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by

Brian Joseph Cuevas
To my loving wife, Cheska Cuevas; parents, Elbert and Darnell Cuevas; sister, Amy Cuevas; and grandparents, for their unconditional love, support, and patience throughout my educational experiences
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<tr>
<td>MMAC</td>
<td>Murine Mammary Adneocarcenoma.</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance.</td>
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<tr>
<td>ASTM</td>
<td>America standard testing method.</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier.</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin.</td>
</tr>
<tr>
<td>CAB</td>
<td>Cellulose acetate butyrate.</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system.</td>
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<td>CMC</td>
<td>Sodium carboxymethyl cellulose.</td>
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<tr>
<td>CMS</td>
<td>Casein microspheres.</td>
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<tr>
<td>CSF</td>
<td>Blood-cerebrospinal fluid barrier.</td>
</tr>
<tr>
<td>Cx</td>
<td>Donates the concentration of crosslinker (mg/ml).</td>
</tr>
<tr>
<td>D/C ratio</td>
<td>Dispersed to continuous phase volume ratio.</td>
</tr>
<tr>
<td>DCE</td>
<td>1,2-Dichloroethane.</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubelco’s Minimial Essential Media.</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleac acid.</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid disodium salt: dehydrate.</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-Nnitrosourea.</td>
</tr>
<tr>
<td>EVAc</td>
<td>Poly(ethylene-co-vinylacetate).</td>
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<tr>
<td>GCMS</td>
<td>Gelatin/carboxymethyl cellulose mesospheres.</td>
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GEN  Genipin.

G₀  Nonproliferating phase.

G₁  Intermitotic phase.

G₂  Premitiotic phase.

GF  Growth fraction.

GMS  Gelatin mesospheres.

GTA  Glutaraldehyde.

HDCT  High-dose chemotherapy.

HPLC  High performance liquid chromatography.

HSA  Human serum albumin.

IA.  Intraarterial.

IP  Intraperitoneal administration.

IT  Intratumoral administration.

IV  Intravenous administration.

KDa  Kilo Daltons.

M  Mitotic phase.

mM  Millimolar.

Mₚ  Molecular weight of gelatin’s repeat unit (g/mol).

MTT assay  Colorimetric assay.

Mₖw_{gelatin}  Weight average molecular weight of gelatin (g/mol).

MXN  Mitoxantrone.

MXN-GCMS  In situ mitoxantrone loaded gelatin/carboxymethyl cellulose mesospheres.

MXN-GMS  In situ mitoxantrone loaded gelatin/carboxymethyl cellulose mesospheres.
<table>
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<tr>
<td>mOsm</td>
<td>Milliosmoles.</td>
</tr>
<tr>
<td>MXN</td>
<td>Mitoxantrone hydrochloride or mitoxantrone.</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride.</td>
</tr>
<tr>
<td>OR</td>
<td>Ommaya Reservoir.</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline.</td>
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<tr>
<td>PDI</td>
<td>Polydispersity index.</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane.</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate).</td>
</tr>
<tr>
<td>PS</td>
<td>Poly(styrene).</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet –visible spectrometry</td>
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<tr>
<td>RG-2</td>
<td>Rat Glioma 2.</td>
</tr>
<tr>
<td>S</td>
<td>DNA synthesis phase.</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy.</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid.</td>
</tr>
<tr>
<td>W\text{dry}</td>
<td>Dry weight.</td>
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<tr>
<td>W\text{hydrated}</td>
<td>Hydrated weight.</td>
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<tr>
<td>%MEQ</td>
<td>Percent molar equivalence.</td>
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SYNTHESIS AND PROPERTIES OF PROTEIN MICRO/MESOSPHERE-DRUG
COMPOSITIONS DESIGNED FOR INTRATUMORAL CANCER THERAPY

