ECM or the removal from the circulation by the kidneys, as discussed in Chapter 2. Average size around 100 nm is optimal as stated in many previous publications [70,71]. Therefore, the first approach to fabricate PLGA nanoparticles, which would be used as the core of the final nanocarriers, at desired sizes was to utilize the commonly used solvent displacement method. This method allows the fabrication of PLGA-NPs of various sizes by adjusting the concentration of PLGA added into the water. The parameters for the solvent displacement method were chosen according to the examples in previous publications [66, 71, 72]. As discussed in Chapter 2, this is a low-cost method that is easy to set up.

After PLGA-NPs are fabricated they are co-extruded with RBC membranes through a 100 nm extrusion membrane to obtain the final nanocarriers. The size and the zeta-potential of the
PLGA-NPs as well as the RBC membrane vesicles are monitored via DLS and Zeta-Sizer before and after the co-extrusion as shown in Table 5-1. PLGA-NPs fabricated with the final concentration of 0.625 mg/mL of PLGA in water have an average particle size of 77 nm, whereas the RBC membrane vesicles alone have an average size of 160 nm. When they are co-extruded the RBC vesicles are forced to break apart and coat the PLGA-NPs as they go through the extrusion membrane together. As a result, after extrusion the RBC-PLGA-NPs have a higher average size than PLGA-NPs alone and smaller average size than RBC membrane vesicles. This phenomenon of RBC membranes coating PLGA-NPs can also be seen from the zeta potentials of each sample. PLGA-NPs have an average zeta potential of -51 mV whereas RBC vesicles have an average zeta potential of -35 mV. The zeta potential after co-extrusion is -40 mV, which is higher than the average zeta potential of PLGA-NPs alone, showing that the PLGA-NPs are coated with RBC membranes.

Table 5-1. Average sizes of PLGA-NPs and RBC vesicles before extrusion and the resulting RBC-PLGA-NPs after coextrusion

<table>
<thead>
<tr>
<th></th>
<th>Avg. Size</th>
<th>PDI</th>
<th>Z-Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA-NPs</td>
<td>77</td>
<td>0.18</td>
<td>-51</td>
</tr>
<tr>
<td>RBC Vesicle</td>
<td>160</td>
<td>0.18</td>
<td>-35</td>
</tr>
<tr>
<td>RBC-PLGA-NPs</td>
<td>133</td>
<td>0.17</td>
<td>-40</td>
</tr>
</tbody>
</table>
5.1.2. PLGA-NP Fabrication with Modified Solvent Displacement Method

The size of the PLGA-NPs obtained with the aforementioned solvent displacement method varied significantly when the procedure was repeated without changing parameters, which was not desired. Therefore, the initial solvent displacement method was modified to achieve a more reliable method for PLGA-NP fabrication. As it can be seen from Table 5-2 the average size of the PLGA-NPs fabricated with the modified solvent displacement method at a concentration of 2.5 mg/mL PLGA in water was 75 nm. The PLGA-NPs fabricated using the modified method were mixed and extruded with RBC membranes through 200 nm extrusion membrane to obtain RBC-PLGA-NPs as shown in Figure 5-1.

![Figure 5-1 - TEM micrographs of RBC-PLGA-NPs (2.5 mg PLGA/mL), covered with RBC membrane, fabricated with the modified solvent displacement method. Nanoparticles consist of hard PLGA core and RBC coating. Scale bars are 100 nm.](image)

As mentioned earlier extruding the RBC membrane vesicles with PLGA-NPs results in RBC coated PLGA-NPs with average sizes larger than the PLGA-NPs alone, yet smaller than RBC
membrane vesicles. As shown in Table 5-2, the final RBC-PLGA-NPs obtained using PLGA-NPs fabricated with the modified method resulted in an average nanocarrier size around 100 nm which is target size for the final nanocarriers. The average size of RBC membrane vesicles mixed with PLGA-NPs before extrusion was also measured to confirm extrusions role in refining the size of final nanoparticles.

