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CURCUMIN AND RESVERATROL LOADED CALCIUM ALGINATE NANOPATICLES FOR ANTI-CANCER THERAPY

By

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A THESIS

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ABSTRACT

Curcumin and resveratrol are naturally occurring polyphenolic compounds having anti-cancer potential. However, their poor aqueous solubility and bioavailability limits their clinical use. Entrapment of hydrophobic drugs into hydrophilic nanoparticles such as calcium alginate presents a means to deliver these drugs to their target site.

Curcumin and resveratrol loaded calcium alginate nanoparticles were prepared by emulsification and cross-linking process. The nanoparticles were characterized for particle size, zeta potential, moisture content, physical state of the drugs, physical stability, and entrapment efficiency. An UPLC method was developed and validated for the simultaneous analysis of curcumin and resveratrol. The in vitro release of drugs from the nanoparticles was studied using the dialysis membrane. The cytotoxicity and cellular uptake of the formulation was studied in DU145 prostate cancer cell line.

The particle size of the nanosuspension and freeze dried nanoparticles was found to be 12.53±1.06 nm and 60.23±15 nm, respectively. The zeta potential of blank as well as drug loaded nanoparticles were in the range of -13 to -25 mV. The moisture content of blank and drug loaded nanoparticles was 5.9±0.8% and 4.8±0.2%, respectively, as determined by Karl Fisher
titrimetry. Both DSC and powder XRD studies indicated that curcumin as well as resveratrol were present in a non-crystalline state, in the nanoparticles. The entrapment efficiency for curcumin and resveratrol was found to be 49.3±4.3% and 70.99±6.1%, respectively. Resveratrol showed a higher release than curcumin (87.6±7.9% versus 16.3±3.1%) in 24 hours. Curcumin was found to be taken up by the cells from solution as well as the nanoparticles. Resveratrol had a poor cellular uptake. The drug loaded nanoparticles were found to exhibit cytotoxic effects on DU145 cells with a 47.2±7.7% cell survival, 72-hours post-treatment. Drug solution exhibited greater toxicity than nanoparticles at the highest concentrations. The formulation was found to be safe for intravenous administration. This formulation needs further testing for its efficacy in other cells lines such as prostate cancer, breast cancer and pancreatic cancer cell lines. The possible efflux mechanism for resveratrol transport in these cell needs future investigation.
PREFACE

Poster presented in national/international conferences:


Poster accepted:

Dedicated to my family
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LIST OF ABBREVIATIONS

ADT          Androgen Deprivation Therapy
AR            Androgen Receptor
ATCC         American Type Culture Collection
BCA          Bicinchoninic acid
BCS          Biopharmaceutics Classification System
CDK          Cyclin Dependent Kinase
COX-2        Cyclooxygenase-2
CRPC         Castration Resistant Prostate Cancer
DAPI         4′,6-Diamidino-2-phenyindole
DMF          Dimethyl formamide
DSC          Differential Scanning Calorimetry
EPR          Enhanced and Permeation Retention
FDA          Food and Drug Administration
FAO          Food and Agriculture Organization
GRAS         Generally Regarded As Safe
HPLC         High Performance Liquid Chromatography
HPTLC        High Performance Thin Layer Chromatography
HSS          High Strength Silica
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<tr>
<td>LC-MS</td>
<td>Liquid Chromatography- Mass Spectrometry</td>
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<td>LDL</td>
<td>Low Density Lipoproteins</td>
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<tr>
<td>LOX</td>
<td>Lipooxygenase</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor κB</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
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<td>SCID</td>
<td>Severely Compromised Immunodeficient</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>TGA</td>
<td>Thermogravimetric Analysis</td>
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<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor α</td>
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<td>UPLC</td>
<td>Ultra Performance Liquid Chromatography</td>
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<td>USP</td>
<td>United States Pharmacopoeia</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>XRD</td>
<td>X-Ray Diffraction</td>
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CHAPTER 1

Introduction
1.1. Cancer and chemotherapy

Cancer is one of the most challenging diseases today, presenting health issues in United States and the entire world. The American Cancer Society estimates occurrence of 1,685,210 new cancer cases and 595,690 deaths in 2016. Prostate cancer and breast cancer lead the new cancer cases in males and females respectively; while the cancer of lung and bronchus remains the leading cause of death\(^1\).

Cancer occurs when the cells of a particular organ undergo abnormal cell division and grow at an uncontrolled rate which ultimately leads to the formation of a tumor. These cells may remain limited to that particular organ or may metastasize to other organs. Benign tumor remains isolated to an organ, whereas malignant tumors metastasize to other organ systems which can be fatal. Cancer can be caused by both external and internal factors. External factors include smoking, alcohol, tobacco, radiation and carcinogens such as asbestos, arsenic, benzene and other xenobiotics. The internal causes of cancer include activation of oncogenes, hormonal causes or immunosuppression. In case of an early detection, surgical removal of the tumor is the preferred treatment. However, with advanced metastases, surgery
cannot be effective and radiotherapy or chemotherapy is the course of treatment.

Chemotherapy involves treatment of cancer using cytotoxic drugs that alter the metabolism of the tumor cells, inhibit various stages of cell division cycle or exhibit a direct tumoricidal effect. The aim of the chemotherapeutic drug treatment is to help decrease the cancer symptoms and to prolong the life of the patient. These act via multiple mechanisms of action. Alkylating agents such as cisplatin, cyclophosphamide, ifosfamide, busulfan, melphalan act by alkylating specific sites in the cell DNA. This interferes in the mitotic process, DNA replication and RNA formation which affects the syntheses of enzymes and other essential proteins.\(^2\)

Targeting the *de novo* DNA synthesis of rapidly dividing cells offers another avenue for treatment. The antimetabolite drugs are analogs of the natural purines and pyrimidines which tend to interact with the enzymes involved in DNA synthesis. Thus, they interfere in DNA synthesis by competing for the natural substrate or by causing chain termination. The common drugs in this category include Mercaptopurine, Methotrexate, 5-Fluorouracil, Gemcitabine.\(^3,4\) Topoisomerase I and II is a vital enzyme necessary for separating the double strands of DNA.\(^5\) Camptothecin, Topotecan (Topoisomerase I) and Etoposide, Doxorubicin (Topoisomerase II)
are some of the drugs that inhibit this enzyme. As Topoisomerase II is expressed more in proliferating cells, use of Topoisomerase II inhibitors offer better selectivity as compared to Topoisomerase I inhibitors. Drugs such as Paclitaxel are microtubule stabilizers. They stabilize the microtubules in their polymeric stage and prevent their disassembly resulting into blockage of mitosis. Vinca alkaloids bind to the β-subunit of the tubulin dimers and tend to block mitosis. Tumors can be treated by targeting the antigens associated with them. A wide range of antigens such as glycoproteins, growth factors, vascular factors can be targeted. Trastuzumab, Bevacizumab, Cetuximab are some of the FDA approved monoclonal antibodies for cancer treatment.

Tyrosine kinase is an important enzyme regulating cell differentiation and growth. Imatinib is a drug used for tyrosine kinase inhibition.

Chemotherapy is largely effective, but it has many adverse effects. Although the drugs are meant to have effects on abnormally active malignant cells, the non-malignant cells are active as well and are subject to the effects of these drugs. The most common adverse reactions of chemotherapeutic drugs include nausea, vomiting, blood disorders, alopecia and disorders of a particular system such as the nervous, cardiovascular, and the reproductive systems. Thus there is an urgent need of a chemotherapeutic agent with minimal side effects. Curcumin and Resveratrol, commonly present in food
and beverages, present an alternative option for anti-cancer chemotherapy with minimal adverse effects.

**1.2. Prostate Cancer**

Prostate cancer ranks second in both cancer diagnosis and cancer deaths in men in the United States\(^1\). The prostate gland secretions aid in the motility and nourishment of the sperm and contribute up to 30% of the semen. Androgen hormones such as testosterone and dihydrotestosterone are required for the normal functioning of the prostate gland. The actions of the androgens are mediated by the Androgen Receptor (AR)\(^{11}\). Signaling via the AR axis is an important mechanism of prostate carcinogenesis. There are multiple ways in which the AR and androgens contribute to the development of prostate cancer. Higher levels of androgens can lead to a progression to prostate cancer. In the initial stages, when the cancer is dependent on the androgen levels, Androgen Deprivation Therapy (ADT) can be used for treatment. ADT includes use of drugs that are Leutinizing Hormone Releasing Hormone (LHRH) antagonists such as Abarelix and Degarelix. They help to reduce the androgens to castrate levels (<0.5 ng/mL)\(^{12}\). However, after a period of ADT, the cancer progresses to a stage where it no longer responds to castrate levels of androgens. At this stage the cancer is called Castration Resistant Prostate Cancer (CRPC). Several factors that lead to CRPC include amplification of AR expression,
mutations in AR, local production of androgens in prostate cells and AR activation by non-traditional ligands\textsuperscript{13}. Prostate Specific Antigen (PSA) is a glycoprotein enzyme secreted in the prostate which can be used as a biomarker for prostate cancer. CRPC is a highly metastatic form that metastasizes to the bones and lymph nodes causing increased fatalities. PSA levels above 4 ng/mL are recommended to be screened for prostate cancer\textsuperscript{14}.

Secondary ADT can be initiated in CRPC. This includes anti-androgens such as Flutamide and Nilutamide, glucocorticoids such as Prednisone for the suppression of steroidogenesis, and the second generation AR antagonist Enzalutamide. Alternatively, biosynthetic pathways of androgen production can be targeted with drugs such as Abiraterone acetate (irreversible inhibitor of CYP17A1). With the increased aggressiveness of CRPC, the treatment gets limited to the use of chemotherapeutic drugs such as Docetaxel and Cabazitaxel, Mitoxantrone, Satraplatin, and tyrosine kinase inhibitors such as Imatinib mesylate. Bone metastasis can be managed using bisphosphonates such as Zoledronic acid and Alendronate that act on the bone remodeling\textsuperscript{12}. Curcumin and Resveratrol have been indicated to exhibit a cytotoxic effect in prostate cancer acting on cell division cycle and inducing cell apoptosis\textsuperscript{15,16,17,18,19}. 
1.3. Curcumin

Curcumin is a yellow colored compound obtained from the rhizomes of *Curcuma longa*, Family: Zingiberaceae (turmeric). Curcumin constitutes about 3-4 % of the total turmeric constituents. It is responsible for the yellow color of turmeric. Curcumin was first isolated in 1815 by Vogel et al. and its diferuloylmethane structure was confirmed by Lampe et al. in 1910\textsuperscript{20}. Turmeric has been used in medication since ancient times for its wound healing and anti-inflammatory properties\textsuperscript{21}.

1.3.1. Physicochemical Properties

![Curcumin structure](image)

**Figure 1.1:** The structure and the structural components of curcumin\textsuperscript{22}.
Curcumin has a molecular formula of $\text{C}_{21}\text{H}_{20}\text{O}_{6}$, a molecular weight of 368.37 g/mol and a melting point of 175 °C. The chemical name of curcumin is diferuloylmethane and can be categorized under polyphenols. It is a bis-$\alpha\beta$-unsaturated $\beta$-diketone. The chemical name of curcumin is $(1E,6E)$-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione$^{23}$. Curcumin is the most abundantly occurring natural analogue at 77 %, followed by demethoxycurcumin (17%) and bis-demethoxycurcumin (3%) in which the methoxy group is absent from one of the phenyl rings and from both phenyl rings, respectively$^{24}$. Collectively, they are termed as curcuminoids. Curcumin exists in two isomeric forms: the enol form and the diketone form. These two forms exist in equilibrium.

![Figure 1.2: Tautomerism of curcumin molecule](image)

The equilibrium between the diketone and the keto-enol form on the heptadienone linkage tends to affect the physicochemical properties of curcumin. The diketone form of curcumin predominates in acidic and neutral
conditions where it acts as a potent H-atom donor. The heptadienone linkage in the diketone form contains a highly activated carbon atom and where the C-H bond is weak due to the delocalization of the unpaired electrons on the two adjacent oxygen atoms. On the other hand, at pH greater than 8, the keto-enol is the predominating form which acts as an electron donor\textsuperscript{25}.

Curcumin is a hydrophobic compound and its logP value is 2.5\textsuperscript{26}. It has an extremely low solubility in water (11 ng/mL)\textsuperscript{27}, but is soluble in organic solvents such as acetone, methanol, ethanol, acetonitrile. The Biopharmaceutics Classification System (BCS) places curcumin in the Class IV compounds that possess low permeability and low solubility\textsuperscript{28}. In the keto-enol form, curcumin has three ionizable protons; two from the phenolic groups and one from the enol group. The experimentally determined pKa values for these ionizable protons are 10.69, 9.30 and 8.54 respectively\textsuperscript{29}.

Curcumin is stable in acidic conditions. The decomposition of curcumin has been found to be pH dependent and occurs faster in neutral-basic conditions, following first-order kinetics. Curcumin color turns from yellow to red in increasingly alkaline solutions. It also undergoes faster degradation in serum-free medium as compared to serum containing medium. Vanillin, ferulic acid and feruloyl methane are the degradation products of curcumin, with vanillin being the major degradation product\textsuperscript{30}. Curcumin is a chromophore that
absorbs strongly in visible wavelength range, and hence susceptible to photodegradation. In solution state, curcumin is sensitive to UV light and can be subject to photochemical degradation\textsuperscript{31}.

Figure 1.3: The degradation products of curcumin\textsuperscript{32}

1.3.2. Pharmacokinetics and Metabolism of Curcumin

The absorption, metabolism, distribution and elimination of curcumin has been studied in rodent models as well as clinical models. Wahlstorm et al., administered curcumin at 1g/kg to rats, showing 75% dose elimination in feces, whereas negligible amount was found to have been excreted in urine.
Biliary excretion measurements showed poor absorption from the gut\textsuperscript{33}. In another study, curcumin was shown to have a 60% absorption in rats. The amount of curcumin present in blood, liver and kidneys of the rats was found to be low. Glucuronide and sulfate conjugated were detected in the urine\textsuperscript{34}. The very low urinary excretion of curcumin and the high fecal elimination of the drug was also confirmed in a study using radioactive \([3H]\)-curcumin\textsuperscript{35}. Holder et al. have reported biliary excretion of curcumin in rats. The major biliary metabolites were found to be glucuronides of tetrahydrocurcumin and hexahydrocurcumin\textsuperscript{36}. A study by Pan et al. demonstrates that curcumin is first converted to dihydrocurcumin and tetrahydrocurcumin, which are then transformed into their glucuronide conjugates\textsuperscript{37}. The preclinical work of Ireson et al. showed presence of glucuronide and sulfate conjugates of curcumin in plasma. This work was also performed on suspensions of isolated human hepatocytes which showed that metabolic reduction of curcumin was a fast process, occurring within minutes\textsuperscript{38}.

