SURFACE MODIFIED SOLID LIPID CURCUMIN NANOPARTICLES FOR ORAL DELIVERY

By

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A THESIS

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ABSTRACT

Curcumin is a naturally available polyphenolic compound that has demonstrated chemotherapeutic effects in several carcinogenic models as well as pre-clinical trials. However, its poor oral bioavailability due to extremely low aqueous solubility, poor permeability and extensive pre-systemic metabolism has been the major limitation for its use as a chemotherapeutic agent. Solid lipid nanoparticulate formulations have been successfully used to enhance the oral bioavailability of several poorly soluble drugs. The objective of the present research was to develop and characterize solid lipid nanoparticles for oral delivery of curcumin using GMO/chitosan based nanoparticulate system containing two different stabilizers.

The oil/water nanoemulsions were prepared by sonication and high pressure homogenization (HPH) using GMO/chitosan system. Two different stabilizers namely, PVA and poloxamer 407 were used for this purpose. The particles size (PS) and zeta potential (ZP) of nanoemulsion were determined at different stages of preparation using zetameter. The physical stability of the nanoemulsion was studied at 25°C over a period of 60 days by determining its PS and ZP. The blank and curcumin loaded nanoemulsions of both PVA as well as poloxamer containing formulations were further lyophilized and characterized for PS and ZP. The surface morphology of drug loaded nanostructures was determined using scanning electron microscopy. The weight loss of these formulations on heating was investigated using thermogravimetry while the moisture content was determined using Karl Fischer titrimetry. Differential scanning calorimetry (DSC) and X-ray diffraction (XRD) analysis was performed to determine the physical state of the drug in the nanostructures. An UPLC method was developed and validated for the analysis of
curcumin. The *in vitro* release of curcumin from the nanostructures was evaluated at 37°C in pH 7.4 buffer containing 0.5% (w/v) Tween 80 by using UPLC. The cellular uptake of curcumin from the solution as well as nanostructures was investigated in Caco-2 cells after 30, 60 and 90 minutes of treatment.

The process of HPH effectively reduced the particle size of the curcumin loaded GMO/chitosan nanoemulsions by 50 to 65% after three cycles. Loading the GMO/chitosan system with a hydrophobic drug caused an increase in its particle size. Poloxamer 407 was found to be a more efficient stabilizer as compared to PVA to stabilize the GMO/chitosan nanostructures because it gives stable nanoemulsions at a % (w/v) concentration five times less than PVA. The DSC and XRD studies confirmed the crystalline nature of drug in the nanostructures and showed that the absence of visible thermal events in the DSC thermograms could be misleading if two opposite thermal events occur at the same temperature range. Use of poloxamer as a stabilizer sustained the curcumin release from the nanostructures when compared with PVA. The percent cumulative release of curcumin from the nanoparticulate formulation with PVA and poloxamer after 171 hours was found to be 73.93 ± 5.25 and 53.15 ± 5.84, respectively. The cellular uptake of curcumin was 2.5-fold higher in nanostructures containing PVA as compared to poloxamer, at all the time points tested. Appropriate selection of stabilizer for the fabrication of GMO/chitosan nanoparticulate system can affect its ZP, surface morphology, rates of release and cellular uptake in Caco-2 cells. However, the PS, moisture content, and physical state of the drug in the system, was unaffected by the type of stabilizers tested.
ABSTRACTS:


Dedicated to my family
ACKNOWLEDGEMENTS

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>ix</td>
</tr>
<tr>
<td>List of figures</td>
<td>xiii</td>
</tr>
<tr>
<td>List of tables</td>
<td>xv</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>xvi</td>
</tr>
<tr>
<td><strong>CHAPTER 1:</strong> <strong>Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>1.1: Cancer chemotherapy and its routes of administration</td>
<td>2</td>
</tr>
<tr>
<td>1.2: Curcumin: Chemistry and pharmacology</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1: Physicochemical properties of curcumin</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2: Pharmacokinetics of curcumin</td>
<td>7</td>
</tr>
<tr>
<td>1.2.3: Pharmacodynamics of curcumin</td>
<td>9</td>
</tr>
<tr>
<td>1.3: Solid lipid nanoparticles</td>
<td>11</td>
</tr>
<tr>
<td>1.3.1: Stabilizers for SLNs</td>
<td>12</td>
</tr>
<tr>
<td>1.3.1.1: D-α-tocopherol polyethylene glycol 1000 succinate</td>
<td>13</td>
</tr>
<tr>
<td>1.3.1.2: Poly (vinyl alcohol)</td>
<td>14</td>
</tr>
<tr>
<td>1.3.1.3: Poloxamer 407</td>
<td>15</td>
</tr>
<tr>
<td>1.3.2: Methods of preparation of SLNs</td>
<td>16</td>
</tr>
<tr>
<td>1.3.2.1: Solvent emulsification/evaporation</td>
<td>16</td>
</tr>
<tr>
<td>1.3.2.2: Microemulsion based preparation</td>
<td>16</td>
</tr>
<tr>
<td>1.3.2.3: High shear homogenization and/or sonication</td>
<td>17</td>
</tr>
<tr>
<td>1.3.2.4: High pressure homogenization</td>
<td>17</td>
</tr>
<tr>
<td>1.3.2.4.1: Hot homogenization</td>
<td>19</td>
</tr>
</tbody>
</table>
1.3.2.4.2: Cold homogenization
1.4.: Glyceryl monooleate (GMO)/chitosan system
1.5.: Objectives, hypothesis and specific aims

CHAPTER 2: Analytical method development and validation

2.1: Introduction
2.2: Materials
2.3: Methods
2.3.1: Chromatography
2.3.2: Preparation of solutions
2.3.2.1: Mobile phase
2.3.2.2: Standard solution
2.4: Calculations
2.5: Results and discussions
2.5.1: Specificity
2.5.2: Linearity
2.5.3: Precision
2.5.4: Accuracy
2.6: Applications of the UPLC method
2.6.1: Determination of drug loading efficiency
2.6.2: In vitro release of curcumin from the formulation
2.6.3: Cellular uptake of curcumin in Caco-2 cells
2.6.4: Caco-2 permeability studies
2.7: Conclusions
CHAPTER 3: Effect of high pressure homogenization and stabilizer on the physicochemical properties of curcumin loaded GMO/chitosan nanostructures

3.1: Introduction 36
3.2: Materials 37
3.3: Methods 38
3.3.1: Formulation of the SLN systems 38
3.3.2: Analysis of particle size, surface charge and physical stability of the nanoemulsion 39
3.3.3: Scanning electron microscopy (SEM) 39
3.3.4: Differential scanning calorimetry (DSC) 39
3.3.5: Thermogravimetric analysis (TGA) 40
3.3.6: Karl Fischer titration 40
3.3.7: X-ray diffraction (XRD) analysis 40
3.3.8: UPLC analysis 41
3.3.9: Determination of drug loading efficiency 41
3.3.10: In vitro release studies 42
3.3.11: Determination of cellular uptake 42
3.3.12: Sub-cellular localization studies 43
3.3.13: Caco-2 permeability studies 43
3.3.14: MTT toxicity assay 44
3.3.15: Statistical data analysis 45
3.4: Results 45
3.4.1: Particle size, zeta potential & physical stability of nanoemulsions 45
3.4.2: Particle size and zeta potential of nanoparticles 47
3.4.3: Scanning electron microscopy analysis 48
3.4.4: Differential scanning calorimetry 49
3.4.5: Thermogravimetric analysis and Karl Fischer titration 52
3.4.6: X-ray diffraction analysis 52
3.4.7: In vitro release of curcumin 54
3.4.8: Cellular uptake of curcumin 55
3.4.9: Sub-cellular localization studies 56
3.4.10: Caco-2 permeability studies 57
3.4.11: MTT toxicity assay 58
3.5: Discussion 60
3.5.1: Formulation of the delivery system 60
3.5.2: Particle size, zeta potential and physical stability of Nanoemulsion 60
3.5.3: Particle size, zeta potential and surface morphology of SLN 62
3.5.4: UPLC analysis 63
3.5.5: In vitro release studies 63
3.5.6: Thermal analysis, moisture content and X-ray diffraction 64
3.5.7: Cellular uptake studies 65
3.5.8: Sub-cellular localization and Caco-2 permeability studies 66
3.5.9: MTT toxicity assay 67
3.6: Conclusions 67

CHAPTER 4: Summary and future directions
4.1: Summary 70
4.2: Future directions 71

References 73
**LIST OF FIGURES**

| Figure 1.1 | Chemical structure of curcumin. | 4 |
| Figure 1.2 | Chemical structure of curcumin showing keto-enol tautomerism | 4 |
| Figure 1.3 | Structures of the degradation products of curcumin at 37°C in pH 7.2 buffer. | 6 |
| Figure 1.4 | Metabolism of curcumin in mice has been represented in two major biotransformation pathways namely reduction and glucuronidation. | 7 |
| Figure 1.5 | Stages in tumor progression inhibited by curcumin by suppressing multiple signaling pathways. | 10 |
| Figure 1.6 | Chemical structure of TPGS. | 13 |
| Figure 1.7 | Structural formula of Poly (vinyl alcohol). | 14 |
| Figure 1.8 | Structural formula of poloxamer. | 15 |
| Figure 1.9 | A schematic representation of hot and cold homogenization technique for preparation of SLNs. | 18 |
| Figure 2.1 | Chemical structure of curcuminoids: curcumin, demethoxycurcumin, bisdemethoxycurcumin. | 23 |
| Figure 2.2 | A representative chromatograms of a) mobile phase and b) sample showing major peaks for curcumin (C), demethoxycurcumin (D) and bisdemethoxycurcumin(B) in the mobile phase. | 27 |
| Figure 2.3 | Standard curve of curcumin showing linearity over a concentration range of 0.015 to 62.5 μg/mL | 28 |
| Figure 3.1 | Effect of HPH cycles, drug load and stabilizer on particle size of nanoemulsion with a) 0.5% PVA and b) 0.1% poloxamer. | 46 |
| Figure 3.2 | Effect of HPH cycles, drug load and stabilizer on zeta potential of nanoemulsion with a) 0.5% PVA and b) 0.1% poloxamer. | 46 |
| Figure 3.3 | SEM micrographs of (a) curcumin nanoparticulate system with PVA (b) curcumin nanoparticulate system with poloxamer. | 48 |
| Figure 3.4 | (a) An overlay of DSC thermograms of curcumin (pure drug), blank | xiii |
Figure 3.4 (b) An overlay of DSC thermograms of blank NP with PVA, curcumin loaded NP with PVA and curcumin loaded NP with blank NP as reference.

Figure 3.4 (c) An overlay of DSC thermograms of curcumin (pure drug), blank NP with poloxamer and curcumin loaded NP with poloxamer.

Figure 3.4 (d) An overlay of DSC thermograms of blank NP with poloxamer, curcumin loaded NP with poloxamer and curcumin loaded NP with blank NP as reference.

Figure 3.5 An overlay plot of XRD patterns of curcumin (pure drug), Blank NP with PVA and curcumin NP with PVA.

Figure 3.6 An overlay plot of XRD patterns of curcumin (pure drug), Blank NP with poloxamer and curcumin NP with poloxamer.

Figure 3.7 The \textit{in vitro} release profiles of curcumin nanoparticles with PVA and poloxamer in pH 7.4 phosphate buffer.

Figure 3.8 A plot of \% cumulative drug released vs. square root of time representing the Higuchi model of release kinetics.

Figure 3.9 The cellular uptake of curcumin from the solution and nanoparticulate system in Caco-2 cells at different time points.

Figure 3.10 Sub-cellular localization of curcumin NP with PVA after a) 15 minutes and b) 60 minutes of treatment.

Figure 3.11 Sub-cellular localization of curcumin NP with poloxamer after a) 15 minutes and b) 60 minutes of treatment.

Figure 3.12 Cytotoxicity profile of curcumin solution, blank nanoparticles and curcumin loaded nanoparticles containing PVA and poloxamer after 24 hours incubation.

Figure 3.13 Cytotoxicity profile of curcumin solution, blank nanoparticles and curcumin loaded nanoparticles containing PVA and poloxamer after 48 hours incubation.

