Modification of gold markers with Doxorubicin as Radiosensitizer encapsulated in sustained release PLGA nanoparticles to enhance Image Guided Radiotherapy (IGRT)

Master’s Thesis

By

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1. SUMMARY

National Cancer Institute (NCI) predicts the number of cancer cases in United States alone by the year 2030 to be 2.3 million which is a 45% increase than the current 1.6 million. Therefore there is an undying need for research and development to aggressively attack this disease. Cancer is a multifaceted disease. So, there is no single approach to the treatment of this disease. Various expeditions have been undertaken in all directions to counter this deadly enemy.

Radiation therapy is one of these expeditions. X Rays were discovered by William Roentgen in 1895. The use of radiation therapy began in the same year when Emil Grubbe used X rays for breast cancer therapy. Radiation oncology has advances leaps and bounds in the last century. One of them was Image Guided Radiation therapy (IGRT). A tumor is often considered a moving target. Prostate cancer tumors change positions due to movements of the prostate gland. This often leads to radiations missing the desired target and affecting healthy tissue. To avoid this, (IGRT) was introduced where inert metallic markers were inserted into the organ to pin point the location of the tumor. In spite of success in targeting the tumor a major hurdle in radiation therapy is the ability of cancer cells to repair damaged DNA. This results in an increase in the cell survival and loss of sensitivity to radiation therapy. One way of countering this problem is use of radio sensitizing agents that block DNA repair and increase the sensitivity of tumor cells to radiations. In this study we aim to design a sustained release platform of doxorubicin that could be used as radiosensitizing tool in radiation therapy.

The long term aim of this study is to modify gold markers with Doxorubicin in order to form a sustained release platform as an adjunct therapy for radiation therapy. To achieve this, the coated gold markers will be first tested for their in vitro and in vivo drug release and cytotoxicity. Pegylated PLGA Nanoparticles loaded with doxorubicin were formulated as the drug carrier. The pegylated nanoparticles were tested for in vitro cytotoxicity. These were dispersed in chitosan (3% aqueous solution) and this
slurry was coated on a gold fiducials. Drug loading in the nano particles was optimized and they were characterized for particle size, zeta potential and morphology. The optimized and characterized nanoparticle formulation was tested for release of free doxorubicin over time. The release of free doxorubicin from the coated fiducial over time was also measured. PC3 human prostate cancer cells and HeLa cells were cultured and intracellular delivery of nanoparticles was determined both qualitatively and quantitatively by fluorescence microscopy and fluorescence spectroscopy respectively.

Initially, fiducials coated with dox-PLGA nanoparticles were implanted inside tumors using a 16G needle. The purpose of doing so was to study the spatial distribution of doxorubicin as it was released over time. We were faced with the problem of interference by tissue autofluorescence. Therefore it was impossible to unmix tissue autofluorescence from the doxorubicin signal. To, eliminate this phenomenon; we formulated PLGA nanoparticles that encapsulated a special kind of dye. This dye had its peak excitation and emission in the Near Infrared Range (NIR). The dye chosen for this study was Cy 7.5, a cyanine dye. These nanoparticles were injected intratumorally and in vivo imaging was carried out.
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2. INTRODUCTION

2.1 Statement of the Problem

The National Cancer Institute estimates that approximately 11.4 million Americans with a history of cancer were alive in January 2006. In 2010, about 549,490 Americans were expected to die of Cancer which is more than 1,500 people a day. 1 in 4 people dies due to cancer which makes cancer is the most common cause of death in Americans. Image guided radiotherapy is a very powerful tool in the treatment of prostate cancer. The physician is able to estimate the movement and deformation of the tumors thereby reducing the received by health tissue. Radiation therapy causes single stranded breaks (ssb) and double stranded breaks (dsb) in the DNA of cancerous cells. The dsb are more lethal to the cells than the ssb. But the cells have indigenous mechanisms by which they repair this damage to their DNA. Moreover, tumors are hypoxic at their centers and express various proteins that again repair damaged DNA\textsuperscript{3}. All these factors reduce the effect of radiotherapy and therefore there is a need to sensitize the tumors to preserve the efficacy of radiotherapy.

2.2 Objectives and Hypothesis

Certain chemotherapeutic agents such as doxorubicin bind to the DNA at the dsb and prevent the repair of the damaged DNA enhancing the effect of radiation therapy. This mechanism is called Radiosensitization\textsuperscript{4}. Doxorubicin has shown act as a readiosensitizer drug\textsuperscript{4}. Image-guided radiation treatments (IGRT) routinely utilize radio-opaque implantable devices, such as fiducials or brachytherapy spacers, for improved spatial accuracy\textsuperscript{5}. In addition to guiding beam delivery, the fiducials could be modified to provide in vivo localized delivery of readiosensitizer to a tumor. This may improve the therapeutic ratio of radiation therapy without introducing any additional patient interventions. Recent work in our group demonstrated that chitosan thin films can be used as a biodegradable matrix on gold
fiducials typically used in IGRT from which drug loaded NPs are released\textsuperscript{6}. This platform of drug delivery is called as biological in-situ Image guided Radiotherapy (BIS-IGRT). These drug-loaded biocompatible polymer-coated gold fiducials are capable of releasing radio-sensitizer over a period of 4 weeks or more for BIS-IGRT\textsuperscript{6}. In this study we will load maximum amount of drug in the nanoparticles. These nanoparticles would be then dispersed in chitosan and a biodegradable matrix will be coated on the fiducial. The \textit{in vitro} and \textit{in vivo} release profiles of the free drug from the fiducials will be tested. \textit{In vitro} cytotoxicity of the nanoparticles and \textit{in vivo} cytotoxicity of the nanoparticles and the coated fiducials will be evaluated.

Our experimental hypotheses for the studies proposed here are as follows.

1) Synthesis of drug loaded nanoparticles coated fiducials in a chitosan biodegradable matrix will perform as a sustained release platform.

2) The release nanoparticles will be taken up by the tumor cells and the drug released inside the cells killing them.

3) The prepared nanoparticles will be blended in a nanoporous matrix. This platform will be used to study in further research to enhance Image Guided Radiotherapy (IGRT).

\textit{2.3 Specific Aims:}

The specific aims that will be evaluated in this MS thesis project are:

\textbf{Aim 1: Synthesis and Characterization of Pegylated PLGA nanoparticles encapsulating Doxorubicin (DOX).}

1. Synthesis of base form of hydrophobic Doxorubicin from Doxorubicin HCl.
2. Synthesis of surface modified PLGA nanoparticles encapsulating Dox and characterization by Dynamic Light Scattering measurements, Zeta potential, encapsulation efficiency and in vitro release Kinetics.

**Aim 2: Coating radiosensitizing Gold Fiducial Marker with**

1. PLGA Nanoparticles encapsulating Dox dispersed in chitosan.
2. Free Dox dispersed in chitosan.

**Aim 3: Comparative assessment of release kinetics from**

1. PLGA Nano particles encapsulating Dox.
2. Gold fiducial marker coated with PLGA nano particles encapsulating dox dispersed in chitosan.
3. Gold fiducial Marker coated with Free Dox dispersed in chitosan.