<table>
<thead>
<tr>
<th></th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Z-Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA-NPs</td>
<td>75 ± 3.4</td>
<td>0.18 ± 0.012</td>
<td>-63 ± 3.7</td>
</tr>
<tr>
<td>RBC Vesicle</td>
<td>139 ± 22</td>
<td>0.14 ± 0.15</td>
<td>-39 ± 4.6</td>
</tr>
<tr>
<td>RBC-PLGA-NP Mix</td>
<td>107 ± 10</td>
<td>0.21 ± 0.013</td>
<td>-45 ± 8.3</td>
</tr>
<tr>
<td>RBC-PLGA-NP Extruded</td>
<td>98 ± 10</td>
<td>0.23 ± 0.064</td>
<td>-33 ± 8.9</td>
</tr>
</tbody>
</table>

Besides DLS size measurements, statistical data of the RBC-PLGA-NP size was also obtained from TEM images. The average size of the RBC membrane coated PLGA-NPs was found to be 80.4 ± 23 nm (mean ± s.d., n=30). The standard deviation in size represents the size difference among nanoparticles prepared in one experiment, while the s.d. in Table 1 is the difference of average diameter of nanoparticle fabricated in 3 independent experiments.

One of the goals of this thesis was to fabricate RBC-PLGA-NPs with HAase on surfaces. Therefore, after the PLGA-NP fabrication method was optimized, PLGA-NPs coated with RBC membranes modified with HAase were prepared. Figure 5-2, shows TEM micrographs of these nanoparticles.
5.1.3. **Comparison of the Two Methods and Conclusions**

PLGA-NPs with average sizes of about 75 nm could be fabricated using either method as shown on Table 5-1 and Table 5-2. However, even though the parameters are kept the same, the average size of the PLGA-NPs obtained from the solvent displacement method varied significantly. Using the same parameters, NPs with average size of 65 nm to 80 nm were obtained. The solvent displacement method involved adding the PLGA solution into water in a dropwise manner while the water/PLGA solution was kept on a stir plate. The stirring speed as well as adding the PLGA solution from a height caused the unwanted variations in the size of the PLGA-NPs. It was found that when the stirring speed was increased the particle size decreased. Apart from the stirring speed, adding PLGA into water in a dropwise manner caused PLGA to agglomerate and form a film on the surface, therefore decreasing the amount of dissolved PLGA in water and making it impossible to quantize the dissolved PLGA concentration in water.

*Figure 5-2 – TEM micrographs of HAase conjugated RBC-PLGA-NPs*
Table 5-1 shows the average sizes of the NPs fabricated with the solvent displacement method, however it doesn’t reflect the variance in the size of the fabricated PLGA-NPs. Figure 5-3, shows the average PLGA-NP size obtained from various trials with the solvent displacement method, whereas Figure 5-4, shows the average PLGA-NP size obtained from various trials with the modified solvent displacement method. The calculated standard deviation as shown by the error bars is smaller for the modified solvent displacement method.

Figure 5-3 - Average sizes of PLGA-NPs prepared with the solvent displacement method and RBC vesicles before extrusion and the resulting RBC-PLGA-NPs after coextrusion (mean ± s.d., n=3).
Average sizes of PLGA-NPs fabricated with the modified solvent displacement method and RBC vesicles before and after mixing and extrusion (mean ± s.d., n=4).

Apart from having better repeatability, modified solvent displacement method also allows the fabrication larger number of PLGA-NPs at once. This due to the fact that to obtain similar size PLGA-NPs (75 nm), 0.625 mg of PLGA was added into water with the solvent displacement method, whereas 2.5 mg of PLGA was added into water with the modified solvent displacement method. Figure 5-5 shows the average size of PLGA-NPs obtained via the solvent displacement method by changing the concentration of PLGA in water. To fabricate more PLGA-NPs more PLGA needs to be added into the water. However increasing the concentration of PLGA added also increases the size of the resulting PLGA. Therefore, the method that allows the fabrication of PLGA-NPs at desired size with a higher concentration of PLGA is the preferred method to obtain more particles. As shown below in the two graphs (Figure 5-5, 5-6), modified solvent displacement method produces more particles compared to regular solvent displacement method when the resulting NP sizes are similar.
Figure 5-5 - Various sizes of PLGA-NPs fabricated with the solvent displacement method obtained at different PLGA concentrations in water (mean ± s.d., n=4).