Figure 3.14 Cytotoxicity profile of curcumin solution, blank nanoparticles and curcumin loaded nanoparticles containing PVA and poloxamer after 72 hours incubation.
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Anti-proliferative effect of curcumin on the cell cycle of different cancerous cells.</td>
<td>9</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Within day and day to day precision for the UPLC analysis of curcumin</td>
<td>29</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Particle size and zeta potential of nanoemulsion studied at 25°C over a period of 60 days.</td>
<td>30</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Particle size and zeta potential of the blank and drug loaded formulations.</td>
<td>47</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Percentage moisture content and weight loss of the blank and drug loaded formulations.</td>
<td>48</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BEH</td>
<td>Ethylene bridged hybrid</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>GMO</td>
<td>Glyceryl monooleate</td>
</tr>
<tr>
<td>HLB</td>
<td>Hydrophilic-lipophilic balance</td>
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<td>HPH</td>
<td>High pressure homogenization</td>
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<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>HPTLC</td>
<td>High performance thin-layer chromatography</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>PCCA</td>
<td>Professional compounding centers of America</td>
</tr>
<tr>
<td>PES</td>
<td>Polyethersulfone</td>
</tr>
<tr>
<td>PS</td>
<td>Particle size</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl) alcohol</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SLN</td>
<td>Solid lipid nanoparticles</td>
</tr>
<tr>
<td>TEER</td>
<td>Trans epithelial electric resistance</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>THC</td>
<td>Tetrahydrocurcumin</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>TPGS</td>
<td>D-α-tocopherol polyethylene glycol 1000 succinate</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-performance liquid chromatography</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopeia</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray Diffraction</td>
</tr>
<tr>
<td>ZP</td>
<td>Zeta potential</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction
1.1. Cancer chemotherapy and its route of administration

Cancer is one of the major public health problems in United States of America as well as throughout the rest of the world. Presently, 1 in 4 deaths occurring in USA is due to cancer. According to the American Cancer Society, about 1,596,670 new cases of cancer are predicted to be diagnosed in USA and about 571,950 Americans are likely to die from cancers in the year 2011 [1]. Cancer is a pathological condition characterized by uncontrolled growth and proliferation of abnormal cells [2]. These uncontrolled cancer cells are collectively termed as tumors. On the basis of its characteristics, tumors could be classified as benign or malignant. Benign tumor conditions are localized and do not invade the neighboring tissues whereas malignant cancers are invasive in nature and metastasize to other parts of body via blood or lymph. Both external factors (tobacco, alcohol, infectious organisms, exposure to certain chemicals and radiation) as well as internal factors (inherited mutations, hormones, and immune conditions) may cause cancer. Surgery, radiation therapy, chemotherapy, hormone therapy as well as combination of these treatments have been used to treat cancers.

Cancer chemotherapy refers to the administration of cytotoxic chemicals to either completely eradicate tumor or at least reduce the tumor burden thereby minimizing the tumor-related symptoms and perhaps prolonging life [3]. Several routes of administration such as intravenous, oral, intraperitoneal, intra-arterial, intrathecal etc. are available for treatment of cancers using chemotherapeutic agents [4-6]. Intravenous route is most commonly used for the administration of anti-cancer agents either as intravenous infusion or intravenous bolus because it allows for better control of dose given to the systemic circulation. However, the intravenous route of administration necessitates continues
medical supervision or hospitalization and, therefore, has low patient compliance. Recently, oral route of administration has received increased attention to harness its advantages in cancer therapy. In 2008, it was estimated that more than a quarter of 400 anticancer drugs in the pipeline were planned as oral drugs [7]. Oral dosage forms are now available for an increased number of anti-cancer drugs such as methotrexate, etoposide, hydroxyurea, idarubicin etc. because it offers several advantages over intravenous route for administering anti-cancer drugs to the patients. The ease of administration along with the cost-effective and non-invasive nature of this route has led to increased patient compliance for oral drugs [8]. Oral route has especially found increased applicability and convenience for chronic regimens of cancer treatment such as maintenance therapy for prevention of metastasis, pain management etc. However, there are certain limitations associated with this route of administration. Orally administered drugs has slow onset of action [9]. Degradation of drugs in acidic and enzymatic environment of gastrointestinal tract is one of the major concerns in orally administering the anti-cancer agents. Poor aqueous solubility or poor permeability of drugs may lead to poor absorption from the gastrointestinal tract into the systemic circulation [10]. Additionally, extensive first-pass metabolism of drugs may also lead to low bioavailability when given orally. Curcumin, a potential anti-cancer agent for treatment of numerous cancers, is a classic example of how low oral bioavailability can limit its therapeutic use for cancers [11].
1.2. Curcumin: Chemistry and pharmacology

Curcumin (or diferuloylmethane) is a naturally available yellow pigment obtained from dried rhizomes (turmeric) of *Curcuma longa*, belonging to the family Zingiberaceae. Turmeric has been traditionally used in Asian countries like India and China as a food additive (spice), preservative and medicine for treatment of common cold, skin diseases, wound healing, abdominal disorders etc. [13]. Turmeric is also an FDA-approved food additive to be used as a flavoring and coloring agent. Curcuminoids are the active ingredients present in turmeric and are responsible for its diverse pharmacological actions.

1.2.1. Physicochemical properties of curcumin

**Figure 1.1:** Chemical structure of curcumin [12].

**Figure 1.2:** Chemical structure of curcumin showing keto-enol tautomerism [11].
Curcumin was first isolated in 1815 by Vogel et al. and its diferuloylmethane structure was confirmed by Lampe et al. in 1910 [14]. Chemically, curcumin (1,7-bis(4-hydroxy 3-methoxy phenyl)- 1,6-heptadiene-3, 5-dione) is a linear diarylheptanoid compound wherein two oxy-substituted aryl moieties are linked together by a seven carbon chain as depicted in Figure 1.1 [15]. This polyphenolic compound has a bis-α,β-unsaturated β-diketone structure that exhibits keto-enol tautomerism. A predominant keto form is seen in acidic and neutral solutions whereas a more stable enol form is observed in alkaline medium as shown in Figure 1.2[11]. The three experimentally determined pK_a values of 8.54, 9.30 and 10.69 have been contributed by the dissociation of the enolic proton and the two phenolic protons, respectively [16]. It has a molecular formula of C_{21}H_{20}O_6 and a molecular weight of 368.37 g/mol with a reported melting point of 183⁰C [14]. Commercially available grade of curcumin is a yellow crystalline powder that consists of three major curcuminoids namely, curcumin (77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%) [17].

Curcumin is a hydrophobic compound with a log P value of 2.5 [18]. It has extremely low solubility in water (11ng/mL) whereas it is soluble in organic solvents like ethanol, methanol, acetone, DMSO etc. [19,20]. Curcumin undergoes pH-dependent first order degradation and the degradation is faster in neutral and alkaline media at 37⁰C as compared to the acidic media [21]. It also degrades rapidly in serum-free media while the stability is better in human blood and media containing 10% fetal calf serum. The increased stability of curcumin in acidic pH conditions has been proposed to be contributed by the conjugated diene structure. At neutral or basic pH conditions, the removal of proton from the phenolic group may lead to the destruction of this conjugated
structure as depicted in Figure 1.3. Vanillin has been reported to be one of the major degradation products of curcumin [21]. Curcumin also undergoes photodegradation that is sensitive to the presence of solvent system and oxygen [22].

![Figure 1.3: Structures of the degradation products of curcumin at 37°C in pH 7.2 buffer](image)

[21].
1.2.2. Pharmacokinetics of curcumin

Figure 1.4: Metabolism of curcumin in mice has been represented in two major biotransformation pathways namely reduction and glucuronidation [23].
The pharmacokinetic studies of curcumin in both animal models as well as humans have been studied in past few decades. In 1978, Wahlstrom et al. first reported the absorption, distribution and excretion of curcumin in Sprague-Dawley rats and showed that curcumin was poorly absorbed from the gut [24]. The apparent reason for poor oral bioavailability of curcumin may be because of its low aqueous solubility, poor gastrointestinal absorption, extensive pre-systemic metabolism and rapid elimination from the body. When administered orally, about 60-66% of given dose was found to be absorbed across the rat intestines [25-27]. The amount of curcumin reaching blood was reported to be negligible after oral administration in rats as well as humans [24,25,28]. The distribution of curcumin in several tissues such as liver, kidney, spleen, brain etc. was found to be low in rats and mice [25,26,29]. Curcumin has also been reported to undergo extensive pre-systemic metabolism by reduction as well as glucuronide- and sulfate- conjugation as depicted in Figure 1.4 [23-25]. Holder et al. reported glucuronides of tetrahydrocurcumin (THC) and hexahydrocurcumin as the major biliary metabolites of curcumin in rats [30]. Also curcumin glucuronoside, dihydrocurcumin-glucuronoside and THC were major metabolites in mice as reported by Pan et al. [29,31]. Similar results were reported by Ireson et al. when investigating the metabolism of curcumin in rats and humans [31]. A majority of orally administered dose is eliminated in feces with only negligible amounts being detected in urine [24-27].
1.2.3. Pharmacodynamics of curcumin

**Table 1.1:** Anti-proliferative effect of curcumin on the cell cycle of different cancerous cells [32].

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Arrested cell cycle phase</th>
<th>Cell cycle-related mechanisms</th>
</tr>
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<tbody>
<tr>
<td>HL-60 (Human acute myeloid leukemia)</td>
<td>G2/M first, then in G0/G1 phase</td>
<td>Inhibition of DNA synthesis</td>
</tr>
<tr>
<td>Human multiple myeloma cells</td>
<td>G1/S</td>
<td>Down regulation of cyclin D1, inhibition of IKK and NF-κB</td>
</tr>
<tr>
<td>CA46 cells (human Burkitt's lymphoma)</td>
<td>G0/G1 or G2/M and S</td>
<td>Inhibition of DNA synthesis</td>
</tr>
<tr>
<td>MDA 686LN (human head and neck squamous cell carcinoma)</td>
<td>G1/S</td>
<td>Down regulation of cyclin D1, inhibition of IKK and NF-κB</td>
</tr>
<tr>
<td>HCT-116 (human colon cancer)</td>
<td>G2/M</td>
<td>P53-and p21-independent</td>
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<tr>
<td></td>
<td></td>
<td>Down regulation of cyclin D and E but not B</td>
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<tr>
<td></td>
<td></td>
<td>Activation of cdc2</td>
</tr>
<tr>
<td>HT-29 and HCT-15 (human colon adenocarcinoma)</td>
<td>G2/M</td>
<td>Prostaglandin independent</td>
</tr>
<tr>
<td>COLO205 (colorectal carcinoma)</td>
<td>G1</td>
<td>Inhibition of Ca²⁺ dependent endonuclease, reduction of p53 gene expression</td>
</tr>
<tr>
<td>Lovo (colon cancer)</td>
<td>S, G2/M</td>
<td>Inhibition of DNA synthesis</td>
</tr>
<tr>
<td>HUVEC (human umbilical vein endothelial cells)</td>
<td>S</td>
<td>Inhibition of DNA synthesis</td>
</tr>
<tr>
<td>ECV304 (immortalized human umbilical vein endothelial cells)</td>
<td>G0/G1 and/or G2/M</td>
<td>Up-regulation of p21/WAF1/CIP1, p27KIP1, and p53</td>
</tr>
<tr>
<td>A7r5 (Rat aortic smooth muscle cell line)</td>
<td>G0/G1 and S</td>
<td>Inhibition of protein tyrosine kinase activity, protein kinase C activity, c-myc mRNA expression and bcl-2 mRNA expression</td>
</tr>
<tr>
<td>PCC4 (mouse embryonal carcinoma cells)</td>
<td>G1</td>
<td>Differentiation characterized by increase of nuclear/cytoplasmic ratio</td>
</tr>
<tr>
<td>MCF-7/1H (multidrug-resistant human breast carcinoma)</td>
<td>G2/M and subG0/G1</td>
<td>Reduction in the expression of Ki67, PCNA and p53 mRNAs</td>
</tr>
<tr>
<td>Human breast cancer cells</td>
<td>S and G2/M</td>
<td>Inhibition of ornithine decarboxylase activity</td>
</tr>
</tbody>
</table>

Curcumin has been reported to exhibit a wide variety of action including antioxidant [33], anti-inflammatory [34], antifungal [35], antithrombotic [36], hepatoprotective [37], hypoglycemic [38] and antirheumatic [39] activities. In the past few decades, there has been increased focus on understanding the chemopreventive and chemotherapeutic potential of curcumin against a variety of cancers. Several studies have
been performed in cancerous cells as well as in animal models to show the anti-cancer effect of curcumin. As represented in Table 1.1, curcumin arrests different phases of cell cycle in order to inhibit the proliferation of cancer cells derived from breast, prostate, colon, liver, kidney, blood and skin [32,40]. It has been reported to induce apoptosis in several malignancies such as leukemia, melanoma, breast, prostate, lung, colon, renal and ovarian carcinomas by interference of multiple signaling pathways and down-regulation of transcription factors such as nuclear factor kappa B (NF-κB) as shown in Figure 1.5 [32,40]. Recently, promising results have been seen during the clinical trials of curcumin in patients with colorectal and pancreatic cancers which shows its potential future use as a therapeutic agent [41,41,42].