**Aim 4: In Vitro uptake and cytotoxicity studies with Prostate Cancer cell line PC3.**

1. Quantitative and Qualitative measurement of cellular uptake studies of dox loaded PLGA nanoparticles in human prostate cancer PC-3 cells and HeLa cells.
2. Therapeutic assays: MTS Assays to measure the efficacy of encapsulated Dox in PLGA nanoparticles on PC3 and HeLa cells.

**Aim 5: In vivo imaging.**

2. In Vivo Imaging: Formulation and characterization of Cy 7.5-PLGA nanoparticles and in vivo imaging.
3. BACKGROUND AND SIGNIFICANCE

3.1. Prostate Cancer

The etymology of cancer goes back to time of Gualen who documented it as a disease that’s looks like a crab due to the enlarged veins on tumors that look like the legs of a crab. Cancer is one of the most prevalent causes of death in the world. In 2008 alone, there were an estimated 12.7 million new cancer cases and 7.6 million deaths worldwide as a result of cancer. In the United States currently 1 in 4 deaths are caused due to cancer and an estimated 569,490 people died of cancer in 2010.

The Prostate gland is an exocrine gland of the male reproductive system below the urinary bladder and in front of the rectum in most mammals. It synthesizes and secretes the seminal fluid that nourishes the sperm. Prostate cancer develops when cells of the prostate gland grow uncontrollably and metastasize to other tissues in the body. These cancer cells also spread to the lymph nodes and if they are detected in this tissue, there is a fair possibility of them having spread to other tissues.

In 2008, prostate cancer was the second most diagnosed type (14% of all cancers) of cancer and ranked sixth in the causes of cancer deaths (6% of all cancers) all over the world\(^2\). There were 217,030 new prostate cancer cases detected in the US and of those 32,050 men died of the disease\(^1\). Figure 1 lists the statistics of (a) prevalence and (b) deaths in both sexes in the US grouped by the type of cancer. Between the years 1975 and 2005, prostate cancer was expected to cause 28% of all incident cancer cases in men\(^1\) (Figure2). The incidence of prostate cancer was highest in males above the age of 80 indicating that this was more prevalent in older men\(^1\). With respect to the race, it was most prevalent and also caused the most deaths in the African-American male population\(^1\).
Figure 1: Estimated (a) new cases and (b) deaths by types of cancers in men and women\(^1\).

In 2008, the estimated new cases of prostate cancer in developed countries were 648,400 as compared to 255,000 in developing countries. This indicated that there was a higher prevalence of prostate cancer in developed countries as compared to developing countries\(^2\). Another way this data can be viewed is that the high incidence rates in developed countries is because of better screening and detection techniques, e.g. detection with Prostate Specific Antigen (PSA). This explains early detections and lower mortality. An example of this is, in North America the incidence rate is 85 per 10,000. But the mortality rate is just 9.9 as compared to say that in West Africa where the incidence rate is 22.2 but the mortality
rate is 18.3. This can be understood using Figure 3. A definite relationship has not been established but prostate cancer was linked to high intake of animal fat and calcium\(^7\). On the other hand, Vitamin E and Selenium have been suspected to be linked with decreasing the risk of prostate cancer.

**Figure 2:** Incidence of types of cancers in men\(^1\).

**Figure 3:** Geographical Distribution of prostate cancer around the world\(^2\).
3.2. Classification of Prostate cancer

a) Cellular classification

Figure 4: Cellular classification of prostate cancer

95% of all cancers that develop in the prostate are adenocarcinomas. As shown in the Figure 4, this type of prostate cancer is further classified into sub categories. In carcinomas there is a co existence of a carcinoma and a sarcoma. The third type is the non epithelial type composed of mesenchymal and lymphomas.

b) Gleason Grading

Gleason Grading involves morphological grading of the tumor growth patterns graded on a scale of 1 to 5. The grading is based on histological architectural features of the neoplastic tissue.
3.3. Diagnosis & Detection of Prostate Cancer

It is essential that prostate cancer is detected early for a better prognosis. Detection of prostate cancer is achieved by a combination of physical, laboratory and imaging techniques. These techniques when applied at the right time in turn affect prognosis rates. Complaints of voiding dysfunctions by patients assist in detection of the disease. The complaints by the patient may not necessarily guarantee early detection as obstructive symptoms and irritation of uro-genital tract do not occur unless the tumor has grown to an appreciable mass.

a) PSA test

The prostate-specific antigen (PSA) test is one of the most popular tests for detection of prostate cancer. The Prostate-specific antigen is a protein made almost exclusively by the prostate gland. The level of this protein is increased in individuals with prostate cancer. However levels of PSA may increase in conditions other than prostate cancer. So, the test might have to be repeated after a particular interval of time for confirmation.

- PSA count of 0-4: below normal
- PSA count of 4-10ng/mL: slightly elevated
- PSA count of 10-20 ng/mL: moderately elevated
- PSA count of >20 ng/mL: highly elevated

b) Digital rectal exam

In this test the doctor inserts a gloved finger into the rectum of the patient to check the prostate. The DRE is not a confirmatory test for prostate cancer but gives the doctor a hint whether further tests are needed to be performed.
Trans-rectal Sonography

An endo-rectal probe is inserted into the rectum of the patient. The probe sends out sound waves that return from the surface of the tissues depending upon the properties of the tissue. This allows the formation of transversal and sagital images. The acoustic electric conversion converts sono-graphic signals into electric impulses seen as black and white dots. This forms an image in various shades of gray.

Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopic Imaging (MRSI)\textsuperscript{10, 11}

The combination of MRI with MSRI is a powerful tool in detection of Prostate cancers. In this technique, metabolic information is provided by MSRI combined with anatomical information by MRI. This enables better assessment of where the cancer is situated, distribution in the prostate, spread in the soft tissue outside the involved node (extra capsular spread) and cancer aggressiveness.

According to Kurhanewicz et al currently, one endo-rectal coil and 4 external coils are used for imaging the prostate. The body coil is used for excitations and the endo-rectal coil for signal reception. This facilitates high spatial resolution and assessment if pelvic lymph node and bone metastases in the same exam.

MRSI utilizes strong magnetic fields to obtain spectra of metabolites in the prostate cancer. In the cancer tissue, the concentration of these metabolites differs as compared to the surrounding healthy tissue. MRSI, specifically detects citrate, choline, creatine and polyamines allowing a comparison of their concentrations between healthy and cancerous tissues.

Positron emission tomography (PET)\textsuperscript{12}

The positron emission tomography measures the metabolic activity and gives the technician a picture of morphological alterations in structures and organs. PET makes use of tracer molecules
to measure metabolic activity. Tracers are tagged with a radioactive molecule and are taken in by tumor cells. Glucose molecules tagged with a fluorine-18 atom is the most commonly used tracer. To avoid disadvantages of high renal elimination and accumulation for this molecule, choline tagged with a carbon-11 atom is used. The rationale for using 18 fluoro-2-deoxy-D-glucose (18 FDG) is the property of increased glucose metabolism by tumors.

**Bone Scintigraphy**

Bone Scintigraphy with technicium$^{99m}$-diphosphonate is done for detecting whether the prostate cancer has metastasized to the bone. It is usually carried out when the PSA count is greater than 50ng/mL.