Clinical studies carried out by Shoba et al. demonstrated poor curcumin bioavailability after administration of a 2g dose to fasting volunteers. Co-administration of piperine (a constituent of pepper) was found to increase the curcumin bioavailability by 2000\%\textsuperscript{39}. A low curcumin bioavailability was also observed in a clinical study performed by Sharma et. al\textsuperscript{40}. In another
clinical study, a single high dose of curcumin was found to result in rapid appearance of glucuronide and sulfate conjugates. This could explain the low amounts of free curcumin present in plasma\textsuperscript{41}.

1.3.3. Safety

Curcumin has a very high therapeutic index. In a phase I clinical study, daily doses up to 3600-8000 mg for 4 months did not cause any discernable toxicity except mild nausea and diarrhea\textsuperscript{42}. In a clinical study for treatment of rheumatoid arthritis, curcumin was found to be well tolerated by subjects at 500 mg daily dose for a test period of 8 weeks\textsuperscript{43}. Turmeric is categorized as Generally Recognized As Safe (GRAS) by the US FDA, and curcumin has been granted an acceptable daily intake level of 0.1–3 mg/kg-BW by the Joint FAO/WHO Expert Committee on Food Additives, 1996\textsuperscript{44}. 
1.3.4. Pharmacology of Curcumin

Curcumin exhibits an array of pharmacologic actions that include anti-cancer, anti-oxidant, anti-angiogenic, and anti-inflammatory effects.

1.3.4.1. Anti-cancer actions
The DNA replication and cell division is a process that occurs in a series of coordinated events called the cell division cycle. This cycle occurs in sequential G1, S, G2 and M phases. The S and M phases are the DNA synthetic phases that are preceded by the gap phases G1 and G2, respectively\textsuperscript{45}. Mutations can cause loss of integrity of the cell division cycle causing a hyper-proliferative stage which eventually leads to cancer\textsuperscript{46}. There are two important cell cycle control mechanisms. The first is the Cyclin and Cyclin Dependent Kinase (CDK) pathway. A host of Cyclin and CDK interactions at various phases of cell cycle regulate the cell proliferation. CDKs are a target in anti-cancer drugs with a rationale of controlling cell division. The second important cell cycle controller is the Tumor protein p53, which serves as a ‘checkpoint’ in the cell cycle. It plays a role in apoptosis and keeps a control over cell proliferation\textsuperscript{47}.

Curcumin has activity as both a blocking agent and as a suppressing agent. It inhibits the initiation step of cancer by preventing activation of carcinogens such as benzene and anthracene derivatives. As a suppressing agent, it inhibits malignant cell proliferation\textsuperscript{48}. Curcumin tends to downregulate the pro-inflammatory cytokines, TNF-\(\alpha\), and the factors regulating pro-inflammatory genes such as nuclear factor \(\kappa B\). Curcumin also has an effect on the factors that regulate apoptosis such as caspases and Bcl-2 family\textsuperscript{49}. Curcumin also
has an anti-angiogenic effect as it decreases the expression of Vascular Endothelial Growth Factor (VEGF)\textsuperscript{50}. Several studies have shown the anti-cancer effect of curcumin in different forms of cancer. The following table summarizes the anti-cancer activity of curcumin in pre-clinical studies.

**Table 1.1:** Anti-cancer effect of curcumin on different cancer cell lines

<table>
<thead>
<tr>
<th>Cell/Cancer Type</th>
<th>Cell Cycle Site of Action</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>LoVo Colon Carcinoma cells</td>
<td>S, G2/M Phase</td>
<td>Cell cycle arrest leading to apoptosis\textsuperscript{51}</td>
</tr>
<tr>
<td>Mammary epithelial carcinoma cells</td>
<td>G2 Phase</td>
<td>Increased p53 expression\textsuperscript{52}</td>
</tr>
<tr>
<td>Human basal cell carcinoma cells</td>
<td>G1 phase</td>
<td>Increased p53 expression\textsuperscript{53}</td>
</tr>
<tr>
<td>HUVEC Human umbilical vein endothelial cell</td>
<td>S Phase</td>
<td>Inhibition of DNA synthesis\textsuperscript{54}</td>
</tr>
<tr>
<td>Ho-8910 Human ovarian cancer cell line</td>
<td>G0/G1 phase</td>
<td>Decrease in expression of Bcl-2, Bcl-X\textsubscript{L} and pro-caspase-3\textsuperscript{55}</td>
</tr>
<tr>
<td>Cell Line</td>
<td>Phase</td>
<td>Effect</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>----------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>LNCaP prostate cancer cell line</td>
<td>S phase</td>
<td>Decreased Bcl-2 expression</td>
</tr>
<tr>
<td>Human multiple myeloma cells</td>
<td>G1/S Phase</td>
<td>Downregulation of NF-κB, Cyclin D1 and Bcl-2</td>
</tr>
<tr>
<td>CA46 Burkitt’s Lymphoma cells</td>
<td>G0/G1 or G2/M and S</td>
<td>Downregulation of NF-κB, Conformational changes in Bax protein</td>
</tr>
</tbody>
</table>

### 1.3.4.2. Other actions

Curcumin exhibits anti-inflammatory effects by inhibiting the enzymes cyclooxygenase 2 (COX-2) and liopoxygenase (LOX). These enzymes are responsible for the production of inflammatory prostaglandins, leukotrienes and thromboxanes. Curcumin is a potent anti-oxidant and acts as a free radical scavenger. It can significantly inhibit reactive oxygen species (ROS) such as superoxide anions, nitrite radicals and peroxides, which can cause oxidative stress and cellular damage. Curcumin also assists in tissue repair which has been the basis of its use for wound-healing.
1.4. Resveratrol

Resveratrol is a polyphenolic phytoalexin which was first isolated from the roots of white hellebore: *Veratrum glandiflorum* O. Loes (Family: Melinthiaceae). It has been identified as an important constituent in various food sources such as skin of red grapes (in red wine), peanuts and mulberries.

1.4.1. Physicochemical Properties

![Chemical structure of trans-Resveratrol](image-url)
The chemical name of resveratrol is trans-3,5,4′-trihydroxystilbene. Resveratrol exists as a diastereomer, in trans-(E) and cis-(Z) isomeric forms. Resveratrol has a molecular weight of 228.24 g/mol and a melting point of 264°C. It is a hydrophobic compound with a logP value of 3.1. It has a very poor water solubility of 0.03 mg/mL. It is soluble in organic solvents such as acetone, ethanol, methanol and DMSO. Resveratrol is categorized in BCS Class II compounds, with high permeability and low solubility. The resveratrol molecule has 3 phenolic protons that can dissociate. The pKa values for these protons have been experimentally determined to be 8.8, 9.8 and 11.4. The trans form of resveratrol is the more biologically active form.

Stability experiments have shown that the trans-resveratrol form is stable for months when protected from light; except in high pH buffers. On the other hand, cis-resveratrol was found to be stable only near neutral pH with complete light protection. Trans-resveratrol was found to be stable in acidic pH but degrades exponentially above pH 6.8. It is rapidly degraded at pH 10 with a half-life of only 1.6 hours. Trans-resveratrol has been shown to degrade at elevated temperatures. It was also found to degrade at a temperature of 4°C as compared at 30°C for a period of 30 days. The possible reason for this could be the oxidative degradation due to the higher solubility of oxygen at lower temperatures. As with pH and temperature, light also affects the
stability of resveratrol. Trans-resveratrol is extremely photosensitive and 
isomerizes to the cis form on exposure to UV or visible light\textsuperscript{69}.

![Isomerization of (E)-resveratrol to (Z)-resveratrol](image)

**Figure 1.7:** Isomerization of (E)-resveratrol to (Z)-resveratrol

### 1.4.2. Pharmacokinetics and Metabolism of Resveratrol

Resveratrol was found to have a low bioavailability; which was largely 
independent of the dose administered. Glucuronide and sulfate were the 
primary conjugates, and sulfate metabolite being the predominant\textsuperscript{70}. The 
metabolism of resveratrol is a fast process and the conjugates are excreted in 
urine\textsuperscript{71}. The conjugated form of resveratrol is predominant as compared to the 
free form, in the blood\textsuperscript{72}.

![Metabolism of Resveratrol](image)

**Figure 1.8:** Metabolism of Resveratrol\textsuperscript{73}
1.4.3. Safety

Resveratrol was found to be well tolerated and non-toxic when given in high (up to 750 mg/kg/day) and low doses to rats and rabbits\(^7^4\). In humans, it is considered to be a safe drug with no marked toxicity. In a multiple dose study of 25, 50, 100, 150 mg of resveratrol, every 4 hour for 48 hours, the only adverse effects observed were headache and dizziness\(^7^5\).

1.4.4. Pharmacology of Resveratrol

Resveratrol is a potent anti-oxidant agent. It also acts as an anti-cancer agent. Some of its other actions include anti-inflammatory, neuro and cardio protective, and DNA repair\(^7^6\).

1.4.4.1. Anti-cancer actions

Resveratrol has multiple intracellular targets that affect cell growth, inflammation, apoptosis, angiogenesis and metastasis\(^7^7\). Resveratrol has been shown to modulate cell cycle mediators causing cell cycle arrest at G1/S phase. It downregulates cyclins D1, D2 and E; CDKs 2,4,6 and induces inhibitor proteins p21WAF1 and p27KIP1\(^7^8\). It also inhibits the cell cycle at S and G2/M phases by inhibition of CDK 7. Resveratrol upregulates the tumor suppressor p53 protein, induces the transcription of p53 by activating the specific genes. It also causes the downregulation of Bcl-2\(^7^7\). Resveratrol
inhibits ribonucleotide reductase and DNA polymerase, thereby directly inhibiting DNA synthesis\textsuperscript{79}.

The apoptosis mediated by resveratrol has been mainly associated with p53 expression. Resveratrol also activates the death receptor Fas/CD95/APO-1. This receptor activates a death signaling cascade after binding to corresponding ligands\textsuperscript{80}. Matrix metalloproteinases (MMP) are the enzymes that allow for metastasis by proteolytic degradation of extracellular matrix. Resveratrol controls metastasis by reducing the expression of MMP-2 and MMP-9\textsuperscript{81}. Resveratrol also controls angiogenesis by suppressing VEGF\textsuperscript{77}. NF-κB is a transcription factor playing an important role in tumorigenesis. It is also activated by certain oncogenes. Resveratrol can inhibit the activation of NF-κB\textsuperscript{82}. Resveratrol also suppress the DNA binding ability of the transcription factor AP-1\textsuperscript{83}.

The following table summarizes the effect of resveratrol on human cancer cells:
### Table 1.2: Studies showing the anti-cancer effect of resveratrol on cancer cell lines

<table>
<thead>
<tr>
<th>Cancer Type and Cell Line</th>
<th>Molecular Target</th>
<th>Cellular Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast Cancer MCF-7</td>
<td>Bcl-2 downregulation, NF-κB inhibition</td>
<td>Apoptosis&lt;sup&gt;84&lt;/sup&gt;</td>
</tr>
<tr>
<td>Prostate Cancer PC-3, LNCaP</td>
<td>Bak, Bad upregulation Cyclins and CDKs</td>
<td>Apoptosis, G0/G1 arrest&lt;sup&gt;85&lt;/sup&gt;,&lt;sup&gt;86&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pancreatic Cancer CD18, S2-013</td>
<td>MIC-1 (Macrophage inhibitory cytokine-1) NF-κB</td>
<td>Apoptosis, Cell growth&lt;sup&gt;87&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon Cancer HT-29</td>
<td>AMPK, ROS, cyclins</td>
<td>Apoptosis, G2 arrest&lt;sup&gt;88&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thyroid Cancer PTC, FTC</td>
<td>p53, p21 upregulation</td>
<td>Apoptosis&lt;sup&gt;89&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acute myeloid leukemia OCIM2, OCI/AML3</td>
<td>NF-κB, IL-1β</td>
<td>S-phase arrest, apoptosis&lt;sup&gt;90&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatoma HepG2</td>
<td>ROS, NF-κB</td>
<td>Apoptosis, cell growth&lt;sup&gt;91&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
1.4.4.2. **Other actions**

Resveratrol is both a free radical scavenger and a potent antioxidant because of its ability to promote the activities of a variety of antioxidant enzymes. It is an inhibitor of reactive oxygen and nitrogen species. Resveratrol also increases the amounts of several antioxidant enzymes, including glutathione peroxidase, glutathione S-transferase and glutathione reductase\(^92\). It also has anti-inflammatory properties and it directly inhibits COX-2 activity and plays an important role in regulation of inflammation in cancer\(^77\). Resveratrol has also been reported to have a cardioprotective action. It was found to modulate vascular cell function, inhibit LDL oxidation, suppress platelet aggregation and reduce myocardial damage\(^93\).
Figure 1.9: Mechanism of action of resveratrol in apoptosis\textsuperscript{94}

1.5. Synergism between curcumin and resveratrol

Curcumin and resveratrol use a similar mechanism of action in their anti-cancer, anti-oxidant and anti-inflammatory effects. In a study comparing the anti-oxidant effects of curcumin, resveratrol and their combination, it was found that the anti-oxidant effect of the combination was about 15% greater than the average of the activities of the individual drugs\textsuperscript{95}. Curcumin and resveratrol were shown to have synergism in their anti-cancer actions as well.
The combination of curcumin and resveratrol was found to be more effective in inhibiting growth of p53-positive and p53-negative colon cancer HCT-116 cells in vitro and in vivo in SCID xenografts of colon cancer HCT-116 cells than either agent alone. Both drugs were found to attenuate NF-κB in this study. Both drugs have been found to be effective as a combination in treatment of hematological malignancies. In a study by Malhotra et al., lung cancer was induced in mice using benzo(a)pyrene exposure. Treatment with individual drugs showed statistically insignificant micronuclei reduction. However, when given in combination, the two drugs produced a reduced number of micronuclei which was statistically significant. The combination of curcumin and resveratrol was also found to modulate the drug metabolizing enzymes such as the cytochrome enzymes as well as the antioxidant enzymes such as superoxide dismutase, glutathione reductase during lung carcinogenesis in mice. Combination of curcumin and resveratrol was more effective in inhibiting in vivo and in vitro cancer growth than the treatment with curcumin alone in head and neck carcinomas. Synergistic effects of curcumin and resveratrol have also been observed in Hepa1-6 hepatocellular carcinoma cells by caspase activation. Liposomal encapsulation of curcumin and resveratrol was found to reduce the incidence of prostate cancer in PTEN knockout mice.
Thus, there are ample *in vitro* and *in vivo* evidences of the effectiveness of using curcumin and resveratrol as a combination therapy for anti-cancer treatment.