By
Brian Joseph Cuevas

August 2003

Chair: Eugene P. Goldberg
Major Department: Materials Science and Engineering

In view of the systemic toxicity and limited efficacy of conventional chemotherapy, new localized cancer treatment modalities are needed. The goal of this research was therefore the synthesis and evaluation of injectable loaded micro/mesospheres using mitoxantrone (MXN), a chemotherapy agent. Processing parameters for the preparation of smooth, spherical albumin, casein (CMS), gelatin (GMS), and gelatin/carboxymethyl cellulose (GCMS) micro/mesospheres were determined using a steric stabilization process. The effects of processing conditions on particle size and particle morphology were evaluated. Crosslinker density effects were examined using various concentrations of glutaraldehyde. In parallel studies, GMS and GCMS synthesis was studied using a novel crosslinker, Genipin. GCMS compositions were also prepared using different concentrations of carboxymethyl cellulose in an attempt to enhance to cationic drug loading and release.
Following initial albumin microspheres process studies and synthesis of 14 w/w % MXN-loaded albumin compositions for evaluation in a 16/C murine mammary adenocarcinoma, emphasis was placed on casein and gelatin compositions. Synthesis studies revealed that particle size decreased with increasing concentrations of GTA for CMS, whereas no effect on particle size was observed with varied crosslinker concentrations for the preparation of GMS and GCMS.

Smooth spherical *in situ* MXN loaded gelatin mesospheres (MXN-GMS) [11 to 12% w/w MXN with varying crosslinker, GTA and GEN and crosslinker concentrations] with a mean particle size of 2 um, respectively, were synthesized. Also, *in situ* MXN loaded gelatin/carboxymethyl cellulose mesospheres (MXN-GCMS) (8 % w/w MXN) with a mean particle size of 2 um were prepared with GTA. These MXN loaded protein micro/mesospheres were designed for localized IT therapies for the treatment of cancer.

Under *in vitro* evaluations, MXN-GMS exhibited a decrease in percent release (78 to 65 % MXN) with increasing GEN concentration and (65 to 50 % MXN) with increasing GTA concentration. MXN-GMS’s MXN release was limited to the first 4 hrs whereas; MXN-GCMS exhibited sustained release exceeding 240 hrs.

*In vitro* MXN treatments of Rat glioma 2 cells were shown to prevent and inhibit cellular proliferation. The greatest decrease in RG-2 proliferation was found to be at high MXN doses and utilizing combinatory treatments (MXN plus MXN loaded mesospheres). Combinations of free MXN with free gelatin or MXN loaded mesospheres surpassed the single therapies (MXN only, MXN-GMS and MXN-GCMS) at hindering RG-2 cellular proliferation and prevented cellular regeneration.
The validity of using MXN-GMS compositions for IT therapies in the treatment of high mortality cancers was further supported with the\textit{ in vivo} 16/C murine mammary adenocarcinoma evaluations, which displayed increased survival with greatly reduced systemic toxicity.
CHAPTER 1
INTRODUCTION

To overcome the deficiencies of conventional cancer treatments, localized delivery of biodegradable devices has been shown to provide high concentrations of therapeutic agents directly to the tumor cells for prolonged periods of time, with minimal systemic toxicity.  

1 A recent review compared conventional systemic chemotherapy to non-systemic treatments.  

Systemic treatments have shown prolonged survival for several cancer types, but for the high mortality cancers, i.e., brain, breast, lung, and colorectal carcinomas, little improvement has been achieved. This is reflected in the Surveillance, Epidemiology, and End Results (SEER) 1973-1997 and Cancer Statistics Review (NCI-NIH 2000).  

An alternative to systemic treatments is the utilization of intratumoral (IT) chemotherapy, which may allow for the administration of higher dose chemotherapy or immunotherapy agents directly into the tumor site with little systemic toxicity.  

Initial research focused on the synthesis of mitoxantrone loaded albumin microspheres and the effects of processing conditions on particle size, drug loading and release, and in vivo safety and efficacy. In vivo preclinical evaluations showed that mitoxantrone-loaded albumin microspheres crosslinked with glutaraldehyde were successfully used in the treatment 16/C murine mammary adenocarcinoma. MXN-loaded microspheres were administered by IT injection at doses as high as 48 mg/kg (LD 50 for intravenous was 6.6 mg/kg) with minimal systemic toxicity. The median day of death for the mice for IT MXN-loaded microspheres at 48 mg/kg was 43 days, and 22 days for
mice treated with IT free drug 8mg/kg and 7.5 days for the untreated controls. The IT free-drug toxic dose for MXN was estimated at 12 mg/kg.