**d) Biopsy**

In a biopsy, a sample of the prostatic tissue is taken and is examined for metastatic cells. It is performed by a urologist or a radiologist using a TRUS probe. A DRE is done prior to the biopsy. The ultrasound probe is inserted into the rectum to perform the TRUS in order to detect abnormalities. A lidocaine jelly is applied and the anal sphincter is dilated. The biopsy is done by a tissue sampling gun with a core type 18 gauge needle in a fraction of a second. The sample is assessed for malignancies and a Gleason grading is applied to determine the stage of cancer if any is present.
3. 4. Therapy

The choice of treatment for cancer depends on the stage of cancer. The patient’s choice may also be taken into consideration. All aggressive treatments affect urinary, sexual and bowel dysfunction.

a) Surgery

Radical prostectomy is conducted to remove the prostate gland, seminal vesicles and surrounding tissue. Surgery is a preferred choice when the tumor is confined to the prostate itself. The decision to carry out surgery depends on

- Whether the surgery will prevent relapse
- Minimal or no effects on sexual, urinary or bowel functions
- Minimal complications

Nerve sparing prostectomy can prevent unwanted effects such as sexual dysfunction. Post operatively a urinary catheter is inserted into the patient and the patient is counseled for its use and care.

b) Hormone therapy

The aim of this therapy is to reduce the levels of male sex hormone testosterone in the body. The cells of the prostate gland depend on testosterone for growth and development. Reducing the supply of testosterone stunts the growth of the cancer. This is done by

1. Administration of female sex hormone ‘estrogen’ counterbalances the effects of testosterone. This has side effects such as gynecomastia.

2. Luteinizing hormone-releasing hormone antagonists reduce the synthesis of testosterone. They have fewer side effects than estrogen.
c) Radiation Therapy

Radiation therapy is indicated at almost all stages of prostate cancer. It is used to prevent spread of early stage cancer, to control locally advanced disease and to alleviate metastatic disease. Radiation therapy is given either externally or internally.

External radiation therapy

A beam of radiation is administered from outside the body. A higher dose of radiation and an altered regimen is needed for cancers that have metastasized to other tissues such as the bone and the lymph nodes\textsuperscript{16}.

Principle

External Beam radiation therapy was first out into practice at Stanford University in 1956\textsuperscript{17}. The final goal of radiation therapy is to kill cancer cells but to spare healthy cells. Cytotoxicity by radiation therapy is achieved by either direct damage to DNA or by damage by reactive oxygen species (ROS).

The most abundantly used ionizing radiations in radiation oncology are X-Rays and gamma rays. Other types of particles that are also used for radiation therapy are protons, neutrons and carbon ions\textsuperscript{18}. Ionizations are not produced randomly but are produced along track whose paths are decided by the type of ionization. The linear energy transfer allows the calculation of amount of ionization brought about by a type of particle. The Linear energy transfer of a particle is dependent on the energy mass and charge of the particle\textsuperscript{18}. The Linear energy transfer causes chromosomal aberrations in the cells and these aberrations on cells and DNA has been explained in further paragraphs.
Brachytherapy\textsuperscript{19}

Radioactive seeds are implanted in the prostate using ultrasound imaging to direct the implant into the cancerous tissue. The seeds administer radiation doses to the cancerous tissue. The implanted seeds can be in the form of permanent implants or temporary implants. The implants are made up of radioactive elements such as I-125, Palladium-103, Gold-198 and iridium-192. There are two methods for permanent implantation of the seeds a) retro-pubic and b) Trans perennial and these are depicted in Figure 5 respectively. Brachytherapy can be administered as mono-therapy or in conjugation with external radiation therapy.

\textbf{Figure 5:} Retro-pubic (left) and trans perineal (right) technique for seed implantation in brachytherapy\textsuperscript{19}

\textit{d) Image Guided Radiotherapy}

Image guided radio therapy utilizes a combination of CT, MRI or X-Ray imaging with an implanted marker such as a gold fiducial or gold seeds. An example of this can be seen in Figure 6. One of the problems associated with external beam radiotherapy are, the motion of prostate gland and variation in imaging. To eliminate errors associated with these problems and to reduce unwanted radiation dose to the healthy tissue in and around the prostate, metallic markers such as gold fiducials are implanted into the prostate\textsuperscript{21}. The positions of these markers are tracked by one of the imaging modalities and the
radiation dose is targeted to that specific region. An X-Ray scan depicting the same can be seen in Figure 7.

d) Chemotherapy

Androgen deprivation therapy was the first line of therapy for prostate cancer. However, the effects of this therapy wade away the cancer remains unabated and ultimately leads to death\(^{20}\). This condition is called **Hormone refractory prostate cancer (HRPC)**\(^{20}\). Therefore at this stage chemotherapy is

![Figure 6: Transverse CT (left) and T2-weighted MR (right). Gold markers appear as bright spots in CT and dark spots in MR\(^{21}\).](image)

![Figure 7: Relative movements of gold markers relative to bone anatomy\(^{22}\).](image)
essential to introduce chemotherapy to counter the growth of the tumor. There are two mechanism by which this androgen insensitivity develops. The first one being upregulation of the androgen receptor and the second is the growth of cancer cells due to neuropeptides secreted by neuroendocrine cells. 

Alternately, the prostate cancer cells may also arise from prostate cancer stem cells that support the growth of cancerous cells independently\textsuperscript{20}. Patients that develop HRPC, are then subjected to chemotherapy. Three drugs have been approved as first line chemotherapy namely Estramustine, Mitoxantrone and Docetaxel\textsuperscript{23}.

Clinical trials for second line agents for HRPC are currently on\textsuperscript{23} although none of the agents have yet been approved by the FDA. Immense interest is being developed recently for targeted therapy that includes targeting Androgen receptor, apoptotic proteins, immunotherapeutic agents to name a few.\textsuperscript{24}

\textbf{Figure 8:} Mechanisms of androgen insensitivity in prostate cancer\textsuperscript{20}
3.5 Doxorubicin as radio sensitizer and rationale

Radiation therapy is a very effective method of killing cancerous cells. The ionizing power of the radiations produce breaks in the DNA. These could be single stranded or double stranded. But the cells have an intrinsic ability to repair the DNA. Single stranded breaks are repaired by direct repair or base excision repair. Double stranded breaks are handled differently. Depending on the extent of damage, the cells undergo repair or apoptosis. Repair takes place in two ways, homologous or non-homologous recombination. The repair mechanisms are depicted in Figures 10 and 11. Moreover, another factor that contributes to the development of resistance to radiation therapy is tumor hypoxia. Hypoxia is a characteristic property of tumors pathophysiology\textsuperscript{25}. The regions within the tumor that are hypoxic have depleted oxygen tension values\textsuperscript{25}. It has been shown that with increase in the age and stage of prostate cancer, there is an increase in the hypoxic conditions in the tumor\textsuperscript{26}. Tumor hypoxia has both direct and indirect effects that ultimately lead to a resistance to radiation therapy. These effects are summarized in Figure 11.

![Figure 9: Effects of hypoxia on radiation therapy\textsuperscript{27}]
When the DNA damage is brought about by ionizing radiations, several chemotherapeutic agents are used to inhibit these repair mechanisms\. Such drugs are called radiosensitizing drugs.