**Figure 1.10:** Synergistic effects of curcumin and resveratrol in cancer

1.6. **Nanoparticles as a drug delivery system**

Novel drug delivery systems in the size range of nanometers can be developed to deliver of chemotherapeutic drugs for treatment of cancers. Nano-formulations can encompass a variety of systems such as polymeric
nanoparticles, nanocapsules, liposomes, nano-conjugates and dendrimers.

1.6.1. Advantages of nanoparticles

The size of this drug delivery system offers several advantages for the delivery of drugs. Due to their smaller size, the nanoparticles have a potential of deep penetration into the tissues via the narrow capillaries. Their small size also allows for better uptake by the cells\textsuperscript{103}. By using appropriate polymer, the desired control over drug release can be attained. This can aid in sustaining the effect of drug achieving time dependent control of drug release. A sustained release drug delivery system helps to reduce the drug dose and the dosage frequency. This would eventually help to alleviate the adverse effects associated with frequent and high doses of drugs.

Nanoparticles can also be utilized to achieve spatial control over drug delivery. Targeting of the nanoparticles can be especially useful in cancer treatment as it helps to achieve minimal adverse effects of the chemotherapeutic drugs. Nanoparticles can be targeted to the tumor cells in a passive or an active manner. The growing tumor cells require oxygen supply and thus, angiogenesis is initiated. The blood vessels in the tumor tissue are generally characterized by abnormalities such as high proportion of proliferating endothelial cells, pericyte deficiency and aberrant basement
membrane. As a result, the blood vessels are more permeable. Nanoparticles of the size range from 5-200 nm can extravasate and accumulate into the interstitial space due to this ‘leaky vasculature’\textsuperscript{104}. Additionally, the tumor tissue has poor lymphatic drainage. Due to the poorly developed lymphatic system, the nanoparticles that enter the tumor environment cannot be easily removed and they get ‘retained’ in the tissue\textsuperscript{105}. This twin effect that exploits the tumor tissue anatomy is called the Enhanced Permeation and Retention (EPR) effect. By utilizing the EPR effect, drug loaded nanoparticles can be passively targeted to the tumor. The tumor area differs from normal tissues in certain properties, such as temperature and/or pH and usually demonstrate some acidosis and hyperthermia. Thus physical targeting can be achieved by developing stimuli responsive carriers that can disintegrate at lower pH values or higher temperatures\textsuperscript{104}. Active targeting involves designing the system so as to target a particular antigen or a marker that is expressed only in the tumor cells. For this purpose, the nanoparticles are conjugated with a ligand such as a peptide or any such molecule that is a ligand for a receptor on the tumor cell. The targeting of a specific ligand in the tumor cells helps achieve a high level of specificity\textsuperscript{106}. Monoclonal antibodies, aptamers (modified DNA/RNA oligonucleotides), folic acid functionalized nanoparticles for folate receptors on tumor cells are some examples of active targeting moieties\textsuperscript{107}. 
Figure 1.11: Differences between the anatomies of normal tissue versus tumor tissue. EPR effect seen with the nanoparticles (in green) in case of tumor tissue\textsuperscript{108}

1.6.2. Preparation methods of nanoparticles

Nanoparticles can be prepared by multiple methods. The method used for preparation depends on the physical properties of the raw materials, that is, the polymer and the drug. Specialized nanoparticles aimed at targeting are prepared by modification in any of the general methods of preparation which are as follows\textsuperscript{109,110}:

A. Emulsification Solvent Evaporation: This is one of the simplest and the most commonly used two-step method for preparation of nanoparticles. In this method, first, aqueous and organic phases are
made separately. The drug and polymer, are added to their respective phases depending on their aqueous solubility. In the second step organic phase is evaporated to induce polymer precipitation and obtain polymeric nanoparticles. The nanoparticles can then be washed and collected using ultracentrifugation. A primary (o/w) or (w/o) emulsion is formed with the aid of an appropriate emulsifier. High speed homogenizer or ultrasonication can be used for the emulsification process. Primary (o/w) emulsion method is generally used for entrapment of hydrophobic drugs.

**B. Double emulsification and evaporation:** Hydrophilic drugs are poorly entrapped by the emulsification and solvent evaporation method. In a modification of that method, a (w/o) emulsion is first formed with the hydrophilic drug. This emulsion is emulsified again with an aqueous phase to form a (w/o/w) emulsion. The organic solvent is subsequently evaporated and the nanoparticles are collected.

**C. Salting-out method:** Salting-out involves separation of a water miscible solvent using a salting-out agent such as an electrolyte. The polymer and the drug are dissolved in a solvent which is then emulsified into an aqueous gel that contains the electrolyte such as calcium chloride. When this (o/w) emulsion is diluted with more aqueous phase,
the solvent diffuses into the aqueous phase forming nanoparticles. This method is largely restricted to lipophilic drugs.

D. Emulsification-diffusion method: In this method, the polymer is dissolved in a partially miscible solvent and then equilibrated with water. Then, this polymer-water saturated solvent phase is emulsified with an aqueous solution that contains an emulsifier. At this point, the solvent diffuses into the external aqueous phase leading to the formation of nanoparticles. The solvent is then removed by evaporation.

E. Nanoprecipitation: Nanoprecipitation involves the precipitation of a polymer from an organic solvent and the diffusion of that organic solvent into aqueous medium. A surfactant may or may not be required in this method. A semi-polar water miscible solvent is used for solubilizing polymer and/or drug which is then emulsified in an aqueous phase. Rapid solvent diffusion leads to the formation of nanoparticles. The solvent is later removed.

1.7. Calcium Alginate as a polymer of drug delivery

Alginites are a group of polyanionic polymers that are obtained from the marine brown algae. It has been frequently used as a thickening and a stabilizing agent in food industry for products such as jellies, ice creams and
other desserts. It has also been used in paper and paint industries. Calcium alginate has been explored as a biodegradable matrix forming polymer for drug delivery by a number of research groups.

1.7.1. Source and structure

Alginates can be obtained from algal or bacterial sources. However, brown algae are the most common source for the commercially available alginates. *Ascophyllum nodosum* and *Laminaria hyperborea* are the prominent algae used to obtain alginates. The naturally occurring material is subjected to alkaline extraction. The resultant viscous mass is treated with mineral acids to obtain free alginic acid. It is then converted to a salt form such as sodium alginate.

Alginates are composed of two basic sugar residues: α-L-guluronic acid and β-D-mannuronic acid, designated as the G and M residues respectively. The G and M residues are linked to each other by 1-4 glycosidic linkages. The polymer constitutes of three types of segments: the GG segment of continuous G residues, the MM segment composed of continuous M residues and the MG segment composed of interspersed M and G residues. The arrangement and proportions of the M and G residue depends on the source of alginate and determines the physical strength and viscosity of the alginate polymer.
1.7.2. Physicochemical Properties and Cross-linking

The molecular weight of alginate polymer can range from 32000 to 400,000 g/mol. In case of solubility of sodium alginate polymer, the proportions of G and M residues influence the solubility. Higher proportion of guluronic acid residues affords a more water soluble polymer. The viscosity of the alginate solutions is directly proportional to the number of monomer units, and thus, the molecular weight of the polymer.

While monovalent ions such as sodium form salts with alginates, divalent ions such as calcium and magnesium form gels by ionotropic gelation. Alginates thus have the ability to undergo sol-gel transformation in presence
of divalent ions, a property of importance in development of drug delivery systems. The gelation of alginates can be external, internal or by cooling. In the external gelation, alginate solution is added to the cross-linking solution by certain means such as atomization. In the internal gelation process, an insoluble divalent ion salt such as calcium chloride is added to the alginate solution and the gel is formed upon liberation of calcium ions. In the cooling method, both the alginate and cross-linking salt are dissolved at a high temperature of about 90°C and then cooled slowly. It allows for the formation of an ordered tertiary structure and a homogenous matrix\textsuperscript{113}.

![Diagram of ionotropic gelation of alginate and the 'egg-box model']

**Figure 1.13:** Ionotropic gelation of alginate and the ‘egg-box model’\textsuperscript{117}
The cross-linking by the multi-valent ions that occurs with the guluronate residues has been termed as the ‘egg-box’ structure. In this model, pairs of helical chains are packed with the calcium ions located between them\textsuperscript{118}. Different ions have different affinity for either G (divalent) or M residues (trivalent). The strength of cross-linking is also affected by the ionic radii of the divalent ions, with barium producing stronger alginate pellets. Since calcium salts are more commonly used, the focus would be on calcium ion cross-linking. The chelation of the G residues by calcium ions results in ionic interaction between the guluronic acid residues. The van der Waals forces between the alginate segments result in three-dimensional gel network. The extent of cross-linking depends upon the composition of alginate. Alginate with higher guluronate composition have a higher affinity for calcium ions and this facilitates gel formation with more rigid mechanical properties. Alginates with higher mannuronic acid content form more elastic gels\textsuperscript{119}. The ionotropic gelation of alginates is a reversible process. The calcium alginate matrices can be destabilized in a citrate or phosphate buffer solution of pH 7\textsuperscript{120}.

Alginates are categorized as GRAS by the FDA. It is a naturally occurring polymer and has a biodegradable nature\textsuperscript{121}. Alginate purified by a multi-step extraction procedure to a very high purity was not found to induce any
significant foreign body reaction when implanted into animals. On subcutaneous injection of gels into mice, no significant inflammatory response was observed.  

1.7.3. Calcium alginate nanoparticles

Many research groups have used calcium alginate as the polymer for designing nanoparticles for drug delivery. Ping Li et al. developed calcium alginate-chitosan nanoparticles for the delivery of the hydrophobic drug nifedipine. Calcium alginate-chitosan-pluronic nanocomposites have also been used for the drug delivery of curcumin to cancer cells. Nesamony et al. developed calcium alginate nanoparticles for the potential delivery of proteins. Zahoor et al. developed inhalable calcium alginate nanoparticles for the delivery of anti-tuberculosis drugs in experimental tuberculosis. Ionotropic pre-gelation of an alginate core followed by chitosan polyelectrolyte complexation was employed to develop nanoparticles for oral delivery of insulin.

1.8. Objective, hypothesis and specific aims

The objective of this study was to develop and characterize a calcium alginate nanoparticulate delivery system loaded with curcumin and resveratrol intended for an enhanced cytotoxic effect on prostate cancer cells.
The underlying hypothesis of the study was:

Two hydrophobic anti-cancer agents- curcumin and resveratrol can be simultaneously incorporated into a hydrophilic calcium alginate nanoparticulate system for enhanced anti-cancer effect in prostate cancer cells.

The specific aims for this investigation were:

1. Development and validation of an UPLC method for the simultaneous analysis of curcumin and resveratrol.
2. Preparation and characterization of curcumin and resveratrol loaded calcium alginate nanoparticles.
3. *In vitro* testing of the calcium alginate nanoparticles on DU145 prostate cancer cell lines.
CHAPTER 2

Development and validation of an UPLC method for the simultaneous analysis of curcumin and resveratrol
2.1. Introduction

Curcumin is obtained from the rhizomes turmeric *Curcuma longa*. It is the most abundant analogue (77%), followed by demethoxycurcumin (17%) and bisdemethoxycurcumin (3%)\(^{128}\). It has been shown to have anti-cancer action. However, its efficacy is often limited due to its poor aqueous solubility. Various analytical methods have been developed for the quantification of curcumin\(^{129-141}\). These range from using spectrophotometry and chromatography to the modern sensitive methods such as mass spectrometry. K. R. Srinivasan developed a liquid chromatography method for the analysis of the curcuminoids in 1953\(^{129}\). Spectrophotometry in the visible light range has also been used frequently for curcumin analysis\(^{130,131}\). The fluorescence properties of curcumin have been used to develop a fluorimetric assay for the drug\(^{132}\). Amongst the planar chromatographic techniques, Thin Layer Chromatography (TLC)\(^{133}\) and High Performance Thin Layer Chromatography (HPTLC)\(^{134,135}\) have also been used for curcumin analysis. In column chromatography, several High Pressure Liquid Chromatography (HPLC) methods for the separation and quantification of curcumin have been used\(^{136,137,138}\). Song et al. developed a supercritical fluid chromatographic method for the separation and analysis of the three curcuminoids\(^{139}\). Sensitive
LC-MS/MS methods have also been developed for the quantification of very low concentrations of curcumin\textsuperscript{140,141}.

Resveratrol is a phytoalexin obtained from \textit{Veratrum glandiflorum} that has anti-cancer actions\textsuperscript{77-83}. Like curcumin, resveratrol also has poor aqueous solubility. Spectrophotometry in the UV range has been utilized for the analysis of resveratrol\textsuperscript{142}. HPLC methods for resveratrol quantification have also been developed by some groups\textsuperscript{143,144,145}. LC-MS/MS has also been used for a sensitive quantification of resveratrol in wines and juices\textsuperscript{146,147}.

Since, the formulation is intended for the combination drug delivery of curcumin and resveratrol, it was important to have an analytical method that could quantify both the drugs simultaneously. Narayanan et al. used a HPLC method for the simultaneous analysis of curcumin and resveratrol in a liposomal formulation\textsuperscript{148}. Coradini et al. also used a HPLC method for the simultaneous determination of resveratrol and curcumin from lipid core nanocapsules\textsuperscript{149}.