**Figure 1.5:** Stages in tumor progression inhibited by curcumin by suppressing multiple signaling pathways [40].

FDA has categorized curcumin under ‘Generally Recognized As Safe’ food additive. Experiments on various animal models (rats, mice, guinea pigs and monkeys) as
well as humans have demonstrated that curcumin is extremely safe even at very high oral doses [43-45]. The clinical trials have indicated that curcumin is non-toxic and well tolerated at an oral dose as high as 8 to 12 grams per day [46]. Only minor side effects such as diarrhoea have been reported in human trails with oral administration of curcumin [42].

Despite of the higher efficacy of curcumin against several cancers and its lower toxicity in humans, it has found limited application due to its instability and poor oral bioavailability. In order to overcome these challenges several attempts have been made to increase its aqueous solubility or intestinal absorption by formulating curcumin as polymeric nanoparticles (NP), liposomes, cyclodextrin-complexes, solid dispersions etc. [47-51].

1.3. Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are the colloidal drug carriers that consists of solid lipid particles dispersed in a size range of 10 to 1000nm. SLNs have been reported to have applications in intravenous, intramuscular, oral, rectal, ophthalmic, dermal and other routes of administration [52]. They have been claimed to combine the advantages of other colloidal systems such as polymeric nanoparticles, fat emulsions, liposomes and micelles while simultaneous avoiding their disadvantages. Some of the proposed advantages of using SLNs include:

- Increased stability of drugs
- Enhanced oral bioavailability of poorly soluble drugs
- Controlled release of drug
- Drug targeting
- High drug payload
- Minimized toxicity
- Minimal use of organic solvents
- Ease of scale up for large scale production

The general components of SLNs include solid lipid(s), emulsifier(s) and water. The lipid component of SLNs may include triglycerides (e.g. tristearin, tripalmitin), partial glycerides (e.g. glyceryl monostearate, glyceryl monooleate), fatty acids (e.g. stearic acid, palmitic acid), steroids (e.g. cholesterol) and waxes (e.g. cetyl palmitate). Several emulsifiers with different charge and molecular weights (e.g. Soybean lecithin, polysorbates, PVA) have been used for the emulsification and stabilization of lipid dispersions. Occasionally, the surface properties of SLNs are altered by surface modification or coating with polymers in order to avoid phagocytic uptake by macrophages and/or to improve the pharmacokinetics of these colloidal carriers [53,54].

**1.3.1. Stabilizers for SLNs**

In the SLNs, there is a high interfacial tension at the surface of the lipid particles that are dispersed in the aqueous medium. Owing to the small particle size of the dispersed lipid and the resulting enormous surface area, there is increased free energy of the system which cause SLNs to be thermodynamically unstable. In order to decrease the surface free energy, these colloidal particles have a tendency of flocculation, aggregation and crystal growth. Stabilizers are, therefore, used to reduce the free surface energy of the particles by decreasing the interfacial tension and prevent particle aggregation by electronic repulsion or steric stabilization [55]. A variety of surface active agents such as
bile salts, phospholipids, poloxamers, polysorbates etc. have been used for stabilization of SLNs [56].

1.3.1.1. D-alpha-tocopheryl polyethylene glycol 1000 succinate

![Chemical structure of TPGS](image.png)

**Figure 1.6**: Chemical structure of TPGS [57].

D-α-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS or TPGS) is a non-ionic emulsifier derived from Vitamin E (α-tocopherol) (**Figure 1.6**). It is a water soluble form of Vitamin E that has been used as a dietary supplement. It has been approved by FDA as a drug solubilizer in oral, parenteral, topical, nasal as well as rectal/vaginal formulations. It is an o/w emulsifier and has a HLB value of 13 [58]. The polyethylene glycol is the hydrophilic portion whereas the tocopherol succinate is lipophilic portion [57]. *Mu et al.* successfully employed TPGS as an emulsifier for the preparation of paclitaxel PLGA nanoparticles with high entrapment efficiency as compared to poly (vinyl alcohol) (PVA). However, when used alone, TPGS containing nanoparticles gave larger particle size as compared to PVA containing nanoparticles. The
smallest particle size of the nanoparticles was obtained when both TPGS and PVA were used in the formulation [59].

1.3.1.2. Poly (vinyl alcohol)

Poly (vinyl alcohol) or PVA is a water-soluble synthetic polymer which has an empirical formula of \((\text{C}_2\text{H}_4\text{O})_n\) (Figure 1.7). The \(n\) value for the commercially available grades ranges from 500 to 5000 which is equivalent to a molecular weight of approximately 20,000 to 200,000. It is used as a stabilizing agent for the emulsions in concentrations ranging from 0.25% to 3.0% (w/v). It has been extensively used for stabilization of polymeric nanoparticles, microparticles as well as SLNs [56,60]. One of the main advantages of using PVA as an emulsifier is that it efficiently stabilizes the small sized particles. As a result, PVA containing nanoparticles are of relatively small particle size and uniform size distribution [61,62]. However, investigations over past decade have indicated some toxicity issues with the use of PVA. PVA has been reported to be potentially carcinogenic if administered parenterally for a long-term therapy [63]. Therefore, nanoparticles prepared using PVA as a stabilizer may not be satisfactorily biocompatible and may cause systemic toxicity.
1.3.1.3 Poloxamer 407

Poloxamers 407 are non-ionic, block copolymers of ethylene oxide and propylene oxide with a general formula of $\text{HO(C}_2\text{H}_4\text{O)}_a\text{ (C}_3\text{H}_6\text{O)}_b\text{ (C}_2\text{H}_4\text{O)}_a\text{H}$ as shown in Figure 1.8. These are available in a variety of grades that differ in the number of ethylene oxide and propylene oxide units. Poloxamer 407 (or Pluronic F127) is a grade of poloxamer that has an average molecular weight of 12600 that result from about 196 units of ethylene oxide and about 67 units of propylene oxide [64]. It is freely soluble in water and ethanol and has a HLB value of 22. It is a FDA approved stabilizer for oral, parenteral, ophthalmic and dermal formulations [65]. It has been reported to be used as a stabilizing agent to prevent aggregation of nanoemulsions, nanoparticles as well as microparticles [65-67]. Tamilvanan et al. stabilized the castor oil based nanoemulsion using poloxamer 188 and chitosan emulsifier films. The combination of steric stabilization by poloxamer and electrostatic repulsion by cationic chitosan produced a stable nanoemulsion of castor oil [68]. Additionally, stable simvastatin nanoparticles with a sub-200 nm mean particle size were formed with the use of glycercyl monooleate and poloxamer 407 [69].
1.3.2. Methods of preparation of SLNs

A number of methods of preparation have been extensively described in the literature for SLNs. The choice of method for preparation of SLNs depends on the properties of the drug as well as the formulation parameters such as concentration of lipid, type of stabilizers used etc. Some of the widely used methods includes solvent emulsification/evaporation, microemulsion based preparation, high shear homogenization and/or ultrasonication, and high pressure homogenization as described below [52,70,71].

1.3.2.1. Solvent emulsification/evaporation

This method involves precipitation of lipids from (o/w) emulsions to form SLNs. Lipids dissolved in water-immiscible organic solvents such as cyclohexane are emulsified in aqueous phase. Solvent evaporation leads to the precipitation of the lipids in the aqueous phase thereby forming nanoparticulate dispersions. The major advantage of this technique is the avoidance of any heat during the preparation of making it suitable for heat-sensitive drugs. However, this method necessitates the use of large volumes of organic solvents that may cause toxicity issues arising from the residual solvents as well as the environmental concerns. Scaling up of this technique for large scale production can also be challenging.

1.3.2.2. Microemulsion based preparation

This method of preparation of SLNs involves dilution of microemulsion. Microemulsions are prepared by stirring low melting lipid, emulsifier and coemulsifier in aqueous phase at 65-70°C. The hot microemulsion so formed was then dispersed in cold water (2-3°C) under continuous stirring leading to formation of SLNs. The typical volume ratios of the hot microemulsion to cold water are in range of 1:25 to 1:50. Factors
such as composition of microemulsion as well as temperature gradient affect the SLNs prepared by this method. High temperature gradient would facilitate the rapid crystallization of lipid and prevent its aggregation. However, owing to the dilution step required in this method, the lipid contents achievable are considerably low.

1.3.2.3. High shear homogenization and/or ultrasonication

High shear homogenization and ultrasonication were initially used as the dispersing techniques for the preparation of nano-dispersions. Both are easy to handle techniques and are often used alone or in combination to achieve small particle size. However, these dispersions have high polydispersity index with larger microparticle content. Sonication has limited use in formulations with high lipid concentration because it is difficult to uniformly disperse higher fat concentrations by probe sonication. Additionally, small particle sizes are attainable only after long sonication time that may risk shedding of metal contaminants from the metal probe.

1.3.2.4. High pressure homogenization (HPH)

The high pressure homogenization process is a reliable and powerful technique for preparation of SLNs. This method involves mechanical homogenization wherein the liquid formulation is forced through a narrow opening at very high pressure (100 to 2000 bars). The fluid accelerates on a short distance at a very high velocity (over 1000 km/h) and the resulting high shear stress and cavitation causes disruption of particles to submicron range. Some of the advantages of using high pressure homogenization process include narrow particle size distribution, better dispersion of formulations with higher lipid content, avoidance or low volumes of organic solvents, acceptability of homogenization equipment by the regulatory authorities and feasibility of scale-up for
large scale production [52,70,72]. Figure 1.9 gives the schematic representation of two different methods of high pressure homogenization namely, hot homogenization and cold homogenization.

**Figure 1.9:** A schematic representation of hot and cold homogenization technique for preparation of SLNs [70].
1.3.2.4.1. Hot homogenization

In this technique, the lipid component is melted and the drug is dispersed in it. Hot aqueous solution of the surfactant is added to this mixture under continuous stirring to give primary emulsion. This coarse emulsion is then passed through the high pressure homogenizer operated at a temperature higher than the melting point of the lipid. The hot (o/w) nanoemulsion so formed is allowed to cool to room temperature to give SLNs. When a heterogeneous system is cooled down, the average droplet size increases but the number of droplets decreases. Large droplets grow by the condensation of material that is diffused through matrix from small evaporating droplets. This phenomenon is called Ostwald’s ripening[73]. The hot homogenization method of SLN preparation highly relies on the efficiency of the emulsifiers to stabilize the cooled mixture against agglomeration or Ostwald’s ripening. In general, use of higher processing temperatures during hot homogenization produces small particles size due to decreased viscosity of the dispersed phase. However, this technique is unsuitable for formulating thermosensitive drugs as high operating temperatures may accelerate the degradation rate of certain drugs.

1.3.2.4.2. Cold homogenization

In case of cold homogenization, the first step of dispersion of drug in molten lipid remains the same. The drug loaded lipid is rapidly cooled using dry ice or liquid nitrogen and this solidified lipid is milled to give microparticles (50-100 microns in size). This large particles are dispersed in the cold aqueous solutions of surfactants and subjected to the high pressure homogenization at or below room temperature. The two major advantages of cold homogenization technique over hot homogenization is minimized
temperature-induced degradation of drug during processing and minimal partitioning of drug into aqueous phase during homogenization.