**Mechanism of radiosensitization**

One of the reasons for resistance to radiotherapy is that in hypoxic conditions, the dearth of oxygen hampers the formation of free radicals that bring about the damage to the DNA. Thus in their absence, damage to the DNA is hampered\. There are two mechanisms by which doxorubicin brings about radiosensitization of radiation therapy treated cells. The first mechanism of radio sensitization is intercalation of doxorubicin to the double stranded breaks hindering the repair of DNA\. 

![Figure 10: Repair Mechanisms for single stranded breaks](image)

The intercalation brings about a conformational change in the DNA structure that inhibits DNA repair. Doxorubicin is a radiosensitizer drug. In their study Bonner and Lawrence\(^4\), demonstrated that, Doxorubicin caused radio sensitization and increased the mean inactivation radiation dose. The second
mechanism is by stabilizing Topoisomerase II-DNA complex. Topoisomerase II is an enzyme that brings about double stranded breaks in replication forks. This eases the tension in the DNA due to uncoiling during DNA replication. Doxorubicin also stabilizes the Topoisomerase II-DNA complex thereby resulting in permanent double stranded breaks in DNA.

This results in cellular toxicity. Double stranded breaks are most important as far as radiation oncology is concerned. Single stranded breaks are readily repaired by cellular mechanisms and are therefore ineffective in bringing about cell death. If single stranded breaks are not repaired correctly, it leads to mutations and certain mutations are potential hazards themselves.

Figure 11: Cellular Responses to double stranded breaks.
3. 6 Surface modified PLGA nanoparticles and their use.

PLGA and its properties

Poly(L-lactide-co-glycolide) (PLGA) is a biocompatible and biodegradable copolymer of L-lactide and glycolide. It is has been used extensively in nanotechnology to make nanoparticles for delivery of a wide range of drugs. It is approved by the FDA for use in humans. In the body, PLGA undergoes degradation via hydrolysis of the ester bond between lactide and glycolide residues to form glycolic acid and lactic acid. These residues enter the Krebs’ cycle and are metabolized to water and carbon dioxide. The rate of formation of biodegradation products is slow and therefore normal cellular functions in the body are not interrupted. The time for biodegradation depends on the lactide/glycolide ratio.

Figure 12: PLGA [poly(D,L-lactide-co-glycolide)] is a copolymer of poly glycolide and poly lactide.

Poly glycolide  Poly lactide

Doxorubicin loaded PLGA nanoparticles

Doxorubicin is a very potent anti-neoplastic agent. Its use as a radiosensitizer has also been explained earlier. We formulated a nanoparticle formulation with a high drug loading and encapsulation efficiency. Since PLGA has the property of encapsulating hydrophobic drugs, Doxorubicin hydrochloride was converted to hydrophobic doxorubicin by neutralizing the hydrochloride. The preparation of the nanoparticles was done by emulsion solvent dispersion method. The major disadvantage of doxorubicin is that its long term use has been associated with cardiotoxicity. Nanoparticles that are surface modified
with polyethylene glycol called ‘pegylated’ nanoparticles reduce the delivery of doxorubicin to the heart thereby decreasing the doxorubicin induced cardiomyopathy while preserving the anti tumor activity\textsuperscript{33}. Along with protecting the heart from cardiotoxicity, pegylated nanoparticles also prolong the half life of the nanoparticles by preventing opsonization of nanoparticles and rapid removal of nanoparticles from the body by the reticulo-endothelial system\textsuperscript{34}. Use of nanoparticles also allows us the take advantage of the ‘Enhanced Permeability and Retention’ effect (EPR). Due to this, the nanoparticles are readily taken up by the tumor tissue and retained inside the tissue that results in enhanced delivery of doxorubicin to the tumor\textsuperscript{34}.

### 3.7 Chitosan as porous biodegradable polymer

Chitosan \{a (1->4) 2- amino- 2- deoxy- β-D- glucan\} is obtained by alkaline deacetylation of chitin. It is a co polymer of N-acetylglucosamine and glucosamine. Chitosan has a varying degree of deacetylation (40-98%) and molecular weight (50,000-2,000,000 daltons). As a biodegradable polymer chitosan has been used in a number of applications such as wound protection, drug delivery, gene delivery, and even contact lenses. Chitosan implants have been widely used for sustained delivery of drugs. An example of its application as a sustained release platform is the release of Uracil from gluteraldehyde cross linked chitosan implants. These chitosan implants were 5mm in diameter and were able to release the drug in vivo for 3 days\textsuperscript{32}. Other examples for sustained release of drugs are N-Succinyl-chitosan conjugated delivery system, cross linked chitosan microspheres\textsuperscript{32}.

**Figure 13**: Chitosan\textsuperscript{32}
D K Nagesha et al showed that cross linked chitosan on gold fiducial markers could be used for sustained release of doxorubicin. They showed that a sustained release could be maintained for 40 days\textsuperscript{6}. The model of this drug delivery system has been described in Figure 14.

Figure 14: Release of drug-loaded PLGA nanoparticles from chitosan coating of fiducials\textsuperscript{6}.
4. MATERIALS AND METHODS

4.1. Synthesis of base form of hydrophobic Doxorubicin from Doxorubicin HCl.

The commercially available doxorubicin HCl is a hydrophilic molecule. For dispersion of the doxorubicin in PLGA nanoparticles, we synthesized a hydrophobic form of doxorubicin by neutralizing the hydrochloride. First 10 mg doxorubicin HCl was dispersed in 1 mL dimethyl sulfoxide. To this, 100 µL of trimethyl amine and one drop of ammonium hydroxide was added. The solution was sonicated for 10 seconds. The reaction was allowed to take place by stirring overnight to give the hydrophobic doxorubicin. This solution had a basic pH of 10. At this pH the color of the doxorubicin solution changed from red to dark blue.

4.2. Synthesis of surface modified PLGA nanoparticles encapsulating Dox.

Surface modified doxorubicin loaded PLGA nanoparticles were synthesized by the emulsion solvent evaporation method. The steps for the preparation of the nanoparticles are as follows. Structurally, these nanoparticles have a Polylactide-co-Glycolide (PLGA) core and are surface coated with dipalmitoylphosphatidylethethanolamine: Polyethylene glycol (DPPE:PEG) (obtained from Avanti Polar Lipids). The doxorubicin is dispersed in the PLGA matrix.

4.2.1. Formation of primary emulsion.

10 mg of PLGA was accurately weighed in a scintillation vial. 500 µL of 20 mg/mL of DPPE:PEG in chloroform was added to the scintillation vial. A solution of PLGA and DPPE:PEG was formed. The chloroform was evaporated under a steady stream of air. Next, 250 µL of Dichlormethane (DCM) was added to the vial to dissolve the contents. A stock solution of 10 mg/ml of doxorubicin was made of which 100 µL was added to the solution. The solution was sonicated for 5 seconds. This solution had all
the components of the oil phase. The primary emulsion was formed when 400 µL of 2.5% solution of PVA solution was added to the above organic phase under rigorous vortexing for 30 seconds.