Figure 5-6 - Various sizes of PLGA-NPs obtained with the modified solvent displacement method at different PLGA concentrations in water (mean ± s.d., n=4).
5.2. Establishing Chemical Conjugation of Proteins to RBC Membranes via Thiolation

To be able to modify RBC membranes with HAase a chemical conjugation method needed to be established. Thiolation of peptides and proteins to enable attachment to polyethylene glycol (PEG) – Maleimide linker was used as the conjugation method, since the reaction of maleimides with thiols is well established, simple, site-specific and quantitative. Thiolation is sulfhydryl addition as shown in Figure 5-7. Traut’s Reagent (Thermo Scientific), which is a cyclic thioimidate, was used for this reaction.

\[
\text{Sulfhydryl (-SH) groups are introduced as Traut’s Reagent reacts with primary amines (-NH}_2\text{). The charge properties of the resulting molecules are similar to that of the original amino groups (Figure 5-7) [73].}
\]

While proteins were thiolated for conjugation to RBC membranes, RBC membranes were modified with PEG-Maleimide, which react with thiol groups on the thiolated proteins (Figure 5-8).
PEG-Maleimide linker was attached to the RBC membrane through the reaction between the N-Hydroxysuccinimide (NHS) group on the linker with the amine groups on the RBC membrane. NHS acts as an activating reagent for carboxylic acids by reacting with amine groups.

After the PEG-Maleimide linker is attached to the RBC membrane covalently, the sulfhydryl groups that were introduced to the target peptide or protein attach to the maleimide on the PEG-Maleimide linker.

To test the conjugation method, fluorescently labeled bovine serum albumin (FITC-BSA) was used along with a short PEG linker (MW = 425.39) as shown in Figure 5-9, as a cheaper alternative to HAase.

![Figure 5-8 – Chemical representation of the reaction between maleimide and thiolated proteins [74].](image)

![Figure 5-9 – Chemical structure of the short PEG linker](image)
As shown in Figure 5-10, conjugation of FITC-BSA to RBC membranes was achieved through thiolation and subsequent thiol-maleimide reaction.

![Micrograph of FITC-BSA conjugated RBCs, obtained with confocal microscopy.](image)

*Figure 5-10 – Micrograph of FITC-BSA conjugated RBCs, obtained with confocal microscopy.*

Before confocal imaging, phase contrast images were taken to confirm the RBCs were viable, shown in Figure 5-11 (a-d). The control samples were: linker modified RBCs incubated with not thiolated FITC-BSA (Figure 5-11 (B,b)), unmodified RBCs incubated with thiolated FITC-BSA (Figure 5-11 (C,c)) and unmodified RBCs incubated with not thiolated FITC-BSA (Figure 5-11 (D,d)). As expected no signal was obtained from the control samples under the confocal microscope, since conjugation was not achieved. The samples with PEG modified RBCs and thiolated FITC-BSA produced clear images as seen in Figure 5-11.
Figure 5-11 – Micrograph of FITC-BSA conjugated RBC obtained with confocal microscope (A-D) and phase contrast microscope (a-d). (A,a) RBC modified with PEG linker incubated with thiolated FITC-BSA, (B,b) RBC modified with PEG linker incubated with FITC-BSA without thiolation, (C,c) Unmodified RBC incubated with thiolated FITC-BSA, (D,d) Unmodified RBC incubated with FITC-BSA without thiolation. Scale bar 20 μm (A-D), 25 μm (a-d). These images are obtained with two different microscopes.

After the chemical conjugation method was established, HAase enzyme was conjugated onto RBCs instead of FITC-BSA. The long PEG linker (MW = 3400), shown in Figure 5-12, was used to modify RBCs to fabricate HAase-RBC-PLGA-NPs.