1.4. Glyceryl monooleate (GMO)/chitosan system

The Glyceryl monooleate (GMO)/chitosan system is a surface-modified nanoparticulate system consisting of GMO as a lipid component and chitosan as the coating polymer. The GMO/chitosan delivery system was developed by Trickler et al. for the sustained and targeted delivery of both hydrophobic (paclitaxel, dexamethasone) as well as hydrophilic drugs (gemcitabine) [74-76]. These formulations were prepared by multiple emulsion/solvent evaporation technique using large volume of 0.5% (w/v) polyvinyl alcohol (PVA) for stabilization. Recently, Pandit et al. reported sustained delivery and increased stability of ifosfamid in GMO/chitosan system and utilize 0.15% (w/v) Tocopherol polyethylene glycol succinate (TPGS), oleic acid and low volumes of 0.5% (w/v) PVA for the emulsification and stabilization of nanostructures [77]. Due to the toxicity issues associated with PVA, there is emerging concerns about the parenteral applications of this system. Change in the stabilizer of the formulation may modify its physicochemical and/or biological properties. The effect of different stabilizers on nanoparticles have been previously studied on different nanoparticulate systems [78,79]. Additionally, the influence of pressure as well as the number of cycles during high pressure homogenization process on the physicochemical properties of various nanoparticulate systems has been reported [80-82]. However, no information is currently available in the pharmaceutical literature on the effect of the type of stabilizer as well as the optimal high pressure homogenization procedure needed for the preparation of GMO/chitosan nanoparticulate system containing a hydrophobic drug like curcumin.
1.5. Objectives, hypothesis and specific aims

The objective of the present study was to develop and characterize solid lipid nanoparticles for oral delivery of curcumin using GMO/chitosan system with two different stabilizers. The underlying hypothesis for this investigation was that both the process of high pressure homogenization as well as the stabilizer used for preparation of the GMO/chitosan nanostructures may significantly affect its physicochemical properties when loaded with a hydrophobic drug like curcumin. The specific aims for this investigation were:

(i) Development and validation of an UPLC method for quantification of curcumin.

(ii) Development of a curcumin solid lipid nanoparticulate system using GMO/chitosan:

(a) Preparation and characterization of curcumin nanoemulsion containing PVA and poloxamer.

(b) Preparation, characterization and evaluation of curcumin solid lipid nanoparticles containing PVA and poloxamer.
CHAPTER 2

Analytical Method Development and Validation
2.1. Introduction

Curcumin is a naturally available hydrophobic, polyphenolic compound derived from the rhizomes (turmeric) of *Curcuma longa*. Turmeric consists of a mixture of curcuminoids that are natural analogues of curcumin. Commercially available grade of curcumin consists of three major curcuminoids namely, curcumin (77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%) (Figure 2.1). These curcuminoids have been shown to exhibit difference in their pharmacological activities[17].

![Curcuminoids](image)

Figure 2.1: Chemical structure of curcuminoids: curcumin, demethoxycurcumin, bisdemethoxycurcumin.

Various analytical methods have been reported for the quantification of curcuminoids. Earlier, the spectrophotometric methods were widely used to determine the total curcuminoid contents in the sample. However, it was not possible to quantify the individual curcuminoids in the sample by using these spectrophotometric methods. In 1953, liquid chromatographic method was developed for separation and quantification of
curcuminoids [83]. Several chromatographic methods such as thin-layer chromatography (TLC) [84], high performance thin-layer chromatography (HPTLC) [85] [86], high pressure liquid chromatography (HPLC) [87] [88] [89] have been developed for the separation and quantification of curcuminoids. Though HPLC method is most convenient, it has difficulties such as poor resolution, longer retention times, complicated mixtures of mobile phase with gradient elution, low sensitivity etc. Sensitive methods were developed to quantify lower levels of curcumin in plasma using LC-MS/MS techniques [90] [91]. Nevertheless, analysis by LC-MS method is a highly expensive. Recently, the commercially available ultra-performance liquid chromatography (UPLC) technique seems to be a good alternative. UPLC has the ability to withstand high back pressures that enables use of sub 2 µm particles for stationary phase. The UPLC technique has been proven to provide higher sensitivity, better resolution and faster separation of analytes[92]. For fast and simultaneous quantification of the above three curcuminoids, a validated UPLC method was developed by Cheng et al., 2010 [93]. The objective of this study was to develop and validate a sensitive UPLC method for the separation and quantification of curcuminoids using low volume of organic solvents.

2.2. Materials

Commercial grade of curcumin was purchased from Sigma Aldrich (St. Louis, MO). The pure reference standards of curcumin, demethoxycurcumin and bisdemethoxycurcumin were purchased from USP (Rockville, MD). Phosphoric acid, Optima LC/MS grade acetonitrile and water were purchased from Fischer Scientific (Fair Lawn, NJ). The Acquity BEH C18 column (50mm x 2.1mm, 1.7µm) was obtained from Waters (Milford, MA).
2.3. Methods

2.3.1. Chromatography

An UPLC analysis was performed on the curcumin samples using a reversed phase Waters Acquity system (Waters, Milford, MA) equipped with a quaternary solvent delivery pump, an autosampler and a photodiode array detector. The chromatographic separation of curcumin was achieved by isocratic elution at 30°C on a Waters Acquity BEH C \textsubscript{18} column (50mm x 2.1mm, 1.7µm). The mobile phase consisted of 0.05% (v/v) aqueous phosphoric acid:acetonitrile in a ratio of 66:34 (v/v). The apparent pH of the mobile phase was recorded to be 2.5 using an UB-5 UltraBASIC pH meter (Denver instruments, Bohemia, NY). The flow rate was maintained at 0.8mL/minute and the column effluents were monitored at the detector wavelength of 425 nm. The injection volume was 20µL and the total run time for each run was set for 4 minutes.

2.3.2. Preparation of solutions

2.3.2.1. Mobile phase

An aqueous portion of the mobile phase consisted of 0.05% (v/v) phosphoric acid in water. It was prepared by mixing 0.5 mL of phosphoric acid in 1000 mL of water. The solution was then filtered through a 0.2 µm Polyethersulfone (PES) filter (Millipore, Billerica, MA) and degassed for 5 minutes prior to be connected to the aqueous pump of UPLC system. The pH of this solution was recorded to be about 2.3. The aqueous and organic phase were pumped separately using the quaternary solvent pumps. For injection of the mobile phase through the column, the mobile phase mixture was prepared by
mixing 66 mL of 0.05% (v/v) of aqueous phosphoric acid to 34 mL of acetonitrile followed by 5 minutes of degassing. The apparent pH of the mobile phase was about 2.5.

2.3.2.2. Standard solution

A 500 µg/mL stock solution of curcumin was prepared in acetonitrile by dissolving 12.5 mg of curcumin in 25 mL of acetonitrile. Various standards were prepared from this stock solution after appropriate dilution with the mobile phase.

2.4. Calculations

The standard curve was obtained by plotting the peak area of the standards to their concentrations. The unknown concentration of curcumin was determined by interpolating from the regression equation relating to the peak area, obtained from the standard curve.

2.5. Results and discussion

2.5.1. Specificity

The United States Pharmacopeia (USP) defines specificity as the ability of a method to discriminate the analyte from all the potentially interfering substances like impurities, degradation products etc. The specificity of this UPLC method was investigated by comparing the chromatograms obtained from the injection of mobile phase without drug and the chromatogram of the mobile phase containing the drug. The representative chromatogram of the mobile phase without any drug is shown in Figure 2.2 (a) and the representative chromatogram of the mobile phase with the drug is shown in Figure 2.2 (b). The UPLC method successfully separated the three major curcuminoids present in the commercial grade of curcumin. Distinct peaks were obtained
for curcumin, demethoxycurcumin and bisdemethoxycurcumin with a retention time of 3.36, 2.90 and 2.49 minutes, respectively. The identity of these three peaks was confirmed by running standard solutions of each of these three curcuminoids using USP reference standards. No peak from the mobile phase was found to interfere with the drug peak. Thus, the specificity of this UPLC method for quantification of curcumin was confirmed.

**Figure 2.2:** A representative chromatograms of a) mobile phase and b) sample showing major peaks for curcumin (C), demethoxycurcumin (D) and bisdemethoxycurcumin (B) in the mobile phase.
2.5.2. Linearity

The USP defines linearity of an analytical method as its ability to produce test results that are directly proportional to the concentration of analyte in samples within a given range. Linearity was investigated on curcumin standards by injecting seven different standard solutions over a wide range of concentration. Calibration curves were constructed by plotting the peak area against the concentrations. The linearity was assessed by calculating the slope, y-intercept and spearman rank coefficient ($r^2$) using least squares regression. The standard curve for curcumin was found to be linear over the concentration range of 0.015 - 62.50µg/mL. A linear equation of the standard curve was obtained which provided the relation between the concentration of curcumin ($X$) and the peak area ($Y$) of the detector response which is described as $Y = 166185X + 9097.5$, $r^2 = 1$. The spearman rank coefficient ($r^2$) of value > 0.99 is acceptable for an analytical procedure. A high $r^2$ value suggests that there is a strong relationship between the peak area and the concentration in the given concentration range. Figure 2.3 shows a linear standard curve obtained by injecting curcumin standards.

![Standard curve of curcumin showing linearity over a concentration range of 0.015 to 62.5 µg/mL.](image)

**Figure 2.3:** Standard curve of curcumin showing linearity over a concentration range of 0.015 to 62.5 µg/mL.
2.5.3. Precision

As per the USP, precision of an analytical method expresses the degree of agreement between a series of measurements obtained from multiple sampling of a homogenous sample. The higher the precision, the closer are the values to each other on repeated measurements under identical experimental conditions. The precision of the current analytical method was assessed using the within day and day to day precision. For performing within day precision, a set of curcumin standard solutions was prepared and injected four times on the same day. Day to day precision was performed by injecting a set of curcumin standard solutions on five different days over a period of thirty days. The relative standard deviation (RSD) values were calculated for both within day as well as day to day precision and were found to be within acceptable limits. The results from the precision studies for the UPLC method are listed in Table 2.1.

**Table 2.1**: Within day and day to day precision for the UPLC analysis of curcumin

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Within day</th>
<th>Day to day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean peak area</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>0.02</td>
<td>5810.0 ± 270.8</td>
<td>4.66</td>
</tr>
<tr>
<td>0.06</td>
<td>14480.3 ± 247.7</td>
<td>1.71</td>
</tr>
<tr>
<td>0.24</td>
<td>47880.3 ± 323.1</td>
<td>0.67</td>
</tr>
<tr>
<td>0.98</td>
<td>179638.8 ± 962.3</td>
<td>0.54</td>
</tr>
<tr>
<td>3.91</td>
<td>713445.8 ± 6838.1</td>
<td>0.96</td>
</tr>
<tr>
<td>15.63</td>
<td>2698674.0 ± 12642.5</td>
<td>0.47</td>
</tr>
<tr>
<td>62.50</td>
<td>10641756.0 ± 52519.1</td>
<td>0.49</td>
</tr>
</tbody>
</table>
2.5.4. Accuracy

The USP defines accuracy of an analytical method as the closeness of test results obtained by the analytical method to the true value. The accuracy of an analytical method should be established across its range. The accuracy of the given UPLC method was investigated by injecting three quality control samples (0.49 µg/mL, 7.81 µg/mL and 31.25 µg/mL) five times along with the curcumin standards. The accuracy of the assay was determined by comparing the theoretical concentration with the measured concentration value using the following formula:

\[
\% \text{ Accuracy} = \frac{\text{Measured concentration}}{\text{Theoretical concentration}} \times 100
\]

The results of this study were found to be within acceptable limits and are depicted in Table 2.2.