4.2.2. Formation of Nanoparticles

The primary emulsion was sonicated for 30 seconds and then added to 10 mL solution of 0.3% PVA solution in a 20 mL scintillation vial. The resulting dispersion of the emulsion was sonicated for 2 minutes and kept for stirring overnight. The cap of the scintillation vial was kept untightened to facilitate the evaporation of DCM. A final concentration of 18.3 mM(1mg/10mg PLGA) of doxorubicin was obtained. The nanoparticles were dialyzed in cellulose dialysis tubing cellulose membrane bags (Sigma Aldrich,USA) to remove excess unreacted nanoparticle starting material. The nanoparticles in the bags were kept in excess DI water and stirred for 6-8 hrs. Water was recycled every 30 minutes. The nanoparticles were stored at 4°C until further use.

4.3 Characterization of nanoparticles

The prepared nanoparticles were characterized with respect to the hydrodynamic diameters, surface charge (zeta potential) values, drug encapsulation efficiency, and were observed by scanning electron microscopy (SEM).

4.3.1 Particle size of nanoparticles

Particle size and size distribution of the nanoparticles in the blank and drug-containing nanoparticles were determined using the 90 Plus particle size analyzer (Brookhaven Instruments Corporation, Holtsville, NY). It works on the principle of dynamic light scattering (DLS). To measure the particle size, 100 µL of the nanoparticle sample was diluted to 3 mL with deionized water and the particle size was determined at an angle of 90 degree and at 25°C temperature. Polydispersity index was used as a measure for calculating particle size distribution.
4.3.2. **Surface Charge Measurement**

The surface charge (zeta potential) values measure the relative stability of nanoparticles due to electrostatic repulsion. Zeta potential has also been correlated with cellular uptake and biological performance of nanoparticle-based delivery systems. The 90 Plus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY) was used for measurement of zeta potential. The instrument measured the electrophoretic mobility of the nanoparticles.

4.3.3. **Drug Encapsulation Efficiency**

The encapsulation efficiency of doxorubicin was determined by ultrafiltration method using Nanosep® Omega™ centrifugal filter devices (molecular weight cut-off 100kDa, Pall life sciences, Ann Arbor, MI). Nanoparticles (0.4 mL) were placed in the upper donor chamber and the unit was centrifuged at 9,000 rpm for 40 minutes. The percentage of doxorubicin that filtered out through the membrane was calculated by fluorescence at the excitation wavelength of 470nm and emission wavelength of 480nm using FluoroMax-4 (Horiba Scientific, Edison,NJ) and was used to determine the encapsulation efficiency.

4.3.4 **SEM Imaging**

The shape and surface morphology of the produced nanoparticles were investigated by the scanning electron microscopy (Scanning Electron Microscopy/Energy Dispersive Analyzer (Hitachi, Model S-4800). Before observation, a drop of the sample was placed on a cover slip that was fixed to a standard sample stand by a sticky tape. The sample was allowed to dry under a slow stream of air. The sample required a previous coating with conductive carbon, which was performed in an Auto Fine Coater for seven minutes.
4.3.5 In vitro release kinetics

2mL of nanoparticles were taken in a dialysis bag and the bag was kept in a beaker containing 100mL of 1% Tween-80 under continuous stirring. A 3mL aliquot of Tween-80 solution was taken every 24 hours and the fluorescence intensity was calculated at the excitation wavelength of 470nm and emission wavelength of 480nm using FluoroMax-4 (Horiba Scientific, Edison,NJ). All of the Tween-80 solution was recycled at every time point. The cumulative percentage release was calculated against time and a graph of the same was plotted. Figure 16 shows the release of doxorubicin from nanoparticles over time.

4.4 Coating Gold Fiducial Markers and measurement of release of drugs.

The fiducials were 1.5 mm in length and 0.8 mm in diameter. Gold fiducials were coated with the nanoparticles or free doxorubicin. A 3% chitosan solution was used to coat the fiducials. It was prepared by dispersing 300 mg of powdered Chitosan (Sigma) in 10 mL deionized water. Chitosan as such is insoluble in water at neutral pH but is soluble at acidic pH. The solution was made acidic by adding 100µL of 0.2M acetic acid to make the 3% chitosan.

4.4.1 Coating with Free Drug

In order to coat the fiducial with the free drug, 100µL of Doxorubicin HCl was taken from a stock solution of 10mg/mL and dispersed in 50µL of 3% chitosan. The hydrophobic form of doxorubicin is highly basic. At this pH chitosan is insoluble in water and does not interact with water. Due to this the drug is not released from the chitosan. Therefore hydrophilic form (dox HCl) was used. The fiducial was dipped in the drug-chitosan suspension and air dried at 35°C. This process was repeated until all the suspension was coated on the fiducial. The chitosan was cross-linked after every 3 layers of coating. The process of cross-linking is as follows. (a) immersion in the aqueous solution of sodium hydroxide (4M),
(b) washing with methanol, (c) immersion in the methanol solution of acetic anhydride (4M) and (d) washing with methanol. The coated fiducial was dried overnight at 30°C and stored at 4°C until further use.

4.4.2 Coating with Nanoparticles.

In order to coat the nanoparticles first the total volume was reduced from 10 ml to 100μL. This was done by filtering the nanoparticles in 5mL Nanosep® Omega™ centrifugal filter devices (molecular weight cut-off 100kDa, Pall life sciences, Ann Arbor, MI). This concentrated suspension of nanoparticles was dispersed in 3% chitosan and was coated on the gold fiducials and the cross linking step carried out in the same manner as described in section 4.4.1. The coated fiducial was dried overnight at 30°C and stored at 4°C until further use.

4.5 Coated fiducials: In vitro release kinetics studies

The release of free doxorubicin from the coated fiducials was quantified to measure the release kinetics from the fiducial. The release from each of the two types was measured using FluoroMax-4 (Horiba Scientific, Edison, NJ) spectrofluorimeter. The release from the two fiducials was compared to elucidate the difference in their release patterns. The quantitative analysis of released free doxorubicin was used to study the release kinetics of the drug from the fiducials.

4.5.1 Design of release studies

The coated fiducials were placed in a cellulose membrane dialysis bag and the dialysis bags sealed at both ends. In the dialysis bag, 2mL of Lysozyme (1.5 μg/mL) in PBS (pH adjusted to 6.1) was added. The physiological concentration of lysozyme is 1.5 μg/mL and 6.1 is the pH at which the activity of lysozyme is the highest. The dialysis bag with the coated fiducial was placed in a 250mL beaker containing 100mL 1% Tween-80 solution. The beaker was sealed with parafilm. And the beaker was covered by aluminum
foil to protect released drug from light. The apparatus was kept for stirring at 100 rpm for the duration of the study at 25°C. A standard curve of doxorubicin was obtained by determining the fluorescence intensity at different concentrations of doxorubicin dissolved in the dissolution medium. The amount of doxorubicin released was calculated by extrapolating the fluorescence intensity on the standard curve. The amount of drug released was measured at 25°C.

4.5.2 In vitro release from fiducial coated with free drug.

The apparatus was set up as described in the section 4.5.1. The fiducial coated with free drug was placed inside the dialysis bag. A 3mL aliquot of the Tween-80 solution was taken every 24 hours and the fluorescence intensity was calculated at the excitation wavelength of 470nm and emission wavelength of 480nm using FluoroMax-4 (Horiba Scientific, Edison,NJ). A graph of the cumulative release v/s time was plotted.