UV-visible spectrophotometry is a simple analytical technique but has poor sensitivity. On the other hand, although mass spectrometry is very sensitive, it is also an expensive technique. Column chromatography is the best suited technique for achieving a balance between ease of use and efficiency. Ultra-
Pressure Liquid Chromatography (UPLC) provides distinct advantages over HPLC. Although HPLC is convenient to use, it has some disadvantages such as longer retention times, poor resolution and mobile phase preparation difficulties in gradient flow. With UPLC less time consuming protocols can be developed. It has an ability to withstand high system back-pressures and stationary phase with a low particle size (< 2µm) can be used providing a greater surface area for analyte separation. UPLC has also been shown to have better resolution and higher specificity\textsuperscript{150,151}. It is also an economical instrument with respect to the consumption of mobile phase solvents.

The objective of this study was to develop and validate an UPLC method for the separation and simultaneous analysis of curcumin and resveratrol.

2.2. **Materials**

Commercial grade curcumin was purchased from Sigma Aldrich (St. Louis, MO). Commercial grade resveratrol was purchased from Cayman Chemicals (Ann Arbor, MI). Optima grade acetonitrile and water and o-phosphoric acid was purchased from Fischer Scientific (Fair Lawn, NJ). The Acquity HSS-T3 C18 column was obtained from Waters (Milford, MA).

2.3. **Methods**
2.3.1. Chromatography

The UPLC analysis was performed using Waters Acquity UPLC system (Milford, MA). The instrument was equipped with a quaternary solvent pump for delivery of mobile phase, an autosampler and a photodiode array detector. The Acquity HSS-T3 C18 column (1.8 µm, 2.1x50 mm) was used for the chromatographic separation of curcumin and resveratrol. The column temperature was maintained at 30°C. Gradient elution was used for the separation of resveratrol and curcumin and the other curcuminoids. The mobile phase consisted of 0.05% o-phosphoric acid and acetonitrile in a ratio of 66:34 (v:v), gradient to 70:30 (v:v) over a run time of 6 minutes. The apparent pH of the mobile phase was 2.5. The flow rate was maintained at 0.8 mL/minute. Wavelengths for the detection of curcumin and resveratrol were 425 and 320 nm, respectively. The injection volume used was 5µL.

2.3.2. Preparation of solutions

2.3.2.1. Preparation of mobile phase

The aqueous portion of the mobile phase was prepared by adding 0.5 mL o-phosphoric acid to 1 liter of deionized water. This 0.05% (v/v) solution of o-phosphoric acid was then filtered through 0.2 µm filter. The aqueous phase and the organic phase (acetonitrile) were pumped separately into the UPLC
by the quaternary solvent manager. The apparent pH of the mobile phase was 2.5.

2.3.2.2. Preparation of standard solutions

For running the standard curve, a series of standard solutions were prepared starting from a stock solution of 500 µg/mL. The stock solution was prepared by dissolving 5 mg each of curcumin and resveratrol in acetonitrile, in a 10 mL volumetric flask. Serial dilutions were made using the mobile phase. A concentration range of 1.9-250 µg/mL was used and two standard curves in were constructed.

2.4. Calculations

The standard curve was obtained by plotting the peak area of the standards on Y-coordinate against their concentrations on the X-coordinate. The concentration of the unknown samples was determined by interpolation using the equation of the line of the standard curves.

2.5. Results and Discussion

2.5.1. Specificity

According to the USP, specificity is the ability of the analytical method to unequivocally assess the analyte in presence of other components that may be expected to be present which includes impurities, formulation components
and degradation products. The method was tested for specificity by comparing the chromatograms obtained by injecting a mobile phase sample without the drugs and a mobile phase sample containing the drugs. This comparison was carried out for both 425 nm and 320 nm channels. The representative chromatogram at 425 nm for the mobile phase without drugs (blank) and with curcumin is shown in figure 1 (a) and (b) respectively. The UPLC method was found to separate the curcuminoid peaks with the main curcumin peak having a retention time of 4.9 minutes. The identity of the curcumin peak was confirmed by running a USP reference standard for pure curcumin. No interference from the mobile phase peak was seen in the curcumin peak. The representative chromatogram at 320 nm for the mobile phase without drugs and with resveratrol is shown in figure 2 (a) and 2 (b) respectively. Resveratrol was found to have a retention time of 0.5 minutes and there was no interference from the mobile phase peak with the resveratrol peak. Thus, the UPLC method developed was found to be specific for both curcumin and resveratrol.
Figure 2.1: Representative chromatogram at 425 nm for (a) blank and (b) curcumin at 4.9 minutes retention time
Figure 2.2: Representative chromatograms at 320 nm for (a) blank and (b) resveratrol at 0.5 minutes retention time
2.5.2. Linearity

The USP defines linearity of an analytical procedure as its ability to produce results that are directly, or by well-defined mathematical transformation, are proportional to the concentration of analyte within a given range\textsuperscript{152}. In the context of this UPLC method, linearity refers to the direct proportionality between the peak area or peak height and the concentration of the drugs. Linearity was assessed by injecting a series of curcumin and resveratrol standards and plotting the calibration curves of peak area against the concentration. Linearity was ascertained by determining the slope of the standard curve, the $y$-intercept and the Spearman’s rank coefficient ($r^2$) value. The standard curve was plotted for both the drugs in a high and low concentration ranges. The standard curves for curcumin and resveratrol were found to be linear in the concentration ranges of 15.62-250 $\mu$g/mL and 1.9-31.25 $\mu$g/mL. Table 2.1 depicts the linear equations and the Spearman’s rank coefficient ($r^2$) values for curcumin and resveratrol standards for both low and high concentration ranges:
Table 2.1: Linearity for curcumin and resveratrol

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration range</th>
<th>Linear Equation for the standard curve</th>
<th>r² value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>15.62-250 µg/mL</td>
<td>y = 33461x + 70571</td>
<td>0.9999</td>
</tr>
<tr>
<td></td>
<td>1.9-31.25 µg/mL</td>
<td>y = 34880x + 4547.2</td>
<td>1.00</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>15.62-250 µg/mL</td>
<td>y = 43979x + 42278</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>1.9-31.25 µg/mL</td>
<td>y = 44686x + 6887.7</td>
<td>0.9995</td>
</tr>
</tbody>
</table>

A high r² value suggests a strong relationship between the peak area and the concentration. An r² value of > 0.99 is an acceptable measure of the linearity of an analytical procedure. All the r² values for both curcumin and resveratrol in both concentration ranges were > 0.99. Therefore, UPLC method was found to be linear over that concentration ranges. The figure 3 (a) and (b) depicts the standard curves for curcumin, while the figure 4 (a) and (b) depicts the standard curves for resveratrol.
Figure 2.3: Standard curve for curcumin at (a) 15.62-250 µg/mL and (b) 1.95-31.25 µg/mL at 425 nm
Figure 2.4: Standard curve for curcumin at (a) 15.62-250 µg/mL and (b) 1.9-31.25 µg/mL at 320 nm
2.5.3. Precision

The USP defines the precision of an analytical procedure as the degree of agreement among individual test results, when the procedure is applied repeatedly to multiple samplings of a homogenous sample\textsuperscript{152}. The closer the values are for a particular sample after multiple measurements under similar experimental conditions, higher is the precision. The current UPLC method was tested for precision using intra-day as well as inter-day studies. For the intra-day study, a set of standard solutions was made from one stock solution and injected four times within a period of 24 hours. The inter-day precision was studied with six sets of standard solutions performed on different days over a period of one month. The intra-day and inter-day precision of the method was determined by calculating the percent relative standard deviation (\% RSD) for the peak areas of each standard. The percent RSD for all the standards was found to be < 10\% and thus, within the required precision limits as specified by the USP\textsuperscript{152}. The UPLC method was found to be precise for both curcumin and resveratrol. Table 2.2 represents precision study results for curcumin and table 2.3 represents precision study results for resveratrol:
**Table 2.2:** Intra-day and inter-day precision found in quantification of curcumin by UPLC

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Intra-day</th>
<th></th>
<th>Inter-day</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Peak Area (x 10^3)</td>
<td>%RSD</td>
<td>Mean Peak Area (x 10^3)</td>
<td>%RSD</td>
</tr>
<tr>
<td>1.95</td>
<td>79.2 ± 2.4</td>
<td>3.10</td>
<td>75.6 ± 4.4</td>
<td>5.91</td>
</tr>
<tr>
<td>3.90</td>
<td>151.3 ± 4.3</td>
<td>2.85</td>
<td>149.5 ± 8.9</td>
<td>6.01</td>
</tr>
<tr>
<td>7.81</td>
<td>305.4 ± 2.6</td>
<td>0.87</td>
<td>299.9 ± 16.9</td>
<td>5.65</td>
</tr>
<tr>
<td>15.625</td>
<td>604.0 ± 9.7</td>
<td>1.61</td>
<td>589.0 ± 37.2</td>
<td>6.33</td>
</tr>
<tr>
<td>31.25</td>
<td>1199.4 ± 22.6</td>
<td>1.89</td>
<td>1169.9 ± 82.9</td>
<td>7.09</td>
</tr>
<tr>
<td>62.5</td>
<td>2389.5 ± 44.3</td>
<td>1.86</td>
<td>2348.6 ± 143.5</td>
<td>6.11</td>
</tr>
<tr>
<td>125</td>
<td>4737.6 ± 73.7</td>
<td>1.56</td>
<td>4694.8 ± 288.7</td>
<td>6.15</td>
</tr>
<tr>
<td>250</td>
<td>9316.9 ± 131.8</td>
<td>1.41</td>
<td>9183.4 ± 659.8</td>
<td>7.18</td>
</tr>
</tbody>
</table>
Table 2.3: Intra-day and inter-day precision found in quantification of resveratrol by UPLC

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Intra-day</th>
<th></th>
<th>Inter-day</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Peak Area (x 10^3)</td>
<td>%RSD</td>
<td>Mean Peak Area (x 10^3)</td>
<td>%RSD</td>
</tr>
<tr>
<td>1.95</td>
<td>84.0 ± 2.5</td>
<td>2.98</td>
<td>87.7 ± 4.3</td>
<td>5.02</td>
</tr>
<tr>
<td>3.90</td>
<td>155.6 ± 3.4</td>
<td>2.19</td>
<td>171.5 ± 10.9</td>
<td>6.40</td>
</tr>
<tr>
<td>7.81</td>
<td>310.2 ± 2.1</td>
<td>0.69</td>
<td>342.9 ± 20.9</td>
<td>6.11</td>
</tr>
<tr>
<td>15.625</td>
<td>614.2 ± 8.0</td>
<td>1.31</td>
<td>669.8 ± 38.7</td>
<td>5.79</td>
</tr>
<tr>
<td>31.25</td>
<td>1225.4 ± 18.1</td>
<td>1.48</td>
<td>1329.2 ± 55.7</td>
<td>4.20</td>
</tr>
<tr>
<td>62.5</td>
<td>2450.7 ± 41.5</td>
<td>1.69</td>
<td>2671.8 ± 133.3</td>
<td>4.99</td>
</tr>
<tr>
<td>125</td>
<td>4878.8 ± 43.2</td>
<td>0.89</td>
<td>5319.1 ± 277.8</td>
<td>5.22</td>
</tr>
<tr>
<td>250</td>
<td>9673.9 ± 65.8</td>
<td>0.68</td>
<td>10459.5 ± 487.8</td>
<td>4.66</td>
</tr>
</tbody>
</table>
2.5.4. Accuracy

The USP defines accuracy as the closeness of test results obtained by that procedure to the true value\textsuperscript{152}. The accuracy of the UPLC method was determined by injecting three quality control samples in the low, medium and high concentration ranges for curcumin and resveratrol. This was performed five times over a period of one month. The concentrations selected for accuracy were 3.9 µg/mL, 50 µg/mL and 200 µg/mL. The experimental value was then compared with the theoretical value to determine the accuracy. The following formula was used for determination of percent accuracy:

\[
\text{Percent Accuracy} = \frac{\text{Measured Concentration}}{\text{Theoretical Concentration}} \times 100
\]

The percent accuracy was found to be within 10\% standard deviation for curcumin and resveratrol for all three quality control samples. The UPLC method was thus found to be accurate. Table 2.4 and 2.5 represent the accuracy study results for curcumin and resveratrol, respectively:
**Table 2.4:** Accuracy results for curcumin using the UPLC method

<table>
<thead>
<tr>
<th>Theoretical Concentration (µg/mL)</th>
<th>Measured concentration (µg/mL)</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9</td>
<td>3.81 ± 0.08</td>
<td>97.82 ± 2.15</td>
</tr>
<tr>
<td>50</td>
<td>48.73 ± 4.40</td>
<td>97.47 ± 9.03</td>
</tr>
<tr>
<td>200</td>
<td>196.37 ± 8.27</td>
<td>98.18 ± 4.21</td>
</tr>
</tbody>
</table>

**Table 2.5:** Accuracy results for resveratrol using the UPLC method

<table>
<thead>
<tr>
<th>Theoretical Concentration (µg/mL)</th>
<th>Measured concentration (µg/mL)</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9</td>
<td>3.86 ± 0.10</td>
<td>98.97 ± 2.82</td>
</tr>
<tr>
<td>50</td>
<td>48.55 ± 4.31</td>
<td>97.11 ± 8.88</td>
</tr>
<tr>
<td>200</td>
<td>196.05 ± 7.82</td>
<td>98.02 ± 3.99</td>
</tr>
</tbody>
</table>
2.6. Applications of the UPLC method

2.6.1. Entrapment efficiency studies

The percent entrapment efficiency of a drug is defined as the percentage of the ratio of amount of drug extracted from the formulation to the amount of drug initially added to the formulation. The entrapment efficiency for curcumin and resveratrol loaded calcium alginate nanoparticles was calculated by adding about 10 mg of freeze dried nanoparticles in 10 mL of mobile phase. This was then ultrasonicated for 10 seconds and centrifuged at 400 rpm for 15 minutes. The supernatant was collected, filtered through a 0.2 µm syringe filter and analyzed for drug content using the UPLC method. The following formula was used to calculate the entrapment efficiency:

\[
\% \text{ Entrapment Efficiency} = \frac{\text{Amount of drug extracted from the formulation}}{\text{Amount of drug initially incorporated in the formulation}} \times 100
\]

2.6.2. In vitro release studies

The in vitro release of curcumin and resveratrol from the nanoparticulate formulation was studied using a dialysis membrane (Spectrapor cellulose membrane, 6000-8000 daltons cut-off). The release medium used was 25%
(v/v) isopropyl alcohol in 0.1 M phosphate buffer pH 7.4 containing 0.5% (w/v) Tween 80. The study was also performed using a conventional release medium of 0.1 M phosphate buffer pH 7.4 containing 0.5% w/v Tween 80, in which isopropyl alcohol was absent. A 2 mL suspension of nanoparticles was prepared in the release medium and sealed into the dialysis bag. This bag was then inserted into a falcon tube containing 13 mL of release medium (receiver compartment). Sink conditions were maintained during the entire release study. The tubes were placed in the incubator-shaker at 150 rpm (MaxQ 4450, Thermo Scientific) at 37°C, and covered with a box for light protection. Samples were collected at specific time intervals by removing 200 µL from the receiver compartment and by replacing it with 200 µL fresh release medium. The aliquots were filtered through 0.2 µm syringe filter and analyzed for drug content using the UPLC method.