**Table 2.2:** Accuracy results for the UPLC analysis of curcumin

<table>
<thead>
<tr>
<th>Actual concentration (µg/mL)</th>
<th>Measured concentration (µg/mL)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.49</td>
<td>0.46±0.01</td>
<td>94.21±1.81</td>
</tr>
<tr>
<td>7.81</td>
<td>7.91±0.10</td>
<td>101.37±1.31</td>
</tr>
<tr>
<td>31.25</td>
<td>31.01±0.48</td>
<td>99.22±1.53</td>
</tr>
</tbody>
</table>
2.6. Applications of the UPLC method

2.6.1. Determination of drug loading efficiency

The theoretical drug load was calculated by dividing the weight of the curcumin added to the formulation divided by the total weight of the formulation ingredients. The drug loading efficiency of the formulation was calculated experimentally. Briefly, 10 mg of the formulation was dispersed in 12 mL of 0.05% (v/v) phosphoric acid and 8 mL of acetonitrile. This suspension was centrifuged at 4000 rpm for 30 minutes at 15°C. The supernatant was filtered through 0.2µm syringe filter and analyzed for the drug content using UPLC method. The drug loading efficiency (%) of the formulation was determined as follows:

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

2.6.2. In-vitro release of curcumin from the formulation

The in vitro drug release profile of the nanoparticulate formulation with PVA and poloxamer was determined by measuring the % cumulative drug released with time. The in vitro release studies were carried out in water-jacketed side-by-side 3 mL glass diffusion chambers (PermeGear Inc., Hellertown, PA) containing both donor and receiver compartments. Three mL of Phosphate buffer (pH 7.4) with 0.5% (w/v) Tween 80 was placed in both donor and recipient compartments maintained at 37°C. About 10 mg of formulation was dispersed in the donor compartment. The donor and recipient compartments were separated by 0.1µm polycarbonate membrane (Millipore, Billerica,
MA) and the release media in both chambers were stirred using magnetic stirrers. For sample collection, the entire 3 mL of the recipient compartment was removed at specific time intervals and were replaced with equal volume of buffer to maintain the sink conditions. The samples were analyzed for curcumin content using the UPLC method.

2.6.3. Cellular uptake of curcumin in Caco-2 cells

The cellular uptake of curcumin from the solution and nanoparticulate delivery system containing PVA and poloxamer was evaluated in Caco-2 cell lines obtained from American Type Culture Collection, Manassas, VA. Caco-2 cells were cultured in a standard Falcon™ 12-well tissue culture plates at a seeding density of approximately 3x10^5 cells per well. Cells were incubated until confluency in a humidified chamber at 37°C. A 500µg/mL of stock solution of curcumin was prepared in ethanol. Confluent cell monolayers were treated with a single dose of curcumin solution (20 µM) or nanoparticles loaded with equivalent curcumin in Dulbecco’s Phosphate Buffered Saline (DPBS) for 30, 60 and 90 minutes. The cells were washed thrice with ice cold DPBS and lysed with 1% (v/v) triton-X-100. The cell lysates were collected in microcentrifuge tubes and centrifuged at 13,000 rpm for 30 minutes at 20°C (accuSpin Micro R, Fisher Scientific, Fairlawn, NJ). Twenty five µL of the supernatant was assayed for the total cellular protein content using the BCA protein assay (Pierce, Rockford, IL). The amount of curcumin in the supernatent was determined by the UPLC method. The cellular uptake was reported as the mean ± SD of curcumin content (in µg) per mg of total cellular protein (n=3).
2.6.4. Caco-2 permeability studies

Caco-2 cell monolayers were cultured on a polycarbonate filter of diameter 24.5 mm in BD Falcon Transwell 6 wells/plate (Franklin Lakes, NJ) at a seeding density of approximately $3 \times 10^5$ cells per well. The cells were cultured in a humidified chamber with 5% CO$_2$ atmosphere at $37^\circ$C to achieve a consistent monolayer (TEER values in the range of 350–380 $\Omega \text{cm}^2$). On the day of the experiment, these membranes were mounted between the donor and receiver compartments of the diffusion chambers (PermeGear Inc., Hellertown, PA). A stock solution of 500 µg/mL curcumin was prepared in ethanol. Three mL of DPBS (pH 7.4) was placed in the receiver compartment. Curcumin solution (20 µM) or nanoparticles dispersion with equivalent curcumin were prepared in DPBS and placed in the donor compartment. Samples (200 µL) were collected from the receiver compartment at specific time points and replaced with DPBS. The analysis of the samples was done using the UPLC method. The permeability from the apical to the basal compartment was studied for a period of 90 minutes.

2.7. Conclusions

A sensitive analytical method was successfully developed and validated for separation and quantification of curcumin using reverse phase UPLC. The UPLC method reported was capable of giving faster elution of curcumin with good resolution of the three major curcuminoids. The method involved low consumption of organic solvents as compared to other analytical techniques and accurately detected concentrations as low as 15ng/mL. The results were within the acceptable limits when validated for specificity,
linearity, precision and accuracy. Overall, a suitable analytical method was developed for rapid and accurate quantification of curcumin.
Chapter 3:

Effect of High Pressure Homogenization and Stabilizer on the Physicochemical Properties of Curcumin Loaded GMO/Chitosan Nanostructures
3.1. Introduction

Curcumin is a naturally available polyphenolic compound derived from the rhizomes (turmeric) of *Curcuma longa* [17]. It has demonstrated both chemopreventive and chemotherapeutic effects in several carcinogenic models and pre-clinical trials by cell cycle arrest, multiple apoptotic pathways and downregulation of transcription factors such as nuclear factor kappa B (NF-κB) [32,94]. However, its poor oral bioavailability due to extremely low aqueous solubility and extensive pre-systemic metabolism has been the major limitation for its use as a chemotherapeutic agent [11,31]. Additionally, it is photosensitive and degrades rapidly in presence of neutral and alkaline pH conditions [21,22]. In order to overcome these challenges several attempts have been made by formulating curcumin as polymeric nanoparticles, liposomes, cyclodextrin-complexes, solid dispersions etc. [47-51].

In past few decades, increased attention has been focused on the development of solid lipid nanoparticles as a colloidal drug carrier system due to its combined advantages of polymeric nanoparticles, fat emulsions and liposomes. The glyceryl monooleate (GMO)/chitosan system is a surface-modified nanoparticulate system consisting of GMO as a lipid component and chitosan as the coating polymer. Trickler et al. developed the GMO/chitosan delivery system for the sustained and targeted delivery of both hydrophobic (paclitaxel, dexamethasone) as well as hydrophilic drugs (gemcitabine) [74-76]. However, large volume of 0.5% (w/v) polyvinyl alcohol (PVA) was used as a stabilizer for these formulations. Though PVA has been widely used in the preparation of nanoparticles, there is some toxicity issues associated with its use. PVA is especially unsuitable for parenteral formulations due to its long-term carcinogenic potential [63].
Recently, Pandit et al. reported sustained delivery and increased stability of ifosfamide in GMO/chitosan system and utilize 0.15% (w/v) Tocopherol polyethylene glycol succinate (TPGS), oleic acid and low volumes of 0.5% (w/v) PVA for the emulsification and stabilization of nanostructures [77]. Poloxamer 407, a nonionic block copolymer of polyethylene oxide and polypropylene oxide, is a FDA approved stabilizer for oral, parenteral, ophthalmic and dermal formulations [65]. Effect of different stabilizers on nanoparticles have been previously studied on different nanoparticulate systems [78,79]. However, no studies have been reported to investigate the effect of use of HPH process or change in stabilizer on the physicochemical properties of GMO/chitosan system.

The influence of pressure as well as the number of cycles during high pressure homogenization process on the physicochemical properties of various nanoparticulate systems has been reported [80-82]. However, no information is currently available in the pharmaceutical literature on the effect of the type of stabilizer as well as the optimal high pressure homogenization procedure needed for the preparation of GMO/chitosan nanoparticulate system. Thus, the objective of the present study was to investigate the influence of high pressure homogenization procedure and stabilizers on some of the physicochemical properties, especially the particle size and the zeta potential of GMO/chitosan nanoparticulate system using curcumin as a model hydrophobic drug.

3.2. Materials

Commercial grade of curcumin was purchased from Sigma Aldrich (St. Louis, MO). Glyceryl monooleate and poloxamer 407 were purchased from Spectrum Chemicals (New Brunswick, NJ). Low molecular weight chitosan (MW 10000-12000 Daltons) and polyvinyl alcohol (MW 30000-70000) were obtained from Aldrich chemical
Co. (Milwaukee, WI). Tocopheryl polyethylene glycol 1000 succinate NF (TPGS) was sourced from PCCA (Houston, TX). Phosphoric acid, Optima LC/MS grade acetonitrile and water for UPLC analysis were purchased from Fischer Scientific (Fair Lawn, NJ). Caco-2 cells were purchased from American Type Culture Collection (Manassas, VA). The Eagle’s Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA, L-glutamine, sodium pyruvate, and non-essential amino acids were purchased from Invitrogen (Carlsbad, CA).

3.3. Methods

3.3.1. Formulation of the SLN systems

A 2.4% (w/v) chitosan solution was prepared by dissolving 2.4 g of chitosan in 100 mL of 2% (v/v) aqueous acetic acid solution. SLNs were prepared by using ultrasonication and high pressure homogenization followed by lyophilization. Briefly, molten GMO (2gm) and curcumin were dissolved in 5 mL of acetone. This solution was added drop-wise to 96 mL of 0.15% (w/v) TPGS solution and simultaneously sonicated at 18 W for 3 minutes (Microson XL2000 Misonix sonicator, Newtown, CT). To this primary emulsion, 20 mL of 0.5% (w/v) PVA solution or 0.1% (w/v) of poloxamer 407 and 24 mL of 2.4% (w/v) chitosan solution were added one after the other and hand homogenized (Biospec M133, Bartlesville, OK) for 2 min at 7000 rpm. The emulsion was subjected to 10 cycles of HPH (Microfluidics M110P, Newton, MA) at 15000 psi to give the final nanoemulsion. This nanoemulsion was further freeze-dried in a lyophilizer (Millrock Technology, Kingston, NY) to give solid lipid nanoparticles (n=3).
3.3.2. Analysis of particle size, surface charge and physical stability of the nanoemulsion

The particle size (PS) and the zeta potential (ZP) of the formulations were determined using zetameter (ZetaPlus, Brookhaven Instruments Corporation, Holtsville, NY). The PS and ZP of the nanoemulsion were measured at different stages during the preparation of nanoemulsion by diluting 100 µL of emulsion with 3.5 mL of deionized water. For nanoparticles, the lyophilized powder was suspended in deionized water at a concentration of 0.01 mg/mL. Each measurement was performed ten times and the values were reported as mean ± SD. The physical stability of the blank as well as 8% curcumin loaded nanoemulsions with PVA and poloxamer was investigated at room temperature (25°C) over a period of 60 days by determining the PS and ZP of the emulsions in triplicates at specific time points.

3.3.3. Scanning Electron Microscopy (SEM)

The surface morphology of the lyophilized drug loaded formulations was obtained using FEI Quanta 200 Scanning Electron Microscope (Hillsboro, OR). The samples were mounted on metal stubs and sputter coated with gold-palladium alloy (40 nm thickness) using a Hummer VI sputter coater (Anatech, NJ). The visualization was carried out at an accelerating voltage of 25kV under vacuum of approx. 1.22 x 10⁻³ Pascal.

3.3.4. Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (Shimadzu DSC-60, Kyoto, Japan) was connected to a thermal analysis operating system (Shimadzu TA-60WS, Kyoto, Japan). About 3 to 5 mg of sample was crimped non-hermetically in an aluminum pan and heated from room temperature to 300°C at a rate of 10⁰C/minute under nitrogen purge. DSC
studies were also carried out on curcumin (pure drug), blank NP with PVA, curcumin NP with PVA, blank NP with poloxamer and curcumin NP with poloxamer using empty non-hermetically crimped aluminum pan as the reference. Another set of DSC studies were performed on curcumin NP with PVA and curcumin NP with poloxamer using blank NP with PVA and blank NP with poloxamer as the reference, respectively.

3.3.5. Thermogravimetric analysis (TGA)

A thermogravimetric analyzer (Shimadzu TGA-50, Kyoto, Japan) was connected to a thermal analysis operating system (Shimadzu TA-60WS, Kyoto, Japan). About 5 mg sample was weighed into aluminum pans and heated from room temperature to 300°C at a heating rate of 10°C/minute under nitrogen purge. The weight loss in the sample was reported (n=3).

3.3.6. Karl Fischer titrimetry

The moisture content in the blank and curcumin loaded nanoparticles was determined using Karl Fischer titration (Mettler DL18 Karl Fischer titrator, NJ, USA). A biamperometric titration was carried out with about 10-15mg of sample. The percentage moisture content of the nanoparticles was determined in triplicate samples.