4.5.3 In vitro release from fiducial coated with nanoparticles.

The release studies were conducted as per the design described in section 4.5.1. A graph of the cumulative release v/s time was plotted. The readings were taken until there was a fall in the release.

4.6 PC3 human prostate cancer cells HeLa cells in culture.

Wild-type PC3 human prostate cancer cells were acquired from ATCC (VA, USA). The cells were grown in F-12K medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. HeLa cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cell cultures were maintained in an incubator maintained at 5% CO2 and 37°C. Subculturing was done by detaching the monolayer from the culture flask by using 0.05% trypsin/EDTA.
4.6 Confocal Microscopy of uptake of nanoparticles.

PC3 Human prostate cancer cells and HeLa cells were incubated in the culture flask, harvested by trypsinization, and transferred to four 50 mm x 15 mm dish with glass bottom (Matek Corporation, Ashland, MA, USA). The cell density was kept between 20,000 to 22,000 cells per plate and the cells were allowed to adhere overnight. The media was removed and replaced with 200 μL of dox-PLGA nanoparticles in one plate and 200 μL (100 μg/mL) of free doxorubicin in the second. The plates were incubated for 24 hours in the incubator. Differential interference contrast (DIC) and fluorescence microscopy images were obtained at 40X magnification using a Zeiss LSM 700 laser scanning microscope (Carl Zeiss Microimaging LLC, North America). The excitation wavelength was set at 488nm and the emission filter set at 550 nm.

4.7 In vitro Cytotoxicity

Free Doxorubicin (Doxorubicin HCL) and Doxorubicin loaded PLGA nanoparticles were assayed for their dose dependent growth inhibitory effect. Various concentrations ranging from 180 nM to 4 μM doxorubicin equivalent were prepared. Doxorubicin HCl was dissolved in DPBS solution and diluted with growth medium to obtain graded concentrations of their respective solution. Nanoparticles were also diluted with to obtain the graded concentrations. Approximately, 6000 cells per well were seeded into 96-well plates and allowed to adhere overnight. After overnight incubation, the media was replaced with various concentrations of drug solutions and nanoparticles. The negative control was treatment with the growth medium. To test whether any other component of the nanoparticles contributed to the cytotoxicity, blank nanoparticles (no drug encapsulated) were prepared and were incubated with cells as mentioned above. Eight replicates were made for each test condition and plates were incubated for 24 hours. The cytotoxicity was measured using the CellTiter 96® AQueous One Solution (Promega Corporation. Madison, WI). Initially, 1.92ml of the reagent was diluted with 12.48 ml of growth medium.
Then, 150μl of this solution was added to each well of the 96-well plate. The plates were incubated for 4 hours. Living cells reduces the reagent to insoluble formazan crystals which are solubilized in growth medium to give purple color whose absorbance is measured at 490nm using Bio-Tek Instrument’s Synergy® HT microplate reader. The data obtained was statistically compared.

4.8 Quantitative Intracellular Drug Accumulation

To quantitate the amount of drug internalized in the cells, fluorescence spectroscopy and bicinchonic acid (BCA) protein assay (Pierce, Rockford, IL) were performed whereby the amount of the doxorubicin in the cell was normalized to the total amount of proteins in the cell.

4.8.1 Standard Curve for Total Protein Content:

Standard curve was established for the total amount of standard protein (albumin) by measuring the absorbance at different concentrations after reacting them with the working reagent. About 25 μL of protein samples were added to 200 μL of working reagent. Working reagent consists of BCA along with cupric ions in a highly alkaline medium. The alkaline medium aids the conversion of cupric ions to cuprous ions by the proteins, which reacts with BCA in stochiometric proportions to give purple colored solution whose absorbance is measured at 562nm using Bio-Tek Instrument’s Synergy® HT microplate reader.

4.8.2 Standard Curve for Doxorubicin

Doxorubicin is a fluorescently active compound with an excitation wavelength of 470nm and an emission wavelength of 590 nm. Different concentrations of free doxorubicin and doxorubicin loaded
nanoparticles were prepared using deionized water and the fluorescence intensity was measured using FluoroMax-4 (Horiba Scientific, Edison,NJ).

4.8.3 Quantitative Determination of Doxorubicin

About 80,000 cells were seeded in the 6-well plate and were allowed to adhere by incubating the plate overnight. The media was replaced with 400 μL of nanoparticles (drug content 40µg/mL) and 400 µL of free doxorubicin (400 which was diluted to 2 mL with the growth medium. The plates were incubated at 37°C for 6 hours. After 6 hours of incubation, wells were washed twice with 1X PBS solution to ensure complete removal of the non internalized nanoparticles. About 400µL of the lysis buffer was added to each well and the plate was kept on shaker at 4°C until a white film appeared at the bottom which indicates complete lysis. The lysate was collected and micro-centrifuged (AccuSpin Micro R, Fischer Scientific) at 13,000 rpm for 10 min at 4°C. The supernatant layer was collected and evaluated for the total amount of proteins using the BCA assay kit (Pierce, Rockford, IL). To determine the amount of doxorubicin, the supernatant was taken and the fluorescence intensity was measured at an excitation wavelength of 470nm and emission wavelength of 590nm using FluoroMax-4 (Horiba Scientific, Edison,NJ). The amount of total proteins and doxorubicin in the lysate was determined by extrapolating the readings obtained from the standard curve.

4.9 In vivo Imaging

In vivo applications of optical bioimaging are severely limited, owing to the poor tissue penetration of visible light. Therefore, in recent years there has been a surge in the development of new generation of optical probes, which absorb and emit light in the near-infrared (near-IR) range (700–1000 nm). This spectral region is considered as the “optical transmission window” of biological tissues, where there is
less absorption and scattering of the excitation and emitted light as well as reduced autofluorescence, thus allowing penetration of light for deep-tissue imaging with higher contrast\textsuperscript{35}. The recent rise in nanotechnology has further bolstered the prospects of in vivo optical imaging through the development of a variety of near-IR-luminescent nanoformulations, which include quantum dots\textsuperscript{36}, upconverting nanophosphors\textsuperscript{37}, and luminophore-containing nanoparticulate carriers such as liposomes\textsuperscript{38}, polymersomes\textsuperscript{39}, ceramic\textsuperscript{40} or polymeric nanoparticles, etc.

The in vivo imaging was carried out by intratumoral administration of nanoparticles. One of the major hurdles for in vivo fluorescence imaging in the visible range is tissue auto-fluorescence. Initially, the imaging was done with the Dox-PLGA nanoparticles coated fiducials implanted intra-tumorly with a 16G needle. The sole motive of using the imaging modality with the coated fiducial is to show the spatial distribution of the released drug/fluorophore from the coated fiducial over a period of time in the tumor matrix. Since the emission wavelength of the Dox lies in the visible range (~590nm), it was extremely difficult to spectrally unmix the Dox fluorescence from autofluorescence. Therefore, to make our way around this, we formulated nanoparticles that encapsulated a dye that fluoresced in the ‘near infrared’ (NIR) region where there is no interference due to tissue auto fluorescence. The dye selected for this purpose was Cy 7.5, a cyanine dye.