The results for entrapment efficiency studies and in vitro release studies would be discussed in chapter 3.

2.7. Conclusion

A sensitive analytical method was successfully developed and validated for the simultaneous analysis of curcumin and resveratrol using a reverse phase UPLC technique and a gradient flow. It was found to separate resveratrol from
curcumin as well as resolve the three major curcuminoids. The method was tested for specificity, linearity, precision and accuracy. Although the standard curves for low and high concentration ranges have been plotted separately, it should be noted that the precision and accuracy studies were performed using a standard curve plotted for the entire concentration range of 1.9-250 µg/mL. The results for all the tests were found to be within specified USP limits. A shorter run time of 6 minutes allowed for lower consumption of organic solvent per run. The UPLC method was useful in determining the entrapment efficiency, *in vitro* release and cellular uptake of curcumin and resveratrol.
CHAPTER 3

Preparation, characterization and in vitro evaluation of curcumin and resveratrol loaded calcium alginate nanoparticles
3.1. Introduction

Curcumin is obtained from the rhizomes of turmeric, that is, *Curcuma longa*. This naturally occurring polyphenolic compound has been shown to have multiple chemopreventive and chemotherapeutic actions in various *in vitro*, pre-clinical and clinical studies. It acts by multiple mechanisms that include cell-cycle arrest, modulation of apoptotic pathways such that apoptosis is favored and downregulation of transcription factors and enzymes. Resveratrol is a phytoalexin that is obtained from the *Veratrum glandiflorum*, and it has also been shown to have anti-cancer actions in pre-clinical studies. Resveratrol acts on similar lines as that of curcumin. It causes cell-cycle arrest, upregulates suppressor protein p-53, downregulates matrix metalloproteinase and DNA polymerase enzymes. Curcumin and resveratrol are the drugs that are present in food items such as turmeric and wine respectively and have a high therapeutic index. They are considerably less toxic than the other chemotherapeutic drugs and present an excellent alternative to chemotherapeutic agents that are currently in use.

Although both drugs have potent anti-cancer effects, their clinical use is limited due to their poor bioavailability. This is due to low aqueous solubility of both the drugs. Curcumin has been shown to have an aqueous solubility of 11 ng/mL, while resveratrol has a solubility of 30 µg/mL. Both drugs are...
also labile to photodegradation. Curcumin also shows degradation at neutral and alkaline pH. The problems associated with the delivery of curcumin and resveratrol have been addressed by many research groups with the development of drug delivery systems such as solid lipid nanoparticles, polymeric nanoparticles, cyclodextrin complexation and liposomes among others\(^\text{102,124,149,153}\).

Alginate is a polymer obtained from marine brown algae. It is a biodegradable polymer comprising of $\alpha$-L-guluronic acid and $\beta$-D-mannuronic acid residues. Calcium alginate matrices have been used as a material to prepare polymeric nanoparticles. It is a hydrophilic polymer that can be used for delivery of hydrophobic drugs such as curcumin and resveratrol.

Prostate cancer is the second-leading cause of cancer deaths among men and newer treatment options need to be explored. Curcumin and resveratrol have been indicated to be effective in treating prostate cancer. The objective of the current study was to prepare calcium alginate nanoparticles loaded with curcumin and resveratrol; to characterize the nanoparticles and to evaluate the \textit{in vitro} efficacy of the delivery system on prostate cancer cell line.
3.2. Materials

Commercial grade curcumin was purchased from Sigma Aldrich (St. Louis, MO), while resveratrol was purchased from Cayman Chemicals (Ann Arbor, MI). Sodium alginate (medium viscosity) and calcium chloride were purchased from Sigma Aldrich. Tween 80 was obtained from Fisher Chemicals (Fair Lawn, NJ). Optima grade solvents such as water, acetonitrile, acetone and isopropanol were also purchased from Fisher Chemicals. Maltrin M100 (maltodextrin) was obtained from Grain Processing Corporation (Muscatine, IA).

The DU145 prostate cancer cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA). The Eagle’s Minimum Essential Medium, penicillin-streptomycin, L-glutamine, non-essential amino acids, sodium pyruvate were purchased from CellGro® (Manassas, VA). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Gibco® 0.25% Trypsin-EDTA was purchased from Thermo Fisher Scientific. Fluoroshield™ with DAPI was purchased from Sigma. LysoTracker® Red DND-99 was obtained from Thermo Fisher Scientific. PROTOCOL® 10% Neutral Buffered Formalin was obtained from Fisher Diagnostics (Middletown, VA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. Blood used
for hemolysis study was single donor human whole blood purchased from Innovative™ Research (Novi, MI).

3.3. Methods

3.3.1. Preparation of curcumin and resveratrol loaded calcium alginate nanoparticles

A 0.6 mg/mL solution of medium viscosity sodium alginate was made in deionized water with overnight stirring. One hundred mg Tween 80 was added to 20 mL sodium alginate solution to make a 0.5% (w/v) Tween 80 solution forming the aqueous phase. Five mg each of curcumin and resveratrol were dissolved in 1mL acetone to form the organic phase. The organic phase was mixed with the aqueous phase, and then emulsified using ultrasonication (Misonix Sonicator 3000, Farmingdale, NY) at 42 watts with pulse mode (4 seconds on, 2 seconds off) for 10 minutes to form an (o/w) emulsion. A 0.052% (w/v) solution of calcium chloride was added to the emulsion using a syringe pump under continuous ultrasonication at 42 watts for a period of 15 minutes to form calcium alginate nanoparticles. The nanoparticle suspension was then ultracentrifuged at 20000 rpm for 15 minutes to remove any larger aggregates and impurities. The centrifugal force was optimum such that the nanoparticles remained in the supernatant as a suspension. The nanosuspension was subjected to freeze drying using the Millrock Technology
LD85 freeze dryer (Kingston, NY). 1% (w/v) Maltrin M100 was added prior to freeze drying as a cryoprotectant.

### 3.3.2. Measurement of particle size and zeta potential

The particle size and zeta potential of the nanoparticles was measured before and after freeze drying. The measurements were carried out using Brookhaven Zetameter (ZetaPlus, Brookhaven Instruments Corporation, Holtsville, NY). One hundred microliters of the nanoparticles in suspension was diluted to 10 mL with 0.45 µm filtered water, and the particle size and zeta potential was measured. The particle size and zeta potential measurement for freeze dried nanoparticles was carried out by making a 0.1 mg/mL solution in filtered water. Each sample was measured five times. All measurements were performed in triplicates.

### 3.3.3. Atomic Force Microscopy (AFM) imaging

Aqueous suspensions of blank and drug loaded nanoparticles were prepared in Optima grade water at a concentration of 1 mg/mL. The samples were further diluted 25 times. The samples were deposited on mica for imaging. AFM images in air were acquired using MultiMode AFM NanoScope IV system (Bruker Instruments, Santa Barbara, CA) at the Nanoimaging Core Facility of University of Nebraska Medical Center. The
operation was in tapping mode with 1.5 Hz scanning rate. TESPA probes from Bruker were used for tapping mode imaging.

3.3.4. Determination of moisture content by Karl Fisher Titration

The moisture content of the blank and drug loaded freeze dried nanoparticles was determined using Karl Fisher titration (Mettler DL18 Karl Fischer titrator, NJ, USA). The titration was carried out using about 20-25 mg of sample. The moisture content was determined as a percentage by weight of the nanoparticles. All measurements were performed in triplicates.

3.3.5. Determination of weight loss by Thermogravimetric analysis (TGA)

The weight loss of the blank and drug loaded freeze dried nanoparticles as a function of temperature was determined using the thermogravimetric analyzer (Shimadzu TGA-50, Kyoto, Japan). The assembly was connected to a thermal analysis operating system (Shimadzu TA-60WS, Kyoto, Japan). A known quantity (approximately 10 mg) of the sample was placed in an aluminum pan and heated from room temperature to 300°C at the rate of 10°C/minute in a nitrogen environment (flow rate 20 mL/min). The percent weight loss of the sample was reported (n=3).
3.3.6. Determination of physical state of the drug in the nanoparticles by Differential Scanning Calorimetry (DSC)

The physical state of curcumin and resveratrol in the formulation was analyzed using DSC (Shimadzu DSC-60, Kyoto, Japan) with a thermal analysis operating system (Shimadzu TA-60WS, Kyoto, Japan). About 4-5 mg of sample was weighed in an aluminum pan and crimped which was used as the sample pan. An empty crimped aluminum pan was used as the reference pan. The pans were heated from room temperature to 300°C at the rate of 10°C/minute in a nitrogen environment (flow rate 20 mL/min). The DSC study was performed using pure curcumin, pure resveratrol, blank calcium alginate nanoparticles, and curcumin and resveratrol loaded calcium alginate nanoparticles.

3.3.7. Characterization of physical state by X-ray diffraction (XRD)

The physical state of curcumin and resveratrol in the nanoparticles was also studied using X-ray diffraction. The XRD analysis was performed at University of Nebraska- Lincoln. Blank and drug loaded calcium alginate nanoparticles were analyzed and the scan was compared with the XRD scan of pure curcumin and resveratrol. The data in the 2θ range 5–60 degrees was collected in focusing geometry using PANalytical Empyrean Diffractometer
(Almelo, the Netherlands), exposed to Cu Kα radiation at 40 kV and 45 mA. Thin layer of powder sample was placed on a zero background silicon plate and the sample holder was continuously spun at the rate of 90 deg/s during the measurement. Solid state PIXcel³D detector was scanned at a rate of 0.135 deg/s to collect data.

3.3.8. Testing of nanoparticles for particle size and physical stability

The particle size and zeta potential stability of nanoparticles were measured over a period of 60 days. The measurements were made after 7, 14, 21, 28 and 60 days after preparation, using the Brookhaven Zetameter. The stability of the formulation was also tested by analyzing the physical state of the drugs in the nanoparticles after a period of 60 days by performing DSC and XRD analysis on drug loaded nanoparticles.

3.3.9. UPLC analysis

A reverse phase UPLC method was developed for the simultaneous analysis of curcumin and resveratrol. A gradient elution was used on a Waters Acquity system using an Acquity HSS-T3 C18 column maintained at 30°C. The mobile phase used was 0.05% (v/v) o-phosphoric acid and acetonitrile in a ratio of 66:34, gradient to 70:30 over a run time of 6 minutes. A flow rate of 0.8 mL/min was used. The effluents were monitored using a photodiode array
detector at wavelengths of 425 nm and 320 nm for curcumin and resveratrol, respectively.

3.3.10. Determination of drug entrapment efficiency

The percent entrapment efficiency of a nanoparticulate formulation is the percentage of the ratio of amount of drug extracted from the formulation to the amount of drug initially added to the formulation. The entrapment efficiency for curcumin and resveratrol loaded calcium alginate nanoparticles was calculated by adding about 10 mg of freeze dried nanoparticles in 10 mL of mobile phase. This was ultrasonicated for 10 seconds and centrifuged at 400 rpm for 15 minutes. The supernatant was collected, filtered through a 0.2 µm syringe filter and analyzed for drug content using the UPLC method. The following formula was used to calculate the entrapment efficiency:

\[
\text{% Entrapment Efficiency} = \frac{\text{Amount of drug extracted from the formulation}}{\text{Amount of drug initially incorporated in the formulation}} \times 100
\]

3.3.11. In vitro release

The in vitro release of curcumin and resveratrol was studied using a dialysis membrane (Spectrapor cellulose membrane, 6000-8000 daltons cut-off). The release medium used contained 25% (v/v) isopropyl alcohol in 0.1 M
phosphate buffer pH 7.4 with 0.5% (w/v) Tween 80. The study in vitro was also performed using a conventional release medium which was identical to the release medium mentioned earlier but without isopropyl alcohol. A 2.5 mg/mL suspension of nanoparticles was prepared in the release medium. Two mL of this suspension was sealed into the dialysis bag which was inserted into a 15 mL Thermo centrifuge tube containing 13 mL of release medium (receiver compartment). The tubes were placed in the incubator-shaker (MaxQ 4450, Thermo Scientific) at 37°C, and covered with a box for light protection. Samples were collected at specific time intervals by removing 200 µL from the receiver compartment and by replacing it with 200 µL fresh release medium. The aliquots were filtered through 0.2 µm syringe filter and analyzed for drug content using the UPLC method.

3.3.12. Cellular uptake studies

The cellular uptake of curcumin and resveratrol from solution and from the nanoparticle formulation was studied using DU145 prostate cancer cell line obtained from American Type Culture Collection (ATCC), Manassas, VA. The cells were cultured in 6-well plates at a seeding density of 1.8x10^5 cells per well and incubated in a humidified chamber at 37°C until they reached confluence. A 1 mM solution of curcumin and resveratrol was prepared in ethanol and diluted to 20 µM using Minimum Essential Medium (MEM) used
for growing the cells. A known quantity of nanoparticles containing equivalent amounts of curcumin and resveratrol was suspended in MEM. The confluent cell monolayers were treated with 2 mL of either drug or nanoparticle solutions. The treatment was removed at specific time intervals (0.5, 1, 2, 4 and 6 hours). The supernatant was collected for analysis. The cells were washed thrice with cold 0.1 M phosphate buffer pH 7.4. The cell layer was scrapped off using a scraper. The cell mass was collected in a micro-centrifuge tube and ultrasonicated at 30 watts for 10 seconds. The solution was analyzed for curcumin content by visible spectroscopy at 425 nm and fluorescence spectroscopy at 480 nm excitation and 528 nm emission wavelengths. The resveratrol content was analyzed by UV spectroscopy at 320 nm wavelength. Simultaneously, drug solution and nanoparticle suspension, which was not treated on the cells, was also analyzed at the same time points and using the same analytical parameters. Twenty microliters of cell sample was used to determine the total protein content, using the BCA assay. The Dixon’s test for outliers was used to determine any outliers in the data.