3.3.7. X-ray diffraction (XRD) analysis

The X-ray diffraction analysis of the curcumin (pure drug), blank NP with PVA, curcumin NP with PVA, blank NP with poloxamer and curcumin NP with poloxamer was performed at room temperature. The sample was filled in a copper holder and exposed to Cu Kα radiation (40 kV x 40 mA) in a wide angle X-ray diffractometer (Bruker D8 Advance, Madison, WI). The instrument was operated in a step-scan mode, in 0.05°
20 increments, and counts were accumulated for 1.0 second at each step over the angular range of 5-40° 2θ.

### 3.3.8. UPLC analysis

An UPLC method reported by Cheng et al. with modifications was used to quantify the curcumin content in the samples using a reversed-phase Acquity UPLC system (Waters, MA, USA) [93]. The chromatographic separation of curcumin was achieved by isocratic elution at 30⁰C on an Acquity BEH C₁₈ column (50mmx2.1mm, 1.7µ). The mobile phase consisted of 0.05% (v/v) aqueous phosphoric acid:acetonitrile in a ratio of 66:34 (v/v). The flow rate was maintained at 0.8 mL/minute and the column effluents were monitored at the detector wavelength of 425 nm. The total run time was set to be 4 minutes.

### 3.3.9. Determination of drug loading efficiency

The theoretical drug load was calculated by dividing the weight of the curcumin added to the formulation divided by the total weight of the formulation ingredients. The drug loading efficiency of the formulation was calculated experimentally. Briefly, 10 mg of the formulation was dispersed in 12 mL of 0.05% (v/v) phosphoric acid and 8 mL of acetonitrile. This suspension was centrifuged at 4000 rpm for 30 minutes at 15⁰C. The supernatant was filtered through 0.2µm syringe filter and analyzed for the drug content using UPLC. The drug loading efficiency (%) of the formulation was determined as follows:
Drug Loading Efficiency

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

3.3.10. In vitro release studies

The in vitro drug release profile of the nanoparticulate formulation with PVA and poloxamer was determined by measuring the percentage of cumulative drug released with time. The in vitro release studies were carried out in water-jacketed side-by-side 3 mL glass diffusion chambers (PermeGear Inc., Hellertown, PA) containing both donor and receiver compartments. Three mL of phosphate buffer (pH 7.4) with 0.5% (w/v) Tween 80 was placed in both donor and receiver compartments maintained at 37\(^{\circ}\)C. About 10 mg of formulation was dispersed in the donor compartment. The donor and receiver compartments were separated by 0.1\(\mu\)m polycarbonate membrane (Millipore, Billerica, MA) and the release media in both chambers were stirred using magnetic stirrers. For sample collection, the entire 3mL of the recipient compartment was removed at specific time intervals and were replaced with equal volume of buffer to maintain the sink conditions. The samples were analyzed for curcumin content using the UPLC method.

3.3.11. Determination of cellular uptake

The cellular uptake of curcumin from the solution and nanoparticulate delivery system containing PVA and poloxamer was evaluated in Caco-2 cell lines. Caco-2 cells were cultured in a standard Falcon\textsuperscript{TM} 12-well tissue culture plates at a seeding density of approximately 3x10\(^5\) cells per well. Cells were incubated until confluence in a humidified chamber at 37\(^{\circ}\)C. A 500\(\mu\)g/mL of stock solution of curcumin was prepared in ethanol.
Confluent cell monolayers were treated with a single dose of curcumin solution (20 µM) or nanoparticles loaded with equivalent curcumin in Dulbecco’s Phosphate Buffered Saline (DPBS) for 30, 60 and 90 minutes. The cells were washed thrice with ice cold DPBS and lysed with 1% (v/v) triton-X-100. The cell lysates were collected in microcentrifuge tubes and centrifuged at 13,000 rpm for 30 minutes at 20°C (accuSpin Micro R, Fisher Scientific, Fairlawn, NJ). Twenty five µL of the supernatant was assayed for the total cellular protein content using the BCA protein assay (Pierce, Rockford, IL). The amount of curcumin in the supernatant was determined by the UPLC method. The cellular uptake was reported as the mean±SD of curcumin content (in µg) per mg of total cellular protein (n=3).

3.3.12. Sub-cellular localization studies

The sub-cellular localization of the delivery systems was evaluated in Caco-2 cells. Briefly, Caco-2 cells were cultured on BD Falcon 8-chamber slides at a seeding density of 60,000 cells per chamber and incubated overnight in a humidified chamber at 37°C. The cells were treated with curcumin nanoparticles (20 µM) containing PVA and poloxamer in DPBS spiked with lysotracker red (25 nM) for 15 and 60 minutes. The adherent cells were washed three times in cold DPBS and fixed with 1% (v/v) glutaraldehyde. The wells were removed and the cells were further stained with mounting media consisting of DAPI and sealed with coverslips. The slides were viewed on a multi-photon confocal microscope (Carl Ziess, Germany) at the Nebraska Center for Cell Biology, Creighton University.
3.3.13. Caco-2 permeability studies

Caco-2 cell monolayers were cultured on a polycarbonate filter of diameter 24.5 mm in BD Falcon Transwell 6 wells/plate (Franklin Lakes, NJ) at a seeding density of approximately $3 \times 10^5$ cells per well. The cells were cultures in a humidified chamber with 5% CO$_2$ atmosphere at 37$^\circ$C to achieve a consistent monolayer (TEER values in the range of 350–380 Ωcm$^2$). On the day of the experiment, these membranes were mounted between the donor and receiver compartments of the diffusion chambers (PermeGear Inc., Hellertown, PA). A stock solution of 500 µg/mL curcumin was prepared in ethanol. Three mL of DPBS (pH 7.4) was placed in the receiver compartment. Curcumin solution (20 µM) or nanoparticles dispersion with equivalent curcumin were prepared in DPBS and placed in the donor compartment. Samples (200 µL) were collected from the receiver compartment at specific time points and replaced with DPBS. The analysis of the samples was done using the UPLC method. The permeability from the apical to the basal compartment was studied for a period of 90 minutes.

3.3.14. MTT toxicity assay

The cytotoxicity of the curcumin solution and nanoparticulate formulation was determined in Caco-2 cells using the MTT assay. Caco-2 cells were seeded in 96 well plates at a seeding density of 12000 cells per well in 100 µL of MEM media supplemented with 20% FBS, 10% L-glutamine, 10% sodium pyruvate, 10% non-essential amino acids and 10% penicillin streptomycin. The cells were incubated overnight in a humidified chamber with 5% CO$_2$ atmosphere at 37$^\circ$C. The cells were treated for 4 hours with different concentrations of curcumin solution and nanoparticles.
containing equivalent amount of curcumin prepared in DPBS. Following the 4 hours of exposure time, the treatments were removed and cells were supplied with fresh MEM media and allowed to incubate in the humidified chamber for 24, 48 and 72 hours. After incubation period, the cells were treated with 30 µL solution of MTT reagent (5 mg/ml) in DPBS and incubated for 4 hours. The treatment was removed and the cells were lysed using 100 µL of lysing reagent (20% (w/v) SDS solution: Dimethyl formamide in 1:1 ratio) and incubated for 30 minutes. The plates were mixed on a shaker for 15 minutes and the absorbance was analyzed on a microplate reader at 550 nm.

3.3.15. Statistical data analysis

The statistical analysis of the experimental data for the purpose of comparison was performed using single factor ANOVA. The test was considered to be statistically significant if P< 0.05.

3.4. Results

3.4.1. Particle size, zeta potential and physical stability of nanoemulsions

The PS and ZP of nanoemulsion with 0%, 4% and 8% (w/w) curcumin loading were determined at several stages of preparation of nanoemulsion (primary emulsion, emulsion before HPH, after 3 cycles of HPH and after 10 cycles of HPH) as shown in Figure 3.1 and 3.2.
Figure 3.1: Effect of HPH cycles, drug load and stabilizer on particle size of nanoemulsion with a) 0.5% PVA and b) 0.1% poloxamer*

Figure 3.2: Effect of HPH cycles, drug load and stabilizer on zeta potential of nanoemulsion with a) 0.5% PVA and b) 0.1% poloxamer*

*Stage 1= Primary emulsion  Stage 3= HPH 3 cycles
Stage 2= Nanoemulsion before HPH  Stage 4= HPH 10 cycles

The PS of the emulsion was found to increase as the drug loading was increased from 0% to 8% in both PVA as well as poloxamer containing nanoemulsions. In all the cases, the PS of the primary emulsion increased after the addition of chitosan. A 50 to 65%
reduction in PS of nanoemulsion was found merely after 3 cycles of HPH. After passing through a total of 10 cycles of HPH very modest reduction in PS was observed. There was no significant difference in the PS of nanoemulsions with PVA and poloxamer (P>0.05). In all the cases, the ZP of the primary emulsion was found to be negative and it became relatively positive after addition of chitosan to the formulation. As shown in Table 3.1, the PS and ZP of all the nanoemulsions stored at room temperature over a period of 60 days showed only negligible or no changes indicating the physical stability of this system.

Table 3.1: Particle size and zeta potential of nanoemulsion studied at 25°C over a period of 60 days.

<table>
<thead>
<tr>
<th>Days</th>
<th>Blank NP with PVA</th>
<th>Blank NP with poloxamer</th>
<th>Curcumin NP with PVA</th>
<th>Curcumin NP with poloxamer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS</td>
<td>ZP</td>
<td>PS</td>
<td>ZP</td>
</tr>
<tr>
<td>0</td>
<td>220.4±6.4</td>
<td>15.81±0.4</td>
<td>217.8±1.6</td>
<td>4.21±2.0</td>
</tr>
<tr>
<td>7</td>
<td>244.5±4.6</td>
<td>11.93±1.3</td>
<td>209.9±1.0</td>
<td>3.82±0.9</td>
</tr>
<tr>
<td>14</td>
<td>226.8±1.8</td>
<td>18.63±1.2</td>
<td>195.2±1.3</td>
<td>5.49±1.5</td>
</tr>
<tr>
<td>21</td>
<td>244.9±6.7</td>
<td>18.63±1.1</td>
<td>197.1±1.5</td>
<td>6.77±0.6</td>
</tr>
<tr>
<td>28</td>
<td>217.9±2.9</td>
<td>14.13±1.0</td>
<td>207.7±1.2</td>
<td>7.84±0.9</td>
</tr>
<tr>
<td>35</td>
<td>237.4±5.2</td>
<td>13.92±1.0</td>
<td>217.2±1.6</td>
<td>4.22±0.8</td>
</tr>
<tr>
<td>60</td>
<td>222.2±4.2</td>
<td>12.43±1.2</td>
<td>191.1±1.2</td>
<td>5.21±1.1</td>
</tr>
</tbody>
</table>

3.4.2. Particle size and zeta potential of nanoparticles

The particle size and zeta potential of blank and drug-loaded nanoparticulate system is summarized in Table 3.2. These nanoparticles (NP) were prepared by lyophilization of the nanoemulsions described earlier. The curcumin loaded NP showed significantly larger particle size than the respective blank NP. The increase in particle
size from blank to drug loaded nanoparticles varied from 1.2 to 1.6 times. Irrespective of the drug loading, there was no significant difference in the particles size of NP with PVA and NP with poloxamer. Also, there was no significant effect of drug loading on the zeta potential of the nanoparticles with PVA and poloxamer. However, the NP with PVA was found to have more positive zeta potential as compared to NP with poloxamer.

**Table 3.2:** Particle size and zeta potential of the blank and drug loaded formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank NP with PVA</td>
<td>297.1±25.4</td>
<td>23.3±1.9</td>
</tr>
<tr>
<td>Blank NP with poloxamer</td>
<td>262.2±10.8</td>
<td>14.7±2.3</td>
</tr>
<tr>
<td>Curcumin NP with PVA</td>
<td>354.7±27.3</td>
<td>24.1±3.7</td>
</tr>
<tr>
<td>Curcumin NP with poloxamer</td>
<td>428.2±43.1</td>
<td>12.8±1.3</td>
</tr>
</tbody>
</table>

**3.4.3. Scanning electron microscopy analysis**

**Figure 3.3:** SEM micrographs of (a) curcumin nanoparticulate system with PVA at a magnification of 3000x showing a highly porous structure, (b) curcumin nanoparticulate system with poloxamer at a magnification of 3000x showing a smooth surface.