These studies were co-investigated with Drs. Almond and Hadba\textsuperscript{4,5} and demonstrated that high dose chemotherapy can be administered with minimal systemic toxicity to achieve prolonged survival of mice bearing high mortality tumors. The following research was sparked based on the initial successes achieved using albumin microspheres.

**Specific Aims**

The overall goal of this research was to optimize synthesis methods in the preparation of novel casein, and gelatin micro/mesospheres and evaluate mitoxantrone loaded micro/mesosphere compositions for use in localized IT chemotherapies. In view of the systemic toxicity and limited efficacy of conventional chemotherapies, new treatment modalities are needed. Specific aims of this research were the following:

**Aim 1: Synthesis and Properties of Casein and Gelatin Micro/Mesospheres**

**Casein synthesis.** Processing parameters were optimized, i.e., (concentrations, dispersing energy, solvents, washing, and drying) for steric stabilization process used in the synthesis of casein microspheres. After optimization studies were completed, the effects of varying stirring speeds (1000, 1250, and 1500 rpm) and glutaraldehyde concentrations (1.0, 2.5 and 4.0 wt %), \([80, 120, \text{ and } 300 \% \text{ MEQ}]\) on dry particle size and morphology were evaluated.

**Casein characterization.** The average dry particle size, distribution, and morphology were qualitatively observed with SEM, and the average dry particle size and distribution were quantitatively measured with a particle size analyzer.
Gelatin synthesis. Processing parameters were optimized, i.e., (concentrations, dispersing energy, solvents, washing, and drying) for steric stabilization process used in the synthesis of gelatin mesospheres. Crosslinked gelatin films were synthesized to evaluate the validity of genipin as a chemical crosslinker. The modulus, tensile strength, and the percent swellability were determined with varying crosslinkers, genipin and glutaraldehyde, and crosslinker concentrations, 2.3 (C1); 11.3 (C2); and 20.3 (C3) [20; 100; 200 %MEQ] and 2.5 (C1); 4.5 (C2) and 6.5 wt % (C3) [100, 200, and 300 %MEQ], respectively.

After optimization studies were completed, the effects of varying crosslinker (genipin and glutaraldehyde) and crosslinker concentrations 2.3 (C1); 11.3 (C2); 20.3 (C3) and 29.3 wt % (C4) [20, 100, 200, and 300 %MEQ] and 0.5 (C1); 2.5 (C2); 4.5 (C3) and 6.5 wt % (C4) [20, 100, 200, and 300 % MEQ], respectively, on dry particle size, distribution, and morphology were evaluated.

An anionic polysaccharide, carboxymethyl cellulose, was blended with a gelatin solution to produce blended mesospheres. The effects of varying GEN and GTA [200 % MEQ], respectively, and the concentration of CMC (0.0, 5.0, 9.0, and 13.0 w/w %) on dry particle size and distribution, and particle morphology were evaluated.

Gelatin characterization. The modulus and tensile strength of crosslinked gelatin films were determined by tensile measurements made according to ASTM D 412-97. Percent swelling was determined by weighing dry and hydrated gelatin films.

The average dry particle size, distribution, and morphology of gelatin mesospheres were qualitatively observed with scanning electron microscopy and the average dry particle size and distribution were quantitatively measured with a particle size analyzer.
Aim 2: Preparation and Characterization of *In situ* Mitoxantrone Loaded Gelatin Micro/Mesosphere Compositions

**Synthesis.** Based on pre-established conditions, *in situ* mitoxantrone loaded gelatin and gelatin/carboxymethyl cellulose mesospheres were synthesized. The effects of varying the crosslinker (genipin and glutaraldehyde) and crosslinker concentrations 2.3 (C1) and 20.3 (C2) wt % [20 and 200, % MEQ], respectively, and 0.5 (C1) and 4.5 (C2) wt % (C4) [20 and 200 % MEQ], respectively, in the synthesis of MXN-GMS on dry particle size, distribution, and morphology; percent loading; swellability; and percent release were evaluated. The optimized conditions of 13.0 w/w % carboxymethyl cellulose and 4.5 wt % glutaraldehyde [200 % MEQ] were used in the synthesis of gelatin carboxymethyl cellulose mesospheres and the dry particle size, distribution, and morphology and percent loading; and release were determined and compared to comparable gelatin mesospheres.