The co-delivery of a fluorescent probe along with a therapeutic agent provides a key challenge. However, using nanoparticles this can be done by co-encapsulating the two agents in the nanoparticle platform. Once the two probes are encapsulated into the matrix of the nanoparticles, the delivery at a specific target is usually governed by the nanoparticle’s physico-chemical properties. The systemic circulation of nanoparticles with encapsulated probes usually results in accumulation in the tumor via the EPR effect, whereas a considerable accumulation in the non target organs like liver and spleen is also observed. Here, to exploit the advantages associated with the co-encapsulation we have used PLGA
nanoparticles that encapsulated both doxorubicin and the Cy 7.5 and injected into the tumor. This formulation allowed simultaneous delivery of a therapeutic agent and a dye that facilitated imaging and tracking of the distribution if nanoparticles inside the tumor.

4.9.1 Formulation of nanoparticles

4.9.1.1 Formulation of nanoparticles encapsulating Cy 7.5.

A stock solution of 5 mg/mL of Cy 7.5 was made in DMSO. The nanoparticles were synthesized according to the same procedure that has been mentioned in section 4.2. Of the stock solution, 50µL (125µg Cy 7.5) was added to make the nanoparticles.

4.9.1.2 Formulation of nanoparticles encapsulating both doxorubicin and Cy 7.5

Nanoparticles were synthesized so as to contain both doxorubicin and Cy 7.5. The loading was set at 125µg for the Cy 7.5+dox loaded PLGA nanoparticles.

4.9.2 Characterization of nanoparticles.

The prepared nanoparticles were characterized with respect to the hydrodynamic diameters, surface charge (zeta potential) value and their optical properties.

4.9.3 Generation of Tumor Xenografts.

Tumor xenografts were grown in BALB/c nude mice. Approximately 1*10^7 PC3 prostate cancer cells would be suspended in BD Matrigel™ (BD Biosciences) and the suspension was injected subcutaneously in the mice. Tumor growth was monitored every 24–48 h until a tumor size of approximately 5 mm in diameter was observed.
4.9.4 Administration of nanoparticles into the tumor and imaging.

Each formulation of nanoparticles encapsulating were injected into the tumor using. The imaging was done using an IVIS Lumina II imaging system (Caliper Life Sciences).
5. RESULTS AND DISCUSSIONS

5.1 Preparation and Characterization of Nanoparticles.

Doxorubicin in its hydrophobic form was encapsulated in PLGA nanoparticles that were surface modified with a Pegylated lipid (DPPE:PEG). The pegylation reduces the interaction of the nanoparticles with the immune system in vivo. This increases the residence time of the nanoparticles in vivo. PLGA being a biocompatible and biodegradable polymer degrades inside the body without forming toxic degradation products. The prepared nanoparticles were characterized for their hydrodynamic diameters, surface charge (zeta potential) values, drug encapsulation efficiency, and were observed by scanning electron microscopy (SEM).

5.1.1 Particle Size Analysis: The mean hydrodynamic diameter of the prepared nanoparticles was in the range of 110-130 nm with a narrow distribution as seen from the polydispersity index in Table 1.

5.1.2 Surface Charge Measurement: The average zeta potential values of the blank and drug-containing nanoparticles are listed in Table 1. The zeta potential values for all three different nanoparticles were found to be negative possibly due to presence of some residual polyvinyl alchohol.

5.1.3 Drug Encapsulation Efficiency: The encapsulation efficiency, as analyzed using centrifugation and mass balance technique, was found to be 96.43%. These results are shown in Table 1. This high drug encapsulation efficiency could be attributed to the ability of hydrophobic form of doxorubicin to be easily dispersed in PLGA.

5.1.4 SEM Analysis: Figure 15 is the representative SEM images of nanoparticles. It shows the smooth and spherical morphology of the nanoparticles.
Table 1: Characterization of blank and dox-PLGA nanoparticles.
*mean±SD

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Number Diameter (nm)*</th>
<th>Polydispersity</th>
<th>Zeta Potential (mV)</th>
<th>Doxorubicin Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank NP</td>
<td>120±10.30</td>
<td>0.17</td>
<td>-12.73±0.54</td>
<td>--</td>
</tr>
<tr>
<td>Doxorubicin loaded nanoparticles</td>
<td>108±15.90</td>
<td>0.191</td>
<td>-14.17±0.54</td>
<td>96.43</td>
</tr>
</tbody>
</table>

5.1.5 In vitro release kinetics

Figure 16 shows the release of doxorubicin from nanoparticles over time. The amount of doxorubicin released was calculated with respect to time. Initially, the nanoparticles showed an increase in release which stabilized over time. It can be observed that the nanoparticles steadily release approximately 16 µg of doxorubicin per day.

Figure 15: Scanning Electron Microscopy images of Dox loaded PLGA nanoparticles (left), and Blank PLGA nanoparticles (right).
Figure 16: Release of free doxorubicin from nanoparticles function of fluorescence intensity (left) and as concentration (right).

5.2 Coated Fiducials: \textit{In vitro} release kinetics.

5.2.1 Standard curve for measurement of release.

A standard curve (Figure 17), was obtained by preparing various dilutions of doxorubicin in dissolution medium and measuring the fluorescence intensity.

Figure 17: Standard curve of hydrophobic doxorubicin.
5.2.2 *In vitro release from fiducial coated with free drug.*

Using the data from the fluorescence measurements, a graph (Figure 18) of the cumulative release v/s time was plotted. It can be inferred from the graph that when free drug is coated onto the fiducials, there is a burst release. Therefore this supports the use of nanoparticles to be used for sustained release of the drug.

![Figure 18: Release from fiducial coated with free doxorubicin.](image)

5.2.3 *In vitro release from fiducial coated with nanoparticles.*

PLGA nanoparticles encapsulating doxorubicin were coated on the gold fiducials and the release of the free drug was measured. In Figure 19, the graph on the left shows the release of the drug as a function of fluorescence intensity. The graph on the right shows the release of the drug as a function of concentration.
Figure 19: release of free doxorubicin form fiducials as a function of fluorescence intensity (left) and the release of the drug as a function of concentration (right).

From the graphs it can be inferred that a steady release is attained and approximately 11 µg of drug was released per day.

Figure 20 is an overlay figure that compares the release of doxorubicin from only nanoparticles and from fiducials. It is clear that the fiducials release a constant amount of drug even after 40 days.

Figure 20: Overlayed graph of release of doxorubicin from nanoparticles and Fiducials.
5.3 Confocal Microscopy of uptake of nanoparticles.

The nanoparticles were taken up by both PC-3 and HeLa cells. A difference in the pattern of uptake of nanoparticles and free drug can be seen. The free drug stains the nucleus immediately after treatment. The nanoparticles however cannot pass through the nuclear membrane and therefore the nucleus remains unstained. **Figure 21** shows the difference in uptake of nanoparticles and free drug. **Figure 22** shows the uptake of nanoparticles at two different time points.

![Figure 21: DIC and Confocal Images of uptake of doxorubicin loaded PLGA nanoparticles and free drug in PC3 and HeLa cells.](image)

The overlaid DIC and fluorescence microscopy images were presented in **Figure 22**. From the figures, it is evident that both the nanoparticles and the free doxorubicin were internalized by both the cell lines.
Figure 22: Overlaid Images of PC3 cells and Hela cells with respective treatments at 2 time points (2 hours and 24 hours).