Figure 5-12 - Chemical structure of the long PEG linker (MW= 3400)
Previous studies indicated that increasing the chain length of PEG linkers could increase the enzymatic activity [75, 76]. Therefore Maleimide PEG linker with MW of 3400 was used to conjugate HAase to RBCs for enzymatic and in vitro studies, discussed in detail in the following chapter.

5.3. In Vitro Studies

The primary goal of this thesis was to develop biomimetic nanocarriers with HAase on surfaces and demonstrate its potential of enhanced diffusion in tumors. The hypothesis was that HAase on NP surfaces would degrade the dense HA on their diffusion path, allowing drug nanocarriers, to reach cancer cells. Therefore, the fabricated RBC-PLGA-NPs with HAase on surfaces needed to be tested in vitro to determine if the hypothesis was confirmed. In vitro studies were important to determine the next steps of this research such as in vivo animal studies.

To investigate the effective enzymatic activity of the RBC-PLGA-NPs with HAase on surfaces compared to free enzyme and RBC-PLGA-NPs without HAase on surfaces, in vitro studies were done. In vitro studies consisted of treatment of PC3 cells which express dense HA, with the fabricated NPs. After treatment the samples were fixed and imaged to visualize the overall NP uptake and to quantify the effective activity using the fluorescent intensity of the labeled NPs and Image J software.

5.3.1. Quantification of HAase Enzyme Activity on RBC-PLGA-NPs

Before the in vitro studies of NP uptake in PC3 cells could be done, the enzymatic activity of HAase-RBC-PLGA-NPs, needed to be quantified. This quantification was done by an ELISA-like microtiter-based assay. Enzyme assays for HAase used to be based on measuring the changes in the viscosity or the turbidity of samples containing HA [77 - 80]. Besides measuring loss of turbidity and viscosity, Stern et al. developed an assay measuring the generation of new reducing
$N$-acetylamino groups [78]. However, these assays were neither simple nor efficient and they either lacked specificity or sensitivity. The ELISA-like assay used in this thesis, which was developed by Frost et al., doesn’t require complex bioreagents, can be performed using shorter incubation periods than aforementioned methods, and it is very resistant to pH variations while having high sensitivity [77].

This assay involves the biotinylation of the free carboxyl groups of hyaluronic acid as shown in Figure 5-13, which is then immobilized on 96 well-microtiter plate covalently.

![Chemical representation of biotinylated HA (bHA).](image)

After the bHA is conjugated onto the wells in a 96 well microtiter plate, samples, such as RBC-PLGA-NPs with conjugated HAase and intact RBCs modified with HAase, were added into the wells in triplicates after being diluted 5 times. All the test samples contained same concentration of modified whole blood per mL of sample. Apart from test samples a set of standards was generated by diluting 1 mg/mL HAase solution with original activity of 116000 U/mg to a series
of solutions with HAase activity increasing from 1 U/ml to 1000 U/ml. Lastly, PBS was also added in triplicate as the negative control (blank). The plate was incubated at 37°C for 45 minutes to let samples react with the bHA conjugated onto the wells as shown in Figure 5-14.

Figure 5-14 – Schematic representation of the ELISA-like microtiter-based assay steps. A) bHA is added into 96 well-plate. B) bHA is covalently conjugated onto the wells. C) After sample incubation the catalyzed HA fragments are washed away. D) HRP-streptavidin is added which binds to the biotin on bHA. E) Once the HRP-substrate is added color change occurs depending on the amount of remaining uncatalyzed bHA.

After 45 minutes of incubation in 37°C, the reaction was terminated with Gunidine-HCl followed by a washing step. A horseradish peroxidase (HRP) conjugated streptavidin solution was added into the wells. The streptavidin conjugates to the biotin on the remaining uncatalyzed bHA conjugated onto the wells after the removal of the enzyme containing samples. HRP conjugated streptavidin is a commonly used reagent for the detection of biotinylated antibodies in ELISA assays; however, in this assay it is used to detect the bHA rather than an antibody, hence the name
ELISA-like assay. After the HRP-streptavidin solution is incubated in the wells for 30 minutes at room temperature, the plate was washed 5 times to make sure there are no remaining free enzymes or free floating bHA within the wells. Before the absorbance was read at 492 nm using TECAN, OPD was added to the wells which was followed by a sulfuric acid solution 5 minutes later.