**Characterization.** 1) The average dry particle size, distribution, and morphology were qualitatively observed with SEM and the average dry particle size and distribution quantitatively measured with a particle size analyzer. Particle percent swellability was quantitatively measured with optical microscopy and image analysis software. 2) An enzymatic digestion buffer was used to determine the MXN content of *in situ* MXN-loaded gelatin mesosphere compositions. After enzymatic degradation, the supernatant was analyzed using a UV-Visible Spectrophotometer to determine drug concentration. 3) MXN-loaded, gelatin mesosphere compositions were incubated in phosphate buffered saline. Aliquots collected from the *in vitro* release studies were analyzed using a UV-Visible Spectrophotometer to determine the percent release.
Aim 3: *In vitro* and *In vivo* evaluations of MXN loaded Gelatin Mesosphere Compositions

**In vitro treatments.** Various *in vitro* evaluations were designed to study the efficacy of using free MXN and bound MXN delivery systems in the treatment of rat glioma cells (RG-2). These studies examined the dose response of MXN (25.0; 12.5; and 0.5 ppm) and delivery modality (free or bound). There were twelve different treatment groups excluding respective controls: 1) MXN only, 2) MXN and gelatin, 3) MXN loaded GTA C1 crosslinked gelatin mesospheres, 4) MXN loaded GTA C2 crosslinked gelatin mesospheres, 5) MXN loaded GEN C1 crosslinked gelatin mesospheres, 6) MXN loaded GEN C2 crosslinked gelatin mesospheres, 7) MXN-GCMS, 8) Free MXN plus MXN loaded GTA C1 crosslinked gelatin mesospheres, 9) Free MXN plus MXN loaded GTA C2 crosslinked gelatin mesospheres, 10) Free MXN plus MXN loaded GEN C1 crosslinked gelatin mesospheres, 11) Free MXN plus MXN loaded GEN C2 crosslinked gelatin mesospheres, 12) Control (no treatment and/or MXN).

**In vitro characterization.** Cytotoxicity of the treatments was observed by monitoring the cell proliferation using colorimetric assay (MTT based) and morphology using bright field optical microscopy, respectively.

**In vivo evaluations.** *In vivo* evaluations were designed to study the efficacy of using MXN loaded gelatin micro/mesosphere compositions for IT therapies in a 16/C Murine Mammary Adenocarcinoma (MMAC). MXN-GMS *in vivo* evaluations were co investigated with Amanda York and Shema Freeman.
CHAPTER 2
BACKGROUND

Introduction

Each year in the United States, 1 million people are diagnosed with cancer. Of those people diagnosed, 500,000 die, which is approximately one person every sixty two seconds. About 76,000 children between the ages of 3 to 14 perish. Cancer will strike 3 out of 4 families, which results in substantial physical, emotional, and financial burdens. It is estimated that the total cost of cancer in the United States is 72 billion dollars a year, 22 billion in indirect medical costs, and 50 billion in lost wages and productivity.

Cancer is a debilitating disease whereby normal cells are transformed genetically. The transformation leads to cells which grow and divide uncontrollably. The overwhelming growth of the mutant cells results in the invasion and destruction of surrounding and distant tissue. Although cancer has similar characteristics of a bacterial infection, it is not recognized as a foreign body, and the body can only mount a partial immune response. The immune system’s inability to recognize mutant cells is in part due to the fact that mutant cells arise from normal cells and have a very similar cell surface biochemistry. Eventually, the sheer number of dividing mutant cells overwhelms the immune mechanisms and continues to grow.