5.4 In vitro cytotoxicity

The cell viability studies were carried out using three types of treatment groups. Free doxorubicin, Doxorubicin encapsulated in PLGA nanoparticles and Blank PLGA nanoparticles. Two cell lines were used namely PC-3 and HeLa. The number of live cells was measured after treatment using the CellTiter 96® AQReus One Solution (Promega Corporation. Madison, WI). The percentage viability was calculated and plotted against concentration.

From the graphs (Figure 23) it could be observed that the blank PLGA nanoparticles themselves did not impart any toxicity. Therefore it could be concluded that the cytotoxicity was solely from the action of the drug and not from any other component of the formulation. Doxorubicin loaded PLGA nanoparticles had dose dependant cytotoxicity with maximum cytotoxicity at 2.5 µg/mL (4µM) of doxorubicin in both cell lines. Equivalent free doxorubicin concentrations were used to measure the cytotoxicity in HeLa cells. It can be observed that after an initial decline the cytotoxicity of all the concentration remains
almost constant. This could be the result of activation of efflux mechanisms such as increased P-glycoprotein expression in the cells. Further studies confirming this need to be carried out.

Figure 23: Cytotoxicity of different treatments in HeLa cells (left) and PC-3 cells (right).

5.5 Quantitative Intracellular Drug Accumulation.

Image 24: Standard curves of Free Doxorubicin and Dox-PLGA nanoparticles.
Standard curves (Figure 24) for intracellular concentration of free doxorubicin and dox-PLGA nanoparticles were plotted to measure the concentrations of unknown samples. The results for the amount of intracellular drug accumulation in PC-3 cells are summarized in Table 2. The uptake of drug when encapsulated in PLGA nanoparticles is 22 times higher than drug administered in free form. This data clearly suggests that the formulation in use enhances the delivery of the drug inside the cells. Table gives the summary of the drug accumulation inside PC-3 cells. In Figure 25, this data is presented as a bar graph as a visual aid for differentiating between the two treatment types.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total drug accumulation (µg)</th>
<th>µg drug/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Doxorubicin</td>
<td>0.443±0.0007</td>
<td>0.529±0.0253</td>
</tr>
<tr>
<td>Doxorubicin Loaded PLGA nanoparticles</td>
<td>10.209±1.655</td>
<td>11.649± 2.81</td>
</tr>
</tbody>
</table>

Table 2: Summary of Quantitative intracellular drug accumulation.

Figure 25: Comparison of accumulation of free doxorubicin and dox-PLGA nanoparticles.

5.6 In vivo imaging

5.6.1 Characterization of Nanoparticles.

The prepared nanoparticles were characterized with respect to the hydrodynamic diameters, surface charge (zeta potential) value and their optical properties. The data for particle size and zeta potential is summarized in the Table 3. The absorption and emission spectra for the nanoparticles is shown in the
**Figure 26.** The dye had peak excitation and emission in the Near Infra Red (NIR) region of the spectrum with peak emission at 825 and peak excitation at 800nm.

**Figure 26:** Absorption and emission spectra for Cy 7.5 loaded nanoparticles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Hydrodynamic diameter</th>
<th>Polydispersity</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy 7.5 loaded PLGA nanoparticles</td>
<td>126.65±9.12</td>
<td>0.257±0.02</td>
<td>-20.37±4.81</td>
</tr>
<tr>
<td>Cy 7.5+doxorubicin loaded PLGA nanoparticles</td>
<td>120.12±8.45</td>
<td>0.213±0.05</td>
<td>-16.63±3.23</td>
</tr>
</tbody>
</table>

**Table 3:** Characterization of Cy 7.5 loaded PLGA nanoparticles and Cy 7.5+dox PLGA nanoparticles.

### 5.6.2 In vivo Imaging

Following the in vitro assays for therapeutic efficacy, we have further stretched the applicability of the PLGA nanoparticles in in vivo systems. As evident in **Figure 27**, a bright fluorescence signal was observed from the tumor with absolutely zero autofluorescence. The absence of autofluorescence would certainly provide the advantage of using the Cy7.5-PLGA nanoparticles coated fiducials. We presume that these NIR fluorophore nanoparticles coated fiducials when implanted in the tumor would show up as bright
point source fluorescence which when released from the fiducial will progressively ‘paint’ the entire tumor and show a spatial distribution of the released dye in the tumor matrix.

\[ \text{Figure 27: Autofluorescence free in vivo imaging with Cy7.5-PLGA nanoparticles.} \]

Left panel shows the absorbance and fluorescence spectra of the Cy7.5-PLGA nanoparticles and right panel shows the live animal images of the PC3 xenografted nude mice injected intra-tumorly with Cy7.5-PLGA nanoparticles. Images were acquired 24h post injection. The autofluorescence image was acquired using the 488nm excitation filter set (similar to the settings used for imaging Dox-PLGA nanoparticles coated fiducials) whereas for Cy7.5-PLGA nanoparticles, 745 nm filters were used.
6. CONCLUSION

The result of this study has shown that polymeric nanoparticles have the potential to be used as drug carriers in Image Guided Radiotherapy (IGRT). These nanoparticles when coated onto fiducials act as sustained release platforms. PLGA nanoparticles encapsulating doxorubicin in its hydrophobic form were within a desired size range of 110nm and showed a sustained release of the encapsulated doxorubicin. These nanoparticles embedded in the chitosan matrix, when coated on the fiducial markers showed a dual release mechanism of the drug in a sustained manner. In vitro cellular uptake was confirmed by confocal fluorescence microscopy where a difference in the pattern of uptake of nanoparticles and free drug could be seen. The results suggested that the drug was released from the nanoparticles inside the cells and not inside the media. The quantitative intracellular drug accumulation data showed that drug delivery was enhanced when doxorubicin was administered encapsulated in PLGA nanoparticles than in the free form. This could be due to the activation of efflux mechanisms that draw out free doxorubicin from the cells. Further studies confirming this hypothesis need to be carried out. Although, this was supported by in vitro cell viability data that showed that dox-PLGA nanoparticles were more toxic than the free form of the drug. The major hurdle of this study was in vivo imaging of release of drug from coated fiducial. The in vivo imaging was performed with Cy7.5 PLGA nanoparticles injected intratumorly into the mice with subcutaneous prostate cancer xenograft. The results showed a remarkable increase in fluorescence signal from the Cy7.5 nanoparticles with minimal autofluorescence which was otherwise very difficult with the Doxorubicin coated fiducials implanted due to the elevated autofluorescence levels. Also, we have shown the co-encapsulation of Cy7.5 with doxorubicin inside PLGA nanoparticles. These nanoparticles can further be coated on the fiducial in a similar manner as for doxorubicin nanoparticles and when implanted in the tumor would show a sustained release and a wide distribution of the drug and can be monitored by imaging without interference by autofluorescence. The results of
this thesis could be used as a foundation for further research into modification of gold fiducials to enhance Image Guided Radiotherapy (IGRT).
7. REFERENCES

34. Torchilin, V. P., Nanoparticulates as drug carriers. Imperial College Press; Distributed by World Scientific Pub.: London