A standard curve was generated using the absorbance data from the standards. The enzyme activity for each sample was determined graphically by comparing with the standard curve in units of U, and converted into number of molecules. Approximate effective enzyme conjugation on the surface and the number of molecules per unit area were calculated via the enzyme assay. According to the results of enzyme activity assay, fabricated RBC-PLGA-NPs with HAase on surfaces expressed almost twice the activity per area compared to intact RBCs modified with HAase, as shown in Figure 5-15.

![Bar charts](image)

**Figure 5-15** – Bar charts showing the enzyme activity per area of intact RBCs modified with HAase and RBC-PLGA-NPs with HAase on surfaces. Results are obtained from the enzyme activity assay.
This can be explained by the significant size difference between the cells and NPs. Cells have a very large interaction surface due to their large size, therefore the interface between the HA matrix and the HAase on surfaces of RBCs are limited to only the top layer of the substrate. However NPs are much smaller, therefore they can penetrate into the HA matrix, increasing the surface for the enzyme to react with the substrate hence increasing the effective activity.

5.3.2. PC3 Cell Treatment with HAase Conjugated RBC-PLGA-NPs

After the enzyme activity assay, prostate cancer cells (PC3) were treated with the fabricated HAase-RBC-PLGA-NPs. The HAase modified RBC membranes in the fabricated NPs were from the same batch used in the enzyme activity assay, but a separate batch of PLGA NPs, fluorescently labeled with DiD, were used to extrude with the modified membrane to fabricate the final NPs to allow the visualization of the NPs using confocal microscopy.

It is important to investigate how the fabricated NPs perform in vitro, besides measuring the enzymatic activity via the ELISA-like enzyme assay. Therefore, to test whether HAase on RBC-NPs improve their diffusion, HAase-RBC-PLGA-NPs solution diluted in cell culture media were added onto PC3 cells. Controls for this study were unmodified RBC-PLGA-NP alone, unmodified RBC-PLGA-NPs with 10U free enzyme and unmodified RBC-PLGA-NPs with 1000U free enzyme added onto PC3 cells after diluting in cell culture media. The amount of PLGA NPs in all samples were equalized via fluorescent intensity of DiD (0.25 mg/mL). After the addition of the samples to the cells, they were incubated at 37 °C for 2 hours. The cells were then fixed and observed under confocal microscope. Figure 5-16 shows example images obtained using confocal microscopy after PC3 cells were treated with different samples as well as the quantitative results.
Figure 5-16 – Micrograph of PC3 cell line treated with RBC-PLGA-NPs with HAase. Cell nuclei are dyed with DAPI and shown in blue, whereas DiD labeled PLGA NPs are shown in green. A) Unmodified RBC-PLGA-NPs without enzyme. B) Unmodified RBC-PLGA-NPs with added free enzyme (10U). C) Unmodified RBC-PLGA-NPs with added free enzyme (1000U). D) Modified HAase-RBC-PLGA-NPs (10U). Scale bars: 20μm. E) Bar chart showing relative fluorescence of the NP uptake per cell for samples. (Values are mean ±s.d., n=10. **, p<0.001, analyzed by student’s t test).
Prostate cancer cells are surrounded by a HA layer. This HA coating prevents large molecules, cells or particles from reaching the cancer cells. Thompson et al. demonstrated this phenomenon by performing a size exclusion assay using RBCs and PC3 cell lining [81]. The micrograph on the top left in Figure 5-17 shows PC3 cell lining surrounded by a dense HA coat. The RBCs which are 7 μm in size cannot penetrate the HA. The micrograph on the top right shows the similar effect on DU145 prostate cancer lining. After the administration of HAase (PH20) the peritumor matrix is degraded and RBCs can surround the PC3 cells as shown in the bottom images.