Cancer

The average healthy American has approximately 350 billion cells dividing on any given day. The heritable change in one of these cells may lead to the production of daughter cells that are missing the feedback control system, which signals the cells to
stop growing. After the transformation, the mutant cells may continue to divide \textit{(ad infinitum)} and never fully reach a differentiated state. The genes coding differentiation may be suppressed and genes coding proliferation may be left “on.” The growth may be limited only by supply of nutrients and immunological responses.\textsuperscript{10}

The growth of small tumors may be limited by diffusion of oxygen, nutrients, and the removal of waste by surrounding capillaries. Capillaries have to be $\sim 150$ $\text{um}$ from the tumor cells.\textsuperscript{11} Due to the diffusion limits, small tumors reach a steady state, whereby cell death and cell growth are constant. The time for these tumors to reach a detectable threshold varies from months to years depending on the type of cancer.\textsuperscript{12} Although the transition to a detectable tumor is not fully understood, tumors have been shown to release tumor angiogenesis factors, which induce the growth of capillaries into the tumor from surrounding tissue. Once the capillaries are formed, mutant cells become parasitic by rapidly consuming the amino acids, carbohydrates, and essential nutrients from the surrounding tissue. The parasitic actions lead to weight loss, which is the first sign of malignant tumor disease.\textsuperscript{13}

The rapid consumption of nutrients is contributed to high populations of dividing cells. The population of dividing cells, termed growth fraction (GF), is the fraction of total viable cell populations that are actually in an active division cycle.\textsuperscript{14, 15} Tumor cells have GFs between 20 and 70 %. The high GF makes tumor cells very susceptible to the cytotoxic effects of chemotherapy drugs. Unfortunately, distinguishing between normal (e.g., bone marrow, gastrointestinal, and hair follicles) and tumor cells with high GFs is very difficult. For example, bone marrow, which produces granulocytes and platelets,
has a GF of 30% and is susceptible to the damaging effects of chemotherapy, which leads to bleeding and infections.¹⁵

Systemic toxicity is not the only limitation for the utilization of chemotherapy treatments. The effectiveness of the chemotherapy drugs is limited by tumor size. Small tumors are more assessable for anticancer treatments due to their high GF and lower chance of metastases.¹⁴,¹⁶ On the other hand, larger solid tumors have a considerable amount of dead tissue. This tissue can reduce the efficacy of the treatment by preventing complete tumor perfusion. Larger, solid tumors also have been shown to have a smaller GF, a greater probability for metastases, and an overwhelming number of malignant cells, which inhibit the uptake of the drugs and allows for continuing cell growth. The cell cycle is divided into an intermitotic phase, \( G_1 \); DNA synthesis phase, \( S \); premitotic phase, \( G_2 \); mitotic phase, \( M \); and population of viable cells that are not proliferating, \( G_0 \). Tumor cells are made up of a population of cells that are proliferating; proliferating but dormant; and dying. There are also a certain number of viable cells that only divide if they are stimulated, and these are more likely to survive chemotherapy and regenerate.¹⁰ The cytotoxic effects of chemotherapy drugs are only effective against proliferating cells. Therefore, prolonged release of anticancer drugs is needed. Prolonged release can be obtained by intratumoral (IT) injection of biodegradable protein mesospheres loaded with anticancer agents.⁴,⁵,¹⁷,¹⁸ Protein mesospheres may maximize the uptake of the drug and inhibit tumor regeneration, with minimal systemic toxicity.

Cancer Therapies

The success of cancer therapies is determined by its effectiveness to kill the malignant cells with minimal systemic damage. The first recorded systemic treatment of cancer was in 1865, when a patient with leukemia showed positive results after being
treated with potassium arsenite, (Flower’s solution).\textsuperscript{19} Ironically, the first effective anticancer drug, nitrogen mustard, was developed for use as a weapon of mass destruction, and not for treating cancer. Nitrogen mustard, which was synthesized from sulfur mustard, War Gas, was first used in World War I as a chemical weapon. Sulfur mustard caused extreme irritation and had severe toxic effects which produced leucopenia, aplasia of bone marrow, dissolution of lymphoid tissue, and ulceration of the gastrointestinal tract.\textsuperscript{20} These toxic effects are indicative of cellular attacks of cells with elevated GF, therefore ideally suited for attacking malignant cells. Another very interesting facet was in 1931, the initial studies utilized intratumoral (IT) injections of sulfur mustard to treat squamous carcinomas. The rationale at that time for IT therapies was that sulfur mustard was too toxic for systemic injections.\textsuperscript{21} Now, some seventy years later, studies are underway to change the “Standard of Care” from systemic to localized IT treatment of high mortality cancers because of the toxicities associated with systemic therapies, which had been shown by these initial studies but largely overlooked until recently.