The surface morphology of the lyophilized curcumin nanoparticulate formulations with PVA as well as poloxamer was studied using scanning electron microscopy. **Figure**
3.3(a) represents the SEM micrograph of curcumin NP with PVA while Figure 3.3(b) depicts the SEM micrograph of curcumin NP with poloxamer. The SEM micrograph in both the cases showed aggregation of NP after lyophilization. However, the lyophilized NP with PVA was found to have highly porous morphology compared to poloxamer. The formulation containing poloxamer showed a smooth surface as compared to PVA formulation.

3.4.4. Differential Scanning Calorimetry

![DSC thermogram](image)

**Figure 3.4(a):** An overlay of DSC thermograms of curcumin (pure drug), blank nanoparticles with PVA and curcumin loaded NP with PVA.

An overlay of DSC thermograms of curcumin (pure drug), blank NP with PVA and curcumin NP with PVA is represented in Figure 3.4(a). Curcumin showed a sharp endothermic peak at 179.5°C that corresponds to its reported melting point. The blank NP with PVA showed a diffused endothermic peak around 100°C due to possible dehydration and an exothermic peak around 184°C possibly due to the degradation of chitosan. Curcumin loaded nanoparticles with PVA also showed a diffused endothermic
peak below 100°C. However, neither the melting peak of curcumin nor the exothermic peak around 180°C was visible in the thermograms of drug loaded formulation.

**Figure 3.4(b):** An overlay of DSC thermograms of blank nanoparticles with PVA, curcumin loaded NP with PVA and curcumin loaded NP with blank NP as reference.

The thermograms of curcumin loaded formulations with PVA using blank NP as the reference is depicted in **Figure 3.4(b)**. Blank nanoparticles were used in the reference pan to compensate for the thermal activities of all the formulation additives in the nanoparticulate formulation. When blank NP with PVA was used as reference material for heat flow, the curcumin nanoparticles with PVA showed an endothermic peak at 179.2°C. A similar trend was seen in thermograms of curcumin NP with poloxamer as shown in **Figure 3.4(c)** and **Figure 3.4(d).**
**Figure 3.4(c):** An overlay of DSC thermograms of curcumin (pure drug), blank nanoparticles with poloxamer and curcumin loaded NP with poloxamer.

**Figure 3.4(d):** An overlay of DSC thermograms of blank nanoparticles with poloxamer, curcumin loaded NP with poloxamer and curcumin loaded NP with blank NP as reference.
3.4.5. Thermogravimetric analysis and Karl Fischer titrimetry

**Table 3.3:** Percentage moisture content and weight loss of the blank and drug loaded formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>% Moisture content by Karl Fischer titration (n=3)</th>
<th>% Weight loss by TGA analysis (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank NP with PVA</td>
<td>3.58±1.43%</td>
<td>4.29±0.21%</td>
</tr>
<tr>
<td>Curcumin NP with PVA</td>
<td>3.54±0.97%</td>
<td>3.55±1.13%</td>
</tr>
<tr>
<td>Blank NP with poloxamer</td>
<td>3.35±1.71%</td>
<td>4.29±0.45%</td>
</tr>
<tr>
<td>Curcumin NP with poloxamer</td>
<td>3.35±1.10%</td>
<td>3.38±0.97%</td>
</tr>
</tbody>
</table>

The percentage weight loss detected by TGA analysis and the moisture content (in %) determined by Karl Fischer titration is reported in **Table 3.3**. There was no significant difference in the percentage moisture content of all the formulations tested. Similarly, no significant difference was found in the % weight loss for all the formulations analyzed.

3.4.6. X-ray diffraction analysis

An overlay of powder XRD patterns of curcumin (pure drug), blank NP with PVA and curcumin NP with PVA is shown in **Figure 3.5**. The XRD analysis of curcumin gave a unique fingerprint pattern. The curcumin loaded NP with PVA was shown to have several identical characteristic peaks of curcumin. Similarly, the curcumin loaded NP with poloxamer showed several characteristic peaks of curcumin as depicted in **Figure 3.6**. These characteristic fingerprints of curcumin were absent in the blank NP of both the formulations.
**Figure 3.5**: An overlay plot of XRD patterns of curcumin (pure drug), Blank NP with PVA and curcumin NP with PVA.

**Figure 3.6**: An overlay plot of XRD patterns of curcumin (pure drug), Blank NP with poloxamer and curcumin NP with poloxamer.
3.4.7. *In vitro* release of curcumin

![Graph](image)

**Figure 3.7:** The *in vitro* release profiles of curcumin nanoparticles with PVA and poloxamer in pH 7.4 phosphate buffer.

The drug loading efficiency of the curcumin nanoparticulate delivery system with PVA and poloxamer was experimentally determined to be 72.06±9.8% and 77.85±1.6%, respectively. There was no significant difference in the drug loading efficiency of the formulations by change in the stabilizer from PVA to poloxamer. The *in vitro* release pattern of curcumin from the formulation is depicted in **Figure 3.7** as a plot of % cumulative drug release vs. time. In order to understand the mechanism of drug release from the formulation, the release data was plotted into several kinetic models. The best linearity was found by fitting the data in Higuchi’s square root equation plot as shown in **Figure 3.8**. The plot of % cumulative drug release vs. square root of time was found to be linear with $r^2$ value of 0.9811 and 0.9862 for curcumin NP with PVA and poloxamer, respectively. The release of curcumin from NP with poloxamer was significantly slower compared to release from NP with PVA.
**Figure 3.8:** A plot of % cumulative drug released vs. square root of time representing the Higuchi model of release kinetics.

### 3.4.8. Cellular uptake of curcumin

**Figure 3.9:** The cellular uptake of curcumin from the solution and nanoparticulate system in Caco-2 cells at different time points.
The \textit{in vitro} cellular uptake of curcumin in Caco-2 cell line following the treatment with free curcumin solution, curcumin NP with PVA and curcumin NP with poloxamer is presented in \textbf{Figure 3.9}. The cellular uptake of curcumin from the NP with PVA was significantly higher than the free drug solution at all the time points. The uptake of curcumin from the NP with poloxamer was significantly higher than the free drug solution at 60 and 90 minutes. It was found that the amount of curcumin accumulated in Caco-2 cells per mg of protein was approximately 2.5-fold higher in NP with PVA as compared to NP with poloxamer at all the time points. The uptake of curcumin from the solution was found to be decreasing with time, at the time points investigated. However, in case of both the nanoparticulate formulations, there was no significant difference in amount of curcumin accumulated per mg of protein between 60 and 90 minutes of treatment.

\textbf{3.4.9. Sub-cellular localization studies}

\begin{figure}[h]
\centering
\begin{subfigure}{0.45\textwidth}
\includegraphics[width=\textwidth]{figure3a.png}
\caption{a)}
\end{subfigure}
\begin{subfigure}{0.45\textwidth}
\includegraphics[width=\textwidth]{figure3b.png}
\caption{b)}
\end{subfigure}
\caption{Sub-cellular localization of curcumin NP with PVA after a) 15 minutes and b) 60 minutes of treatment.}
\end{figure}
The sub-cellular localization of curcumin nanoparticles containing PVA and poloxamer is depicted in Figure 3.10 and 3.11. The curcumin nanoparticle appeared green due to the fluorescence of curcumin while the DAPI stained the cell nucleus blue. The lysosomes appeared red due to staining from lysotracker red. The fluorescence signal from curcumin was found to be too weak. In Caco-2 cells with 15 and 60 minutes treatment, the delivery system appeared to be internalized in the lysosomes because of co-localization of the green fluorescence of curcumin with lysotracker red.

3.4.10. Caco-2 permeability studies

The permeability of curcumin from apical to basal compartment across the Caco-2 cells was investigated. However, no curcumin was detected by the UPLC method for both solutions as well as nanoparticulate formulations being investigated for the 90 minute studies.

Figure 3.11: Sub-cellular localization of curcumin NP with poloxamer after a) 15 minutes and b) 60 minutes of treatment.
3.4.11. MTT toxicity assay

The percentage cell survival of the intestinal Caco-2 cells after treatment with curcumin solution and nanoparticles is depicted in Figure 3.12-3.14. The dose response curve demonstrated that at higher concentrations GMO/chitosan based nanoparticles containing PVA as well as poloxamer had more cytotoxicity as compared to curcumin solution. The higher cell death was also seen with blank nanoparticles indicating the toxicity to the Caco-2 cells even from the blank formulations.

![MTT assay on Caco2 cells (24 hours)](image)

**Figure 3.12:** Cytotoxicity profile of curcumin solution, blank nanoparticles and curcumin loaded nanoparticles containing PVA and poloxamer after 24 hours incubation.
Figure 3.13: Cytotoxicity profile of curcumin solution, blank nanoparticles and curcumin loaded nanoparticles containing PVA and poloxamer after 48 hours incubation.

Figure 3.13: Cytotoxicity profile of curcumin solution, blank nanoparticles and curcumin loaded nanoparticles containing PVA and poloxamer after 72 hours incubation.
3.5. DISCUSSION

3.5.1. Formulation of the delivery system

An oil-in-water nanoemulsion of curcumin was prepared by using GMO/chitosan system as reported by Pandit et al. with modifications [77]. GMO formed the lipid phase of the emulsion while chitosan was used as a coating material. TPGS, a surfactant for (o/w) emulsions with a HLB value of 13 [58], was used to form the primary emulsion. For stabilization of the nanoemulsion, either 0.5% (w/v) PVA or 0.1% (w/v) poloxamer was used. After formation of a primary emulsion through sonication, further reduction in PS was achieved through high pressure homogenization process. This was one of the biggest challenges while preparing nanoemulsions by sonication only. HPH has been reported to be a more efficient method of preparation of submicron sized solid lipid dispersions as compared to ultrasonication or high shear mixing [70]. Thus, the primary emulsion obtained from ultrasonication was passed through HPH in order to get solid lipid dispersions with smaller PS. For the preparation of the drug loaded nanoemulsions 4%, 8% and 10% (w/w) of curcumin loading was investigated. However, 10% (w/w) loaded curcumin nanoemulsions were difficult to pass through high pressure homogenizer. Hence, 8% (w/w) was found to be an optimal drug load for curcumin in GMO/chitosan system. The effect of change in stabilizers on physicochemical and biological properties of GMO/chitosan system was further investigated.

3.5.2. Particle size, zeta potential and physical stability of nanoemulsions

The PS of the curcumin loaded NP was found to be significantly larger than the blank NP in both PVA as well as poloxamer formulation. This could be explained by the
following two reasons. Firstly, as the drug loading increased there is an increase in the concentration of the drug in the emulsion. This also changes the hydrophobicity of the system since the drug is extremely water insoluble. As a result, stabilizers used for the stabilization of nanoemulsions were not enough for the final emulsion stability. Due to the increase in the concentration of the drug and the hydrophobicity of the particles, the interfacial coverage of surfactant on the dispersed particles is decreased which in turn provides less stability to flocculation and agglomeration of particles [95]. Secondly, increase in the drug loading may lead to increase in the viscosity of the emulsion. It has been previously reported for various types of homogenization device that the breaking of droplets becomes more difficult as the viscosity of the disperse phase increases [96]. The increase in the PS of the primary emulsion after addition of chitosan may be due to coating of chitosan on the surface of GMO particles that causes an increase in the diameter of the particles. Only small reduction in PS was observed after 10 cycles of HPH as compared to PS from 3 cycles. This indicates that 3 cycles of HPH, which produced 50 to 65% PS reduction, is optimal for the preparation of nanoemulsions by this system. The negative zeta potential of the primary emulsion may be contributed by the GMO. Chitosan has a positive charge in acidic solutions due to the presence of protonated amino groups [68]. This explains the relatively positive charge of the emulsion after addition of chitosan to the primary emulsion. The physical stability data indicates that the blank as well as curcumin-loaded nanoemulsions were physically stable at 25⁰C for a period of at least 60 days, irrespective of the stabilizer used.
3.5.3. Particle size, zeta potential and surface morphology of SLN

The increase in PS of the drug-loaded NP as compared to the blank NP can be explained by the increase in droplet size of the curcumin nanoemulsion, as explained earlier. On replacing 0.5% (w/w) PVA as a stabilizer with 0.1% (w/w) poloxamer, there was no significant change in the PS of both blank as well as curcumin loaded nanoparticles. This suggests that poloxamer 407 is more efficient stabilizer for GMO/chitosan system as compared to PVA as the stabilizer concentration (% w/v) could be reduced five times by using poloxamer. However, it should be noted that the molar concentrations of these two stabilizers used were quite close. The molar concentration of PVA was in the range of 71.5-166.7 µM while that of poloxamer was 68.5-101.6 µM. As mentioned earlier, PVA has been reported to be toxic [63]. Reduction in the concentration of a more toxic stabilizer by a substantial amount is not only considered effective but also safe. However, the zeta potential of the NP with PVA was significantly higher compared to that of poloxamer. The SEM micrographs of the delivery systems with PVA and poloxamer showed an obvious difference in the surface morphology. The curcumin NP with PVA were found to be highly porous compared to the curcumin NP with poloxamer. The surface morphology of the formulation has been reported to be dependent on process parameters as well as the type and concentration of stabilizers used [97-99]. Thus, the difference in morphology of the two formulations may be explained by change in stabilizer of the lyophilized nanoparticulate system from PVA to poloxamer.
3.5.4. UPLC analysis

Commercially available grade of curcumin consists of three major curcuminoids namely, curcumin (77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%) [17]. A validated UPLC method was used to separate the above three curcuminoids from the samples. The retention time of curcumin, demethoxycurcumin and bisdemethoxycurcumin was 3.3, 2.9 and 2.4 minutes, respectively. The samples were quantified by analyzing the curcumin peak of the chromatogram.