Figure 5-17 – HA coat around human prostate cancer cell (PC3 and DU 145 lines) excludes RBCs before treatment with HAase [81]
Due to the same reason unmodified RBC-PLGA-NPs could not diffuse through the PC3 cell lining as efficiently as the HAase conjugated RBC-PLGA-NPs or the RBC-PLGA-NPs with added free enzyme as seen in Figure 5-17. Even though RBC-PLGA-NPs are a lot smaller compared to RBCs with an average size of 100 nm, the dense ECM of PC3 cells still hinders the diffusion of NPs. Therefore the presence of HAase increased the diffusion of NPs which was confirmed with confocal images. To further analyze the data, NP uptake was quantized.

5.3.2.1. NP Uptake Quantification

NP uptake quantification was done by analyzing the obtained images with Image J software. First of all, 10 images of different locations within samples were obtained from each sample: modified NPs, unmodified NPs with added 10U free enzyme, unmodified NPs with added 1000 U free enzyme and unmodified NPs alone. As it is shown in Figure 5-16, the PLGA intensity (green color) in each image varies, indicating how much of the NP sample solution could penetrate the ECM and diffuse into the cells. This intensity was measured and divided by the number of cells present in the image for each image and the results were averaged among sample groups. As seen from Figure 5-16 (E), HAase conjugated RBC-PLGA-NPs (modified) had more than twice the effective activity of unmodified NPs. Modified samples had comparable effective activity to unmodified NP samples with added free 1000 U HAase.

The HAase conjugated RBC-PLGA-NP samples exhibiting comparative effective activity to the sample with RBC-PLGA-NPs and free enzyme is important. Administering nanocarriers in junction with free enzymes could be a possibility but it is not desired. The pharmacokinetics of free HAase and therapeutic agents do not match when administered separately. High doses of HAase is needed when it is administered separately which may cause unwanted side effects, such as the enzyme reacting with HA surrounding healthy tissues leading to inflammation. By attaching HAase to the NPs, the catalytic effect of the enzyme can be used to enhance the
diffusion of NPs within tumor matrices with high levels of hyaluronan. Therefore the engineered NPs can release encapsulated drugs while the HAase on the surfaces degrade the dense matrix on its diffusion path, which can lead to increased bioavailability.

5.4. Conclusions

RBC-PLGA-NPs with HAase on surfaces were successfully fabricated. The results of the enzymatic assay and the NP uptake study showed that RBC-PLGA-NPs with HAase on surfaces enhanced nanoparticles diffusion into the pericellular HA layer of PC3 cells. Apart from that, enzymatic activity of HAase is also shown to be higher conjugated onto RBC-PLGA-NPs compared to intact RBCs, which is due to the substantial size difference between RBCs and NPs. As mentioned earlier, the much larger size of the RBCs prevents them from being able to diffuse into the dense hyaluronan matrices, decreasing the surface area that the HAase on surfaces can interact with.

The high effective activity of HAase conjugated to RBC-PLGA-NPs with PEG linker can be attributed to the HAase enzyme having freedom to access HA. The fabricated HAase conjugated RBC-PLGA-NPs successfully increased the penetration and diffusion of nanocarriers within the cancer cell lining in vitro.
6. Future Work

The future work should comprise of in vitro and in vivo studies focusing on two main components: (1) investigation of the circulation half-life of RBC-PLGA NPs in mice and (2) quantifying the biodistribution of RBC-PLGA NPs in mice.

Although high density of ECMDEs may render RBC-PLGA NPs efficient degradation of extracellular matrix, these chemically conjugated ECMDEs may compromise the long circulation time of RBC-PLGA NPs. It is possible that antibody of nanocarriers may be produced in mice gradually. The circulation time after repeated administration of RBC-PLGA NPs with or without ECMDEs should be studied within a set time point.

Apart from circulation half-life, biodistribution and diffusion in tumors also play an important role for nanoparticles to be considered successful. Therefore, biodistribution of NPs should be investigated in animal models.
7. References