In 1942, the first clinical trials were conducted to determine the efficacy of nitrogen mustard in a patient with lymphosarcoma. Due to wartime restraints, its usefulness was not known until a review was published by Gilman and Philips in 1946. This review marks the beginning of chemotherapy.\textsuperscript{22}

After the success of the first clinically effective nitrogen mustards, mechloretamine (NH\textsubscript{2}),\textsuperscript{23} a second class of anticancer drugs, antimetabolites, was investigated. These drugs structurally resembled natural metabolites, which were necessary for cellular functions and disrupted the metabolic functions.
The first successful antimetabolite was aminopterin (methotrexate). Methotrexate was the first reported drug-induced cure for cancer, and still today, it remains one of the most widely used antifolates.

The excitement derived from the initial successes of these anticancer drugs lead to the investigation of a host of new therapeutic agents, which are graphically represented in Figure 2.1. These drugs encompassed a wide array of anticancer activities, i.e., antimitabolites, covalent and noncovalent DNA binding, inhibitors of chromatin function and affecting endocrine function.
**Mitoxantrone**

The class of anticancer drug of interest in this research is the noncovalent DNA-binding drugs, which were originally discovered by screening tests for cytotoxic activity for antibiotics. This research places an emphasis on mitoxantrone. Intercalating drugs form a tight drug-DNA interaction via interaction between the paired bases of DNA, as shown in Figure 2.2.

![Figure 2.2 Schematic diagram of the intercalation of mitoxantrone into DNA](image)

There are several different intercalating drugs routinely used clinically:

- Anthracyclines (Doxorubicin and Daunorubicin); Mitoxantrone; and Dactinomycine.

Mitoxantrone (MXN), (1,4-dihydroxy-5, 8-bis-((2-((2-hydroxyethyl)amino)ethyl)amino)-9, 10-anthracenedione dihydrochloride (Figure 2.3) or its trade name Novantrone®, is a synthetic anthracycenedione, which is available commercially for clinical use. Like the anthracyclines drugs, MXN has the planar polycyclic aromatic ring structure, which permits intercalation into DNA. However, MXN does not have the
sugar moiety and does not exhibit the cardiotoxicity due to the productions of quinine type free radicals of anthracyclines.\textsuperscript{33}

![Chemical structure of mitoxantrone](image)

Figure 2.3 Chemical structure of mitoxantrone

MXN has been shown to be cytotoxic throughout the cell cycle, but cells in the late “S” phase are more sensitive.\textsuperscript{33} The cytotoxicity of MXN is contributed to its ability to bind to DNA via different mechanisms: intercalation\textsuperscript{34} (highest affinity) and electrostatic\textsuperscript{35} (lowest affinity). MXN intercalation into DNA causes protein-linked, double-stranded DNA breaks, due to the disruption of the strand-reunion reaction of topoisomerase II.\textsuperscript{36} Some minor cytotoxicity can be observed from non-protein-linked, single stranded breakage of DNA,\textsuperscript{34, 35} which appears to be contributed to the oxidative activation of MXN.\textsuperscript{37}

MXN has been investigated in the treatment of metastatic and high-risk primary breast cancer by high-dose chemotherapy (HDCT).\textsuperscript{33} MXN was warranted for HDCT due to its lower cardiotoxicity and less severe side effects compared to other anthracyclines.\textsuperscript{38} Intraperitoneal (IP),\textsuperscript{39} intraarterial (IA),\textsuperscript{40} and intratumoral (IT)\textsuperscript{41-43} have also been evaluated as methods to deliver increased concentrations of MXN to tumor sites and decrease systemic damage.
**Chemotherapy Treatment Modalities**

Systemic treatments have shown prolonged survival for several cancer types, but for the high mortality cancers, i.e., brain, breast, lung, and colorectal carcinomas, little improvement has been achieved. Although all high mortality cancers are of interest for localized IT therapies, brain cancer treatment modalities will be reviewed in some detail.

Despite present day advances in chemotherapy and combinatory therapies in the treatment of malignant gliomas, only marginal improvements have been observed. In the U.S., approximately 17,000 residents are diagnosed with brain cancer annually, and of those approximately 12,000 die from the dreaded disease, with roughly half of these cases being gliomas.