3.3.13. Cellular localization studies

The cellular localization studies were performed on DU145 prostate cancer cells. Briefly, DU145 cells were cultured in BD Falcon 4-well slides and
incubated in a humidified chamber at 37°C. The cells were treated with 20 µM solution of curcumin and resveratrol diluted in Eagle’s Minimum Essential Medium. Nanoparticles containing equivalent amounts of curcumin and resveratrol was suspended in MEM. Lysotracker red (0.5 µL) was added to both the drug and nanoparticle solutions prior to treatment. The media in the 4-well slides was removed and 200 µL of drug or nanoparticle solutions were added to 4 wells. At specific time points of 15 minutes, 1 hour, 2 hours and 4 hours the treatment was removed from the wells and the cells were washed twice with cold phosphate buffer pH 7.4. Two hundred µL 10% Neutral Buffered Formalin solution was added to the wells for cell fixation, and removed after a contact time of 10 minutes. The wells were then removed, DAPI was added and the slide was covered with a cover slip. The cells were visualized under Leica DM2500M microscope, fitted with the Leica DFC320 camera.

3.3.14. MTT toxicity assay

The cytotoxicity of blank and drug loaded nanoparticles was studied using MTT cytotoxicity assay. The DU145 prostate cancer cell line was used for this study. Cells were plated in a 96-well plate at a seeding density of 4500 cells per well. Cells were grown in MEM supplemented with 10% Fetal Bovine serum (FBS), 1mM sodium pyruvate, 0.1mM Non-essential Amino
Acids, 2mM L-Glutamine, 100U/ml Penicillin, and 100 µg/ml Streptomycin. After plating the cells, the plates were incubated overnight in a humidified chamber at 37°C with 5% carbon dioxide. On the second day of the experiment, five different concentrations of blank nanoparticles, curcumin and resveratrol loaded nanoparticles, and drug solutions of curcumin and resveratrol were prepared in MEM. Approximately 24 hours after plating, the cells were treated with each of the treatment solutions in triplicates. A blank media treatment was also carried out. A 100 µL of treatment was added to each well. The plates were then incubated in the humidified chamber for 4 hours. After 4 hours, the treatment was removed and 100 µL of fresh MEM was added to each well. The plates were returned to incubation.

After an incubation period of 24, 48 and 72 hours; 30 µL of MTT solution (5 mg/mL) prepared in sterile phosphate buffer pH 7.4 was added. The plate was then incubated for a period of 4 hours. After 4 hours, the treatment was removed and the cells were lysed using a 1:1 solution of 20% (w/v) sodium dodecyl sulfate (SDS) and dimethyl formamide (DMF). The plates were kept on the incubator-shaker (MaxQ 4450, Thermo Scientific) for one hour at 37°C. The analysis was carried out on the micro plate reader (Multiskan MCC) at 540 nm. All the studies were performed in triplicate.
3.3.15. Hemolysis assay

Single donor human whole blood was used for determining the hemolytic effect of the formulation, if any. The blood was centrifuged at 1500 rpm for 10 minutes and the supernatant serum was removed. The levels of residue and total volume were marked. The residue was washed three times using 0.9% normal saline. After the final washing, the supernatant was discarded, volume made up to the initial volume using normal saline solution, and mixed. The blood cells were diluted 1:10 with normal saline, for the test. An approximate dose of 10 mg/kg in human with 5L blood volume was calculated and the drug and nanoparticle solutions were diluted accordingly. One hundred μL of these solutions were diluted with 700 μL of normal saline. Two hundred μL of the diluted blood cells were added to the treatment solutions in a microcentrifuge tube. One percent (v/v) Triton X-100 in normal saline was used as the positive control. Normal saline was used as the negative control. The treated blood cell solution was incubated at 37°C for one hour. After one hour, the tubes were centrifuged for 5 minutes at 13,000 rpm and the absorbance of the supernatants was analyzed at 405nm and 540nm on the micro plate reader (n=4). The percent hemolysis was calculated using the following formula:

\[
\frac{\text{Sample Absorbance} - \text{Absorbance of negative control}}{\text{Absorbance of positive control} - \text{Absorbance of negative control}} \times 100
\]
The percent hemolysis was calculated separately for the two wavelengths.

**3.3.16. Statistical analysis**

The statistical analysis of the experimental data was performed using the two tailed Student’s t-test. A statistically significant difference was termed if the p value was determined to be less than 0.05.

**3.4. Results**

**3.4.1. Particle size and zeta potential**

Particle size and zeta potential measurements were performed on blank and drug loaded nanosuspensions.

**Table 3.1:** Particle size and zeta potential of blank and drug loaded nanosuspensions

<table>
<thead>
<tr>
<th>Nanosuspension type</th>
<th>Particle Size (Mean±SD, n=3)</th>
<th>Zeta Potential (Mean±SD, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>13.4 ± 4.2 nm</td>
<td>-12.82 ± 4.95 mV</td>
</tr>
<tr>
<td>Drug loaded</td>
<td>12.53 ± 1.06 nm</td>
<td>-10.7 ± 2.73 mV</td>
</tr>
</tbody>
</table>

The particle size and zeta potential of blank and drug loaded nanosuspension was found to have no significant (p<0.05) difference.
Particle size and zeta potential measurements were also performed on freeze dried blank and drug loaded nanoparticles.

**Table 3.2:** Particle size and zeta potential of blank and drug loaded nanoparticles

<table>
<thead>
<tr>
<th>Nanoparticle type</th>
<th>Particle Size (Mean±SD, n=3)</th>
<th>Zeta Potential (Mean±SD, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>46.9 ± 11.6 nm</td>
<td>-13.08 ± 1.42 mV</td>
</tr>
<tr>
<td>Drug loaded</td>
<td>57.86 ± 12.61 nm</td>
<td>-22.01 ± 2.17 mV</td>
</tr>
</tbody>
</table>

The drug loaded nanoparticles were found to have a slight increase in particle size as compared to the blank nanoparticles as shown in Table 3.2. The zeta potential of the drug loaded nanoparticles was found to be 1.6 times higher than blank nanoparticles. The particle size of the freeze dried nanoparticles was found to vary as indicated by the high standard deviations. The lyophilized nanoparticles upon dispersion into aqueous medium showed 4 to 5-folds increase in particle size as compared to the nanosuspension. A possible reason for this could be the aggregation of nanoparticles upon redispersion.

### 3.4.2. Atomic Force Microscopy (AFM) imaging
The particle size and morphology of blank and drug loaded nanoparticles was studied by AFM imaging, seen in figure 3.1 and figure 3.2, respectively. Both blank and drug loaded nanoparticles were found to have a roughly spherical shape. A representative nanoparticle was zoomed in to analyze the particle size. The particle size for the blank and drug loaded nanoparticle in the zoomed-in image was found to be 13.09 and 11.54 nm, respectively.

Figure 3.1: (A) AFM images for blank calcium alginate nanoparticles and (B) one example zoomed in
Figure 3.2: (A) AFM images for drug loaded calcium alginate nanoparticles and (B) one example zoomed in

3.4.3. Karl-Fisher titrimetry

The moisture content by weight of blank and drug loaded nanoparticles as determined by Karl-Fisher titration is shown in the following table 3.3:
### Table 3.3: Moisture content by Karl-Fisher titrimetry

<table>
<thead>
<tr>
<th>Sample</th>
<th>Moisture content (% w/w) (Mean±SD, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Nanoparticles</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>Drug loaded nanoparticles</td>
<td>4.8 ± 0.2</td>
</tr>
</tbody>
</table>

The moisture content of blank and drug loaded nanoparticles was found to have no significant difference (p≤0.05).

#### 3.4.4. Thermogravimetric analysis

The TGA thermograms of freeze dried blank and drug loaded nanoparticles is shown in the following figure 3.3.

![TGA thermograms of blank and drug loaded nanoparticles](image)

**Figure 3.3:** TGA thermograms of blank and drug loaded nanoparticles
The blank nanoparticles showed a weight loss of 7.2% while drug loaded nanoparticles had a weight loss of 5.2% when heated from room temperature to 110°C.

3.4.5. Differential Scanning Calorimetry

An overlay of the DSC thermograms of pure curcumin, pure resveratrol, freeze dried blank and drug loaded nanoparticles is shown in figure 3.4. Curcumin shows an endothermic peak at 176.41 °C near its reported melting point of 175°C. Resveratrol shows an endothermic peak at its melting point of 264.28°C. The melting peaks of curcumin and resveratrol were found to be absent in the curcumin and resveratrol loaded nanoparticles. This indicates that the drugs could be present in a non-crystalline state in the nanoparticles. A broad endothermic peak was observed for blank and drug loaded nanoparticles in the range of 100-110°C, due to possible dehydration. Exothermic peaks in the temperature range of 224-232°C were observed in blank and drug loaded nanoparticles indicating a possible degradation of the calcium alginate matrix.
**Figure 3.4**: DSC thermograms for pure curcumin, pure resveratrol, blank nanoparticles, and drug loaded nanoparticles.

### 3.4.6. X-ray diffraction

Powder X-ray diffraction was performed on blank and drug loaded nanoparticles to confirm the results obtained by DSC. Figure 3.5 shows the XRD of curcumin and resveratrol and the XRD of blank and drug loaded nanoparticles. The XRD of pure drugs curcumin and resveratrol gave a unique fingerprint pattern. Such a fingerprint pattern was not observed in case of blank and drug loaded calcium alginate nanoparticles.
Figure 3.5: XRD of curcumin and resveratrol; blank and drug loaded nanoparticles

3.4.7. Physical stability testing of nanoparticles

The particle size and zeta potential measurement data over a period of 60 days is represented in Figure 3.6 (a) and (b). The particle size of the drug loaded nanoparticles showed increase in some measurements. Overall, the particle size of the drug loaded nanoparticles was found to be below 60 nm. The zeta potential was found to remain negative for both blank and drug loaded nanoparticles over a period of 60 days.
Figure 3.6: (a) Particle size and (b) zeta potential over 60 days storage

Figure 3.7 (a) represents the DSC thermograms of pure curcumin, pure resveratrol and drug loaded nanoparticles after storage over a period of 60 days. The DSC thermogram of drug loaded nanoparticles shows similar characteristics as seen in the DSC analysis 60 days prior. The melting peaks
of both curcumin and resveratrol were not seen in drug loaded nanoparticles. The non-crystalline nature of curcumin and resveratrol was confirmed by XRD. The unique fingerprint pattern of either drug was not seen in the drug loaded nanoparticles, as shown in the figure 3.7 (b).

Figure 3.7: (a) DSC curves and (b) XRD plot of pure curcumin, pure resveratrol and drug loaded nanoparticles after 60 days storage
3.4.8. Drug Entrapment Efficiency

The entrapment efficiency of curcumin and resveratrol in the calcium alginate nanoparticles was determined using the UPLC method and the values are listed in table 3.4. The entrapment efficiency for resveratrol was found to be higher than that of curcumin.

**Table 3.4: Entrapment efficiency of curcumin and resveratrol**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Entrapment Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>49.3 ± 4.3 %</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>70.99 ± 6.1 %</td>
</tr>
</tbody>
</table>

3.4.9. *In vitro* release study

The *in vitro* release of curcumin and resveratrol from calcium alginate nanoparticles was plotted as percent cumulative released against time in hours. This study was carried out for a total period of 24 hours. The *in vitro* release of curcumin and resveratrol in a release medium containing isopropyl alcohol is shown in figure 3.8.
Figure 3.8: *In vitro* release of curcumin and resveratrol with 25% (v/v) isopropyl alcohol in the release medium

The release of resveratrol was found to be significantly higher (p<0.05) than that of curcumin in a 24 hour *in vitro* release study. The cumulative amount of resveratrol released in 24 hours was found to be 87±7%. The cumulative amount of curcumin released was found to be only 16.35±3.8 % within 24 hours. An initial lag in the release of curcumin until 2 hours was also seen. A release study without isopropyl alcohol was also performed to determine the effect of isopropyl alcohol on drug release. Figure 3.9 shows the release of resveratrol in the release medium containing 0.5% (w/v) Tween 80 in 0.1M phosphate buffer pH 7.4 over 30 hours.
Figure 3.9: *In vitro* release of resveratrol in a medium without isopropyl alcohol

Curcumin was not detected in the receiver compartment within 30 hours. The cumulative amount of release for resveratrol was found to be 79.9±1.8% within the same time period.

3.4.10 Cellular uptake and localization study

The cellular uptake of curcumin (nanograms) per mg of protein in the cells is depicted in figure 3.10. The curcumin uptake was found to increase from 30 minutes to one hour. However, the amounts of curcumin decreased thereafter up to 6 hours. Each time point had a decreased curcumin amount as compared to the previous time. These results were consistent for curcumin solution as well as the drug loaded nanoparticles. The drug solution had a
higher initial uptake (6.06±1.28 ng/mg of protein) as compared to the nanoparticles (4.11±1.02 ng/mg of protein). However, with time, the difference was found to decrease. At 6 hours, nanoparticles had a higher uptake (2.05±0.71 ng/mg of protein) as compared to the drug solution (1.68±0.37 ng/mg of protein). Resveratrol could not be quantified from the cells.