3.5.5. In vitro release studies

The drug loading efficiency of nanoparticles with PVA and poloxamer showed no significant difference with 8% (w/w) of theoretical drug load. The in vitro release studies of curcumin nanoparticles were carried out in pH 7.4 buffer at 37°C to simulate the intestinal conditions. The release profile was obtained for the two formulations as a plot of % cumulative drug released vs. time. The release patterns show a slower release of drug over the experimental period. The hydrophobicity of curcumin might have prevented the excessive surface binding on hydrophilic chitosan leading to small burst release. To investigate the drug release mechanism from the nanoparticles, several release kinetic models were investigated. The plot of % cumulative drug release vs. square root of time was found to be linear indicating that release of curcumin from these delivery systems followed Higuchi’s square root equation. Therefore, the mechanism of drug release from these delivery systems was by diffusion-controlled release from an insoluble matrix [100]. The release of curcumin from the NP with poloxamer was found to be significantly slower compared to release from NP with PVA. The effect of stabilizer and
process parameters on the drug release profile has been previously reported for nanoparticulate delivery systems [78]. The surface morphology has been reported to be one of the critical parameter that affects the release rate of drug from the formulation [101,102]. The highly porous surface of nanostructures with PVA provided higher surface area as compared to nanostructures with poloxamer. This possibly led to a faster drug release from nanoparticles with PVA because of the relatively shorter diffusion pathway as compared to nanoparticles with poloxamer [103].

3.5.6. Thermal analysis, moisture content and X-ray diffraction

The sharp endothermic peak in DSC thermogram of curcumin at 179.46°C corresponds to the melting of curcumin. The nanoparticulate formulations with both PVA as well as poloxamer showed a diffused endothermic peak around 100°C. To characterize this broad endothermic peak obtained in the DSC thermograms, the weight loss of these formulations was analyzed up to a temperature of 120°C by TGA analysis. However, the weight loss could be due to dehydration or desolvation. Therefore, Karl Fischer titration was performed to determine the moisture content of these formulations. As there was no significant difference in the % weight loss detected by TGA analysis and % moisture content determined by Karl Fischer titration, the endothermic peak in DSC thermograms of the formulations was confirmed to be from the moisture in the formulation. An exothermic peak was observed in blank nanoparticles around 184°C due to possible degradation of the formulation components. Neither the melting (endothermic) peak of curcumin nor the chitosan degradation (exothermic) peak was visible in the thermograms of drug loaded formulation. As these were two opposite thermal events around the same temperature range, the net effect might have been nullified.
In order to test this hypothesis and determine the physical state of the drug in the formulation, the TGA analysis of curcumin loaded formulations was performed using blank NP as the reference. In such a case, any difference in heat flow would be due to presence of drug. When blank NP was used as reference material for DSC analysis, the curcumin nanoparticles showed an endothermic peak around 179°C indicating the melting of curcumin. The powder XRD studies showed curcumin fingerprints in the drug loaded NP which were absent in blank nanoparticulate system. This confirms that the drug (curcumin) was present in a crystalline state in both PVA as well as poloxamer containing nanodelivery systems at a drug load of 8% (w/w) in the formulation. To the best of our knowledge, this is the first experimental report indicating that a misleading conclusion can be drawn from the DSC results if two opposite physical events occur at the same temperature range in the DSC thermograms.

3.5.7. Cellular uptake studies

The cellular uptake of curcumin from the solution and nanoparticulate delivery system was investigated at different time points. The results indicate that the uptake of curcumin from the nanoparticulate system continued somewhat longer (about 60 minutes) as compared to the curcumin solution. The maximum curcumin uptake from the solution was found at 30 minutes, after which it decreased with time. The total amount of curcumin per mg of protein content taken up by the Caco-2 cells was higher in nanoparticulate system at 60 and 90 minutes as compared to the solution. Also, the decrease in the amount of curcumin into the cells decreased faster from the solution as compared to the nanoparticulate formulations. A similar phenomenon was observed by Yadav and co-workers. with curcumin solution and cyclodextrin-complex curcumin in
Caco-2 cells wherein the curcumin concentration peaked in Caco-2 cells at 45 minutes followed by decrease in curcumin concentration within the cells [104]. The higher uptake of curcumin from PVA as compared to poloxamer can be attributed to the higher surface charge of nanoparticles with PVA as compared to poloxamer. It has been reported that the positive charge on the surface of particles enhances its cellular uptake due to increased interaction of particles with the cell membrane [105-107].

3.5.8 Sub-cellular localization and Caco-2 permeability studies

The sub-cellular localization studies showed that the curcumin loaded nanoparticles of PVA as well as poloxamer were co-localized with lysozyme of the cells. Although several mechanisms are available for internalization of nanoparticles, the present work shows that the nanoparticles were possibly taken up into the Caco-2 cells by endo-lysosomal mechanism. The accumulation of nanoparticles within the cells is energy-dependent processes like endocytosis. It has been reported that smaller particle size of the nanoparticles favors their internalization [108,109]. The results from the cellular uptake studies further confirmed the accumulation of curcumin nanoparticles in the Caco-2 cells. The permeability studies showed that the curcumin transported across the Caco-2 cells, if any, was not detectable. This suggests that the permeability of curcumin across these cells is low with both solutions as well as nanoparticulate system. Wahlang and co-workers in 2011 studied the permeability of curcumin through Caco-2 cells and reported that the intestinal first-pass metabolism as well as accumulation of curcumin within the cells played a role in the poor permeability of curcumin [110]. They investigated the permeability of curcumin solution across the Caco-2 cells using 170 µM of curcumin. About 12% of curcumin was reported to be metabolized with >20% being
accumulated in the Caco-2 cells. The investigation of curcumin permeability across the Caco-2 cells with curcumin concentration being as high as 170 µM was not feasible with our work due to the toxicity issues from the nanoparticulate system.

3.5.9. MTT toxicity assay

The MTT toxicity assay on Caco-2 cells indicates the higher toxicity of the nanoparticulate system containing both PVA as well as poloxamer as compared to the solution. The increased toxicity of curcumin nanoparticles may be attributed to higher accumulation of curcumin within the Caco-2 cells as compared to the solution. At higher concentrations, the blank nanoparticles also showed cell death that was approximately constant in 24, 48 and 72 hours. This suggests that the GMO/chitosan nanoparticulate formulation by itself possibly caused necrotic cell death. Also, the percent survival of Caco-2 cells was found to decrease with time in curcumin formulations. The percent cell survival was less after 72 hours as compared to 24 hours incubation suggesting the apoptosis caused by curcumin in Caco-2 cells.

3.6. Conclusions

The effect of process parameters and stabilizers on the GMO/chitosan nanoparticulate system was studied using a model hydrophobic drug, curcumin. The process of HPH was very efficient in reducing the PS of nanoemulsions by 50 to 65% which could not be achieved by sonication alone. Three cycles of HPH were found to be optimal for the preparation of GMO/chitosan nanostructures with a hydrophobic drug loading. Poloxamer 407 was found to be more efficient stabilizer as compared PVA for GMO/chitosan system containing curcumin because it gives stable nanoemulsions at a %
(w/v) concentration five times less than PVA. It can be concluded that the process parameters and the type of stabilizer used for the preparation of GMO/chitosan nanostructures affects several physicochemical properties of the nanoparticulate system such as PS, ZP as well as cellular uptake in Caco-2 cells.
CHAPTER 4:
Summary and Future Directions
4.1. Summary

Stable GMO/chitosan nanoemulsions of curcumin were prepared with two different stabilizers using high pressure homogenization process. Three cycles of high pressure homogenization and 8% (w/w) of curcumin loading was optimal for preparation of curcumin GMO/chitosan nanoemulsion with PVA as well as poloxamer. The process of high pressure homogenization successfully reduced the particle size of the nanoemulsions by 50 to 65% after three cycles. Loading the GMO/chitosan nanoemulsion with a hydrophobic drug like curcumin resulted in an increase in the particle size of both PVA as well as poloxamer containing formulations. A five times reduction in the concentration of stabilizer was achieved without affecting the particle size of the nanoemulsion when the stabilizer was changed from 0.5% (w/v) PVA to 0.1% (w/v) of poloxamer 407.

The blank and curcumin loaded nanostructures with PVA as well as poloxamer were obtained by lyophilization of the respective nanoemulsions. Irrespective of the drug loading, there was no significant change in the particle size or moisture content of nanostructures containing PVA and poloxamer. However, the surface charge and the surface morphology were modified by change in the stabilizer used. The SEM micrographs illustrated a more porous structure of nanoparticulate system containing PVA as compared to poloxamer. The DSC and powder XRD data revealed the crystalline nature of drug in both the nanoparticulate systems. By using blank nanoparticles as a reference in the DSC studies, the present research work illustrated a classic example of the fact that absence of any thermal events in DSC thermograms may be misleading if two opposite thermal events occur at the same temperature range in a formulation.
A sensitive UPLC method was developed and validated for the accurate and precise quantification of curcumin content as low as 15ng/mL. The chromatographic separation was achieved by isocratic elution at 30°C on an Acquity BEH C18 column (50mm x 2.1mm, 1.7µ). The mobile phase consisted of 0.05% (v/v) aqueous phosphoric acid: acetonitrile in a ratio of 66:34 (v/v). The flow rate was maintained at 0.8 mL/minute and the column effluents were monitored at the detector wavelength of 425 nm. The total run time was set to be 4 minutes.

The drug loading efficiency of nanostructures with PVA and poloxamer was found to be 72.06±9.8% and 77.85±1.6% respectively showing no significant difference. The in vitro release studies in pH 7.4 buffer at 37°C revealed that the release of curcumin from poloxamer containing formulation was slower as compared to PVA. Both the formulations were found to follow Higuchi’s square root equation of drug release suggesting that the drug release from these nanodelivery systems follow the diffusion-controlled mechanism. The cellular uptake of curcumin from Caco-2 cells at 60 and 90 minutes was higher in both the nanoparticulate systems than the drug solution. Uptake of curcumin from nanostructures containing PVA was approximately 2.5-fold higher in Caco-2 cells as compared to poloxamer at all the time points. The amount of curcumin taken up by the cells decreased faster in solution as compared to nanoparticulate delivery system.

4.2. Future directions

The present study elucidated the changes in physicochemical properties of GMO/chitosan system when a hydrophobic drug was used for drug loading. However, the
hydrophobicity of drugs used in nanoparticulate system may also modify the properties of nanoparticulate system. Therefore, as a part of future studies, the influence of these process and formulation parameters on the physicochemical and biological properties of GMO/chitosan system should also be investigated with drugs of different hydrophobicity (e.g. using hydrophilic drugs).

The chemical stability of curcumin in these nanostructures should be evaluated at different pH conditions and compared with free drug solutions. Finally, the in vivo studies should be performed on these nanostructures to correlate the results obtained from the in vitro studies. For the animal studies, rodents such as Sprague-Dawley rats or female BALB/c mice should be used to determine the oral bioavailability, efficacy as well as the toxicity of these formulations.


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