![Cellular uptake of curcumin per mg of protein](image)

**Figure 3.10:** Curcumin uptake by DU145 cells expressed as ng/mg of protein

The graphs of percent drug remaining with respect to the initial amount, at each time point were plotted versus time. Curcumin and resveratrol amounts were plotted separately. The plot of each drug depicts the amount of drug
remaining in the supernatant after each time point for drug solution and drug loaded nanoparticles. It also includes the amount of drug remaining in the medium, not treated on the cells, but exposed to similar conditions (control). The results are depicted in figure 3.11 (a) for curcumin and (b) for resveratrol.
(b)

**Figure 3.11:** Percent drug remaining in supernatant with respect to initial concentration for (a) curcumin and (b) resveratrol

From these plots, it can be seen that the concentration of curcumin drug solution and nanoparticles in the controls remains constant throughout the study. However, in the drug solution and nanoparticles that were treated on the cells, the curcumin concentration was found to have fallen to 35.9±9.4% and 57.3±18.0% of the initial concentration. This was indicative of curcumin uptake into the cells. The uptake of curcumin in drug solution was more than curcumin from nanoparticles. On the other hand, in case of resveratrol, the
concentration of all four: control and cell treated drug solution and nanoparticles was found to remain constant. This indicated no uptake of resveratrol from either solution, or nanoparticles.

The images from the cellular localization study for drug solution and drug loaded nanoparticles at various time points are shown in figure 3.12.
Figure 3.12: Cellular localization images for drug solution (left) and curcumin and resveratrol loaded nanoparticles (right) at (A) 15 minutes, (B) 1 hour, (C) 2 hours and (D) 4 hours using DU145 cells.

Cellular localization studies were performed on DU145 cells at different time points of 15 minutes, 1, 2 and 4 hours with drug solution and drug loaded nanoparticles. The blue color in the cells are their nuclei stained with DAPI. The red-yellow stained organelles are the cell lysosomes. This study was based on the inherent fluorescence of curcumin, which is not shown by
resveratrol. Hence, the results are limited to curcumin only. Curcumin can be seen in green color around the cell membrane inside the cells. Both, the drug solution and the drug loaded nanoparticles demonstrated a visible uptake right from the first time point of 15 minutes. The green fluorescence was found to persist up to 4 hours (final time point), indicating a relatively constant localization of curcumin within the cells throughout the study. A similar localization was observed on a qualitative basis for both drug solution and drug loaded nanoparticles.

3.4.10 MTT cytotoxicity assay

The percent survival of the DU145 prostate cancer cells after 24, 48 and 72 hours of post-treatment incubation are represented in figures 3.13 (a), (b), and (c), respectively. The treatment included blank nanoparticles, curcumin and resveratrol loaded nanoparticles and curcumin-resveratrol solution.
Figure 3.13: Percent survival of DU145 cells after (a) 24 hours, (b) 48 hours and (c) 72 hours of incubation post-treatment * Significant difference in cytotoxicity (p < 0.05)

Molar Ratio of Curcumin: Resveratrol is 1:1.6

Curcumin and resveratrol drug solution exhibited a significantly greater toxicity (p<0.05) at higher total drug concentration of 260 µM as compared to drug loaded nanoparticles, 24 and 48 hours post-treatment. In the 72 hours post-treatment study, the drug solutions had a significantly higher toxicity than drug loaded nanoparticles from a 52 µM total drug concentration. Almost complete cell death was seen with the drug solutions at the total drug
concentration of 260 μM. The drug loaded nanoparticles had a significantly higher toxicity (p<0.05) compared to the blank nanoparticles at all concentrations and all time points. The average percent cell survival for blank nanoparticles was found to be 86.77±2.2%. There was no significant difference in the toxicity between the lowest (0.0832 μM total drug) and highest (260 μM total drug) concentrations for blank nanoparticles. The lowest percent survival for curcumin and resveratrol loaded calcium alginate nanoparticles 24, 48, and 72 hours post-treatment was found to be 55.8±12.5%, 52.9±2.1% and 47.2±7.7%, respectively at 260 μM total drug concentration.

3.4.11. Hemolysis assay

The hemolysis assay was performed on five treatments: negative control, positive control, curcumin and resveratrol solution, blank nanoparticles, and drug loaded nanoparticles. The results are represented in Figure 3.14.
Figure 3.14: Percent hemolysis of drug solutions, blank and drug loaded nanoparticles

The results at 405 nm and 540 nm wavelengths were found to be similar. Overall, the raw drug solution of curcumin and resveratrol was found to have a slightly higher percent hemolysis as compared to the blank and drug loaded nanoparticles which showed a negligible percent hemolysis.

3.5. Discussion

3.5.1. Formulation of calcium alginate nanoparticles

Calcium alginate nanoparticles were prepared by emulsification and cross-linking technique. The preparation method was developed based on the method used by Astete et al. in preparation of alginic acid nanoparticles for solubilizing hydrophobic natural colorants\(^1\). This method was subjected to
some modifications with respect to the processing parameters. The aqueous and organic phases were emulsified using ultrasonication to form the emulsion. The aqueous phase consisted of 0.5\% (w/w) Tween 80 in a 0.6 mg/mL sodium alginate solution. Curcumin and resveratrol were dissolved in acetone forming the organic phase. Only acetone was used as the organic phase to prepare blank nanoparticles. Cross-linking of sodium alginate was achieved by addition of calcium chloride solution under continuous ultrasonication. Prior to analysis and freeze drying, the nanosuspension was subjected to ultracentrifugation at 20,000 rpm for 15 minutes. This allowed the aggregates to pellet while the nanoparticles remained suspended in the solution and were separated from the pellet.

Alginate nanoparticles can be prepared by external or internal gelation process\(^{155}\). External gelation process involves delivery of alginate solution to the cross-linking agent. In the internal gelation process, the cross-linking agent is added to a solution of sodium alginate containing the drugs. Both external and internal gelation were tried. However, when the sodium alginate emulsion containing the drugs was added to a larger volume of calcium chloride solution (external gelation), the particle size distribution was varied and the particle size was high. Internal gelation by addition of calcium chloride to the emulsion helped attain a lower particle size. The importance of
ratio of calcium to alginate was investigated by K.L. Douglas and M Tabrizian\textsuperscript{156}. De and Robinson reported that the calcium to alginate ratio should be 0.2 or less for the formation of nanoparticles, and if the ratio is above 0.2, microparticles may be formed\textsuperscript{157}. Considering the results obtained by both groups, a calcium to alginate ratio of 0.22 was maintained. An important challenge in the preparation of nanoparticles was the addition of calcium chloride under continuous ultrasonication in the sound proof chamber. For this purpose, a syringe pump was set up such that calcium chloride was added at the point of ultrasonication (Figure 3.15). This apparatus set-up also helped to decrease the particle size below 60 nm.

![Syringe pump assembly for addition of calcium chloride](image)

**Figure 3.15:** The syringe pump assembly for addition of calcium chloride under continuous ultrasonication
The freeze dried nanoparticles were found to be easily dispersed in an aqueous medium. Ease of redispersion is important so as to obtain a nanoparticle suspension with minimal aggregation. Figure 3.16 shows a visual comparison between the solution of curcumin and resveratrol loaded nanoparticles and pure curcumin and resveratrol in deionized water.

![Figure 3.16: Visual comparison of a suspension of curcumin and resveratrol loaded nanoparticles versus pure curcumin and resveratrol](image)

**3.5.2. Particle size and zeta potential**

The particle size of the nanosuspension was found to be constant within the size range of 12-15 nm. The particle size of the resuspended freeze dried nanoparticles was found to be higher than the nanosuspension. Freeze dried nanoparticles tend to aggregate, as the product undergoes various stresses during the process which results into an increase in particle size. Variability
was seen in the measurements of the freeze dried particles indicated by the high standard deviations. The average polydispersity index was found to be 0.322 indicating a wider particle size distribution. However, all the particle size measurements were always found to be under 60 nm. Effective passive targeting of the nanoparticles by the EPR effect requires particle size to be under 200 nm\textsuperscript{159}. At such a small size, nanoparticles can permeate through the deformed tumor vasculature. With a particle size of well under 100 nm, the current drug delivery system can be used to passively target tumor.

The surface charge on the nanoparticles plays a vital role in their physiological behavior as well as in their stability. The physical stability of particles is enhanced if the particles have a similar surface charge and their size does not increase upon storage. Positively charged particles have been reported to show better cellular uptake, while the negatively charged particles have longer circulation times\textsuperscript{160}. Both, blank and drug loaded nanoparticles showed a negative zeta potential which can be explained by the presence of carboxylic acid groups in the alginate polymer lining the surface of the nanoparticles. The zeta potential of the freeze dried nanoparticles was found to be more negative as compared to the nanosuspension.
3.5.3. Thermal analyses, moisture content and XRD analysis

The DSC thermograms for pure curcumin and resveratrol showed a melting endothermic peak at 174.41°C and 264.28°C, respectively. These endothermic peaks were not observed in the curcumin and resveratrol loaded nanoparticles. This indicated that both the drugs, curcumin and resveratrol were possibly present in a non-crystalline state in the nanoparticles. This possibility was confirmed by performing X-ray diffraction analysis on the blank and drug loaded nanoparticles. Both samples showed a similar XRD pattern. The unique fingerprint pattern of pure curcumin and resveratrol did not match with the pattern of the drug loaded nanoparticles. This confirmed the physical state of curcumin and resveratrol in the calcium alginate nanoparticles as non-crystalline. When drugs are present in a crystalline form, their release may be affected. Thus, if the drug in the matrix is present in a dissolved state, one can expect a better in vitro release as compared to a system where the drug is present in a crystalline state\textsuperscript{161}. Crystallinity also affects the solubility of drugs. The exothermic peak seen in the temperature range of 224-232°C could be assigned to a possible degradation of the calcium alginate matrix. The degradation was visually confirmed by performing hot stage microscopy, wherein charring of the freeze dried material was seen above 230°C.
The blank and drug loaded nanoparticles also showed a broad endothermic peak in the temperature range of 100-110°C. This was due to a possible desolvation event or loss of moisture. The thermogravimetric analysis was performed to check weight loss with temperature. The blank and drug loaded nanoparticles showed a weight loss of 7.2% and 5.2%, respectively. A Karl-Fisher titration was carried out to determine the moisture content. The moisture content of the blank and drug loaded nanoparticles was found to be 5.9% and 4.8% by weight, respectively. The results of Karl-Fisher titrimetry were in close agreement with the percent weight loss determined by TGA.

3.5.4. Physical stability testing of nanoparticles

The stability testing of nanoparticles was done over a period of 60 days. The particle size and zeta potential of blank and drug loaded nanoparticles were analyzed. The freeze dried nanoparticles were re-dispersible over the entire course of study. The particle size was found to be within a size range of 15-45 nm for blank nanoparticles and 15-60 nm for the drug loaded nanoparticles. The zeta potential of blank and drug loaded nanoparticles was found to remain negative, in the range of -15 to -30 mV.

The stability testing was also carried out to check the physical state of drugs after a period of 60 days. The drugs were initially found to exist in a non-
crystalline state. After 60 days, the DSC thermogram of drug loaded nanoparticles did not show the corresponding melting peaks of curcumin or resveratrol. The XRD pattern of the drug loaded nanoparticles also did not match the unique fingerprint patterns of either curcumin or resveratrol. Thus, curcumin and resveratrol were found to exist in a non-crystalline state even after storage over a period of 60 days.

### 3.5.5. UPLC analysis

A reverse phase UPLC method for simultaneous analysis of curcumin and resveratrol was developed and validated. The retention times for resveratrol and curcumin were 0.5 minutes and 4.9 minutes, respectively. The total run time was 6 minutes. Since the formulation consists of both curcumin and resveratrol in the same system, an analytical method for simultaneous determination of both, would be time and cost effective. The simultaneous analysis of both drugs was successfully achieved using the UPLC method.

### 3.5.6. In vitro release study

The very low aqueous solubility of the two drugs, especially curcumin presents a challenge in simulating aqueous body fluids for studying the in vitro release of the drug from the formulation. Hence, some modifications to either the most commonly used release medium of phosphate buffer pH 7.4 or a new
release medium was required where the solubility of drug in the medium was sufficient to maintain a sink condition. Bisht et al. proposed a study utilizing the insolubility of curcumin to quantify released drug\textsuperscript{162}. Curcumin loaded nanoparticles were dispersed in pH 7.4 phosphate buffer. At specific time intervals, the released curcumin was separated by centrifugation and the pellet was analyzed for curcumin. This method was also utilized by other groups for studying the \textit{in vitro} release of curcumin\textsuperscript{163,164,165}. However, this method could not be utilized as resveratrol is not completely insoluble in pH 7.4 phosphate buffer, and also undergoes degradation over a period of 24 hours. The method also required a concentrated nanoparticle suspension. Shaikh et al. used a dialysis bag method to study the release of curcumin from PLGA nanoparticles. The release medium was modified to contain 50\% (v/v) ethanol in water to allow higher solubility of curcumin in the release medium and quantified from receiver compartment\textsuperscript{166}.

The current study used a release medium of 25\% (v/v) isopropyl alcohol in 0.1 M phosphate buffer pH 7.4 containing 0.5\% (w/v) Tween 80. The release profiles for curcumin and resveratrol were found to be very different. The possible reason for it could be the difference in the relative solubility of two drugs in the release medium. Resveratrol has higher solubility as compared to curcumin\textsuperscript{27,63}. A plot of percent cumulative drug released against time was
plotted. Resveratrol showed an 87.6% cumulative release over a period of 24 hours. A peak release of 89.1% was seen at 12 hours, after which the concentrations of resveratrol were found to drop in the receiver compartment. This could be due to the degradation of resveratrol at pH 7.4, as resveratrol is known to degrade at higher pH\textsuperscript{66}. On the other hand, curcumin release had shown a 2 hour lag time during the \textit{in vitro} release study. The cumulative release for curcumin within 24 hours was found to be 16.3%.

The effect of release medium on the release of curcumin and resveratrol was determined by performing release studies in a medium devoid of isopropyl alcohol. This was done in order to closely mimic the physiological conditions during the \textit{in vitro} release studies. The medium consisted of 0.5% (w/v) Tween 80 in 0.1M pH 7.4 phosphate buffer. Resveratrol had a cumulative release of 79.9% after 30 hours and the peak release was found to be 82% after 24 hours, following which the drug underwent degradation. The nanoparticles did not show curcumin release in this release medium, due to the very poor aqueous solubility of curcumin. The release medium devoid of isopropyl alcohol was found to affect the release of both drugs. Resveratrol showed a slower release whereas curcumin release could not be determined.
3.5.7. Cell uptake and localization studies

The cell uptake studies were performed on DU145 prostate cancer cell lines using drug solution in ethanol diluted with cell growth medium and a nanoparticle solution in cell medium. Initially, the UPLC method was used to analyze amount of drugs in the cells. However, the drug concentration was low and could not be quantified. There was also a possible loss of drug in the filter membrane (0.2µ) used for filtration of sample prior to UPLC injection. However, the qualitative analysis of cellular localization proved that curcumin was getting localized within the cells. Hence, fluorescence spectroscopy and UV spectroscopy was used an alternative means to quantify the two drugs. Curcumin was analyzed using excitation and emission wavelengths of 480 and 528 nm, respectively. There was an initial increase (up to 1 hour) in curcumin uptake for both drug solution as well as nanoparticles. The curcumin amounts were found to decrease subsequently (up to 6 hours). There was a considerable decrease in uptake in case of drug solution, such that at 6 hours, the curcumin uptake for nanoparticles was higher. A similar trend was observed by Sun et al. when they studied the uptake of curcumin loaded solid lipid nanoparticles in MCF-7 breast cancer cell line\textsuperscript{167}. Curcumin solution was rapidly taken up by the cells as compared to the nanoparticles. This could be explained from the findings of Yu and Huang, who have reported that
solubilized curcumin permeates across monolayers rapidly by passive diffusion; and this study was performed in Caco-2 cells. The higher curcumin uptake from nanoparticles at 6 hours could also be a possible result of slower release of curcumin from the nanoparticles. Other possible reasons for the decreased amounts with time are degradation of curcumin within the cellular material and lysosomal accumulation of curcumin. Hydrophobic compounds such as curcumin are known to accumulate within the lysosomes, an effect observed by Singh et al. in a Pluronic® based curcumin formulation.

The resveratrol amounts were analyzed at 320 nm after correcting for curcumin absorbance at that wavelength. Using this method, the amount of resveratrol in the lysed cells was again found to be negligible. To confirm that resveratrol had an almost non-existent uptake, the drugs were quantified in the supernatants of the cell medium at specific times after treatment. Curcumin and resveratrol amounts were also determined in the control samples at the same time points, which was the same medium that was not used for treatment on the cells. This was performed in order to eliminate the possibility of degradation or adsorption on to container of either drugs, which would have given false positive results in the cell supernatant samples. By comparing the percent of drug remaining with respect to the initial concentration, it was
found that the resveratrol concentrations for all samples remained constant. Thus resveratrol could not be taken up by the cells from either drug solution or nanoparticles. A possible reason for this could be the efflux of resveratrol from the DU145 cells. Henry et al. studied the uptake and efflux of trans-resveratrol in Caco-2 cell monolayers. The group reported that along with a possible passive mechanism, an active mechanism is also involved in the uptake of resveratrol. The active transport of resveratrol was found to occur via the sodium glucose transporter-protein (SGLT-1). They also reported an efflux of resveratrol from the apical side of Caco-2 cells. This efflux was attributed to the presence of Multidrug Resistance-associated Protein (MRP-2) in the Caco-2 cells. Lançon et al. studied the uptake of resveratrol in HepG2 tumor hepatocytes, wherein an initial uptake was followed by a rapid decrease in resveratrol amounts. They reported that a quick steady state might be attained in the uptake of resveratrol, after which efflux may occur rapidly with the possible involvement of transporters. They also studied the effect of serum proteins on resveratrol uptake, reporting that uptake from a serum-free medium was higher. This indicated that serum protein binding of resveratrol could also play a role in the uptake of the drug by cells. The medium used in the current study contained FBS which might have affected the resveratrol uptake. This analysis confirmed that curcumin demonstrated uptake into the
cells from both, drug solution and nanoparticles. The uptake was more for drug solution than nanoparticles. This could be attributed to the slower release of curcumin from the nanoparticles, whereas in the solution, curcumin was readily available for uptake.

The above result was in conjunction with the localization of curcumin seen visually in the DU145 cells. The curcumin localization was seen at 15 minutes and curcumin could be visualized throughout the 4 hours of study. The images were only for a qualitative verification of localization, and were inconclusive with respect to whether the drug solution or the nanoparticles had a higher localization.

3.5.8. MTT cytotoxicity assay and hemolysis assay

The MTT assay was performed on DU145 prostate cancer cells to determine the cytotoxic effect of the blank nanoparticles, curcumin and resveratrol loaded nanoparticles, and curcumin and resveratrol solution. The molar ratio of curcumin and resveratrol was kept constant at 1:1.6. The highest concentration of curcumin and resveratrol treatment was 100 µM and 160 µM, respectively. The ratio was decided on basis of drug entrapment in the nanoparticles and to keep the drug concentration in all treatments constant. There was a significant difference (p<0.05) between the percent survival for
blank and drug loaded nanoparticles above 52 µM total drug concentration. This indicates that the drug loaded nanoparticles had a greater toxic effect as compared to the blank nanoparticles. The curcumin and resveratrol solution was found to be the most toxic at the highest treatment concentration of 260 µM. The drug loaded nanoparticles were found to have similar toxicity as compared to the drug solutions except at the concentration of 260 µM and 52 µM for 72 hours, where the cytotoxicity of drug solution was significantly higher. Thus, the drug solutions were found to be more toxic than the drug loaded nanoparticles at the highest concentration at all time points. The curcumin and resveratrol solution was made in ethanol and then diluted with growth medium. Due to the complete initial dissolution of the drugs, their bioavailability could have been higher than the drugs released from the nanoparticles. This is a possible reason for the higher toxicity of the drug solution as compared to the nanoparticles. Another possible reason could be the higher proportion of ethanol in the 260 µM concentration of the drug solution, which could be contributing to the cytotoxicity. The LD<sub>50</sub> value for the drug loaded nanoparticles was found to be at 260 µM total drug concentration at 72 hours post-treatment.

Yallapu et al. tested the cytotoxicity of curcumin-cyclodextrin formulation and curcumin solution on DU145 cells<sup>172</sup>. Similar toxicity profile was
observed for both treatments up to a curcumin concentration of 30 µM. With a further increase in concentration, the formulation had a slight increase in cytotoxicity. The same research group developed multi-functional magnetic curcumin nanoparticles and tested them on several cell lines, including PC3 prostate cancer cell line. In this study, the curcumin drug solution was found to have a higher cytotoxic effect as compared to the nanoparticles. This has been attributed to the slower release of the drug from the nanoparticles. The authors have claimed that the inhibition of cell growth was found to be more sustained and effective with only 40% release in 48 hour exposure. If this is co-related to the current study, the possible reason for the lower cytotoxicity of nanoparticles could be the slower release of the two drugs, especially curcumin, as seen from the in vitro release studies. This was an acute toxicity study with a short exposure time of 4 hours. Das et al. developed alginate-chitosan-Pluronic nanoparticles loaded with curcumin. The formulation was tested for cytotoxicity on HeLa cells. Free curcumin was found to show higher toxicity than the curcumin loaded formulation at higher concentrations. Similar results were observed by Kim et al. and Bisht et al. on various pancreatic cancer cell lines after treatment with albumin bound curcumin nanoparticles and polymeric nanoparticles, respectively. Gou et al. attributed the lower cytotoxicity of curcumin loaded polymeric micelles as compared to
free curcumin in C-26 colon carcinoma cell lines to a slower release of curcumin\textsuperscript{176}. Nanoparticle formulation of curcumin was found to show a higher cytotoxicity in DU145, PC3 and LNCaP prostate cancer cells as compared to curcumin solution by Mukerjee and Vishwanatha\textsuperscript{165}.

It was essential to determine the effect of the formulation on red blood cells when injected intravenously. The hemo-compatibility of the formulation was tested by the hemolysis assay. Two wavelengths (405nm and 540 nm) were used for analysis in order to check the reproducibility of results at two separate wavelengths and to eliminate the possibility of interference of any formulation component or drugs in the assay. The results at the two wavelengths were found to match. Neither the blank or the drug loaded nanoparticles showed any significant hemolysis (<0.1%). The percent hemolysis of the drug solutions was slightly higher than nanoparticles (1%). Thus, the formulation was determined to be safe for intravenous administration.

3.6. Conclusions

A calcium alginate nanoparticulate system was successfully developed for the delivery of curcumin and resveratrol. The particle size of the nanoparticles was under 60 nm. Simultaneous analysis of both curcumin and resveratrol was achieved by the development of an UPLC method. The entrapment of
resveratrol was found to be higher than that of curcumin. Resveratrol showed a much higher release as compared to curcumin. The composition of the release medium was found to affect the release of both drugs.

The nanoparticles were seen to be localized in the DU145 prostate cancer cells after 15 minutes of exposure which was similar to the drug solution. In contrast to curcumin, resveratrol did not show any uptake into the cells. The blank formulation was not found to be toxic. The drug loaded nanoparticles exhibited a 40-50% death in the DU145 cells after 4 hours treatment. The formulation was found to be safe for intravenous administration. It can be concluded that calcium alginate polymeric system can be used for entrapment of two hydrophobic drugs, curcumin and resveratrol. The nanoparticles were found to have in vitro efficacy against prostate cancer cells as seen from the MTT assay.
CHAPTER 4:

Summary, future directions and global impact
4.1. Summary

Calcium alginate nanoparticles for the delivery of two hydrophobic anti-cancer agents, curcumin and resveratrol were prepared and characterized. The system was intended for drug delivery to prostate gland in the treatment of prostate cancer. Emulsification and cross-linking process was successfully used for preparation of nanoparticles. The nanoparticles upon freeze drying had a slight increase in particle size. Curcumin and resveratrol were found to exist in a non-crystalline state in the nanoparticles. The nanoparticles were found to be stable over a period of 2 months.

A sensitive UPLC method for the simultaneous determination of curcumin and resveratrol was developed and validated. The chromatographic method used a reversed phase Waters Acquity system using Acquity HSS-T3 C18 column for separation of the two compounds. A mobile phase of 0.05% orthophosphoric acid and acetonitrile in a ratio of 66:34, gradient to 70:30 was used. The flow rate was 0.8 mL/min, with a run time of 6 minutes. Curcumin was monitored at 425 nm while resveratrol was monitored at 320 nm on the PDA detector. The method was specific for curcumin and resveratrol, and was successfully able to quantify a lowest concentration of 1.9 µg/mL. It was validated for linearity, precision and accuracy. This UPLC method was useful
in determining the entrapment efficiency and *in vitro* release of curcumin and resveratrol.

Curcumin and resveratrol were found to have different entrapment efficiency, with resveratrol being the higher at 70.99±6.1%. The entrapment efficiency for curcumin was found to be 49.3±4.3%. The extreme hydrophobicity of the two drugs, especially curcumin, warranted the use of isopropyl alcohol (25% v/v) in the release medium (0.1M pH 7.4 phosphate buffer with 0.5% Tween 80) to study the *in vitro* drug release. In this medium the release of resveratrol was found to be 87.6±7.9% within 24 hours. Curcumin showed an initial lag period of 2 hours, followed by a cumulative release of 16.3±3.1% within 24 hours. The initial lag may be attributed to the low solubility of curcumin. In a medium without isopropyl alcohol, resveratrol had a slower release of 79.92±1.82% within 30 hours, while the insoluble curcumin did not show any release by the developed UPLC method.

The formulation was tested for uptake and cytotoxicity on DU145 prostate cancer cell lines. In the cellular localization studies, the nanoparticles were seen to be internalized in the cells within 15 minutes of treatment. However, qualitatively, the uptake was found to be similar to drug solution made in ethanol and diluted in growth medium. Blank nanoparticles did not exhibit
considerable toxicity in the MTT cytotoxicity assay. The drug loaded nanoparticles showed toxicity in the prostate cancer cells.

4.2. Future directions

The present study involved preparation and characterization of calcium alginate nanoparticles containing two hydrophobic drugs, curcumin and resveratrol. In future studies, the system can be used for loading two hydrophilic or one hydrophilic and one hydrophobic drug. Since the system itself is hydrophilic, there is a possibility of greater drug entrapment for a hydrophilic drug. This system could be tested in vitro for its efficacy on other cancer cell lines such as breast cancer and lung cancer. For prostate cancer treatment, a stem cell inhibitor such as cyclopamine, which is a hydrophobic drug can be used in a combinational therapy with either curcumin or resveratrol. This would help to address the issue of relapse, commonly associated with prostate cancer. A different polymeric drug delivery system such as PLGA can be investigated with the aim of sustaining the drug release of curcumin and resveratrol.

The system could be modified with the use of a different surfactant so that increased entrapment, especially for curcumin can be achieved. Curcumin and resveratrol could be loaded individually in the calcium alginate nanoparticles
to be tested individually along with the combination in order to study any possible synergism between the two drugs. The *in vitro* efficacy studies can be extended to different cancer cell lines. The poor uptake of resveratrol and its possible efflux needs to be investigated by determining the transport mechanisms involved. The *in vitro* cytotoxicity studies can be carried out using a different prostate cancer cell line such as PC3 cells, or a cell line of different cancer type such as breast cancer or lung cancer, in order to reproduce the *in vitro* efficacy of the current system on other cell lines. Correlation of the *in vitro* results can be carried out with in vivo studies. Different animal models can be explored for this purpose such as the SCID (Severely Compromised Immunodeficient) mouse model, transgenic models, and knockout models of mice\textsuperscript{177}. The Lobund-Wistar type rats can also be used in prostate cancer studies\textsuperscript{178}.

### 4.3. Global impact

Prostate cancer ranks second in cancer diagnosis as well as deaths in men. The ever increasing numbers of new cases in older men is a cause for concern. Although the chemotherapeutic treatment for prostate cancer has advanced in the past decade, current chemotherapeutic drugs are not devoid of their adverse effects.
Curcumin and resveratrol have been shown to be effective in prostate cancer treatment. These drugs are constituents of food items consumed worldwide which, have a much better safety profile than current drugs used in cancer chemotherapy. Development of a drug delivery systems such as the one described in current project would be beneficial as it will achieve twin goals of cancer treatment and reduction of adverse effects. Neither drugs are currently used for cancer treatment yet. Clinical testing of the calcium alginate nanoparticulate system in appropriate animal models will give a better insight into its effectiveness. If the system is found to be efficacious in animal models, clinical trials can be initiated.

The anti-cancer actions of curcumin and resveratrol are not limited to prostate cancer. Thus, after suitable in vitro, pre-clinical and clinical testing, the use of curcumin and resveratrol loaded drug delivery system can be used for treatment of a variety of cancers such as breast cancer and lung cancer.
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