The aggressiveness of gliomas is contributed to its ability to recognize, attach to, and migrate through normal tissue barriers. The invasiveness of this tumor reduces the effectiveness of conventional treatments, therefore allowing for the recurrence of the disease.

There are a host of restrictions for the utilization of chemotherapy in the treatment of gliomas. Traditional regimens of intravenous (IV) or oral drugs are not targeted to the central nervous system (CNS). For chemotherapy to be effective, adequate concentrations are needed at the tumor site, with minimal local and systemic toxicity. The restrictions of the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (CSF) require administration of high doses and exclude certain chemotherapy drugs that are effective in treatment of other tumors. The dose escalation to overcome the BBB and CSF causes hematopoietic effects. In order to overcome these effects, antilogous bone marrow rescue is performed after the administration of high dose chemotherapy. Essentially, the patient is brought back from the brink of death.
Although IV administration of chemotherapeutic agents is the most common route for the treatment of gliomas, IA and IT are alternatives to maximize the therapeutic effects with minimal systemic toxicity. IA chemotherapy achieves lower systemic toxic effects per dose due to high capillary permeability, high extraction fraction in the target organ, rapid metabolism and/or excretion.\textsuperscript{54-56}

Several strategies of bypassing the BBB by delivering the drugs directly to the brain have been developed utilizing localized IT therapies. One system administers the drug by direct injection or infusion through a catheter. An example of this technology is the Ommaya Reservoir (OR). The Ommaya Reservoir allows for intermittent, controlled infusion of the drug directly into the tumor. Another technique utilizes a subcutaneous implantable pump.\textsuperscript{57-59} Although a high concentration drug is administered to the tumor site, clinical complications such as infections, mechanical failures, and obstructions are observed.

The next generation of localized IT therapies is polymer based controlled release systems. The first systems were made up of non-biodegradable polymers such as polydimethylsiloxane (PDMS),\textsuperscript{60} poly(ethylene-co-vinylacetate (EVAe),\textsuperscript{61} poly(methyl methacrylate) (PMMA),\textsuperscript{62} poly(styrene) (PS) or ethylcellulose.\textsuperscript{63} Non-biodegradable polymers have very predictable and reproducible release profiles. Release is governed by diffusion processes, which are controlled by molecular weight, charge, and water permeability of the polymer matrix. The major limitation of these devices is the inability to degrade. The implant either remains as a foreign body or a second surgery is required.

Biodegradable technologies were investigated to obscure the limitations of the non-degradable systems. Two different biodegradable polymers have been investigated: 1)
natural polymers, (human or bovine serum (HSA or BSA), gelatin, alginate, chitosan, etc.,\textsuperscript{64-68} and synthetic polymers, (aliphatic polymers and polyanhydrides).\textsuperscript{69-71} In contrast to non-degradable polymers, drug diffusion is controlled by a combination of surface erosion and diffusion.

Gliadel\textsuperscript®} is currently the only commercially available biodegradable drug release device in the treatment of malignant gliomas.\textsuperscript{72} Gliadel\textsuperscript®} is a polyanhydride wafer that is loaded with desired chemotherapy agents and is surgically implanted into the tumor site. Phase I, II and III clinical trials demonstrated that biodegradable polymer drug carriers can be used safely and effectively in the treatment of brain cancer.\textsuperscript{72-74}

Recently, injectable microspheres loaded with various chemotherapy agents have been investigated in the treatment of gliomas.\textsuperscript{64, 75, 76} Injectable microspheres have the ability to perfuse the tumor site and surrounding tissue, and provide sustained release of high therapeutic doses with minimal systemic toxicity. This technology is similar to the commercially available Gliadel\textsuperscript®} device, but injectable microspheres utilize minimally invasive biopsy procedures unlike the surgical procedure necessary for the implantation of the polyanhydride wafer.

Several studies have evaluated the efficacy of local delivery of mitoxantrone for the treatment of malignant brain tumors in rats\textsuperscript{41, 42} and humans, \textit{in vitro}\textsuperscript{77} and \textit{in vivo}.\textsuperscript{43} Malignant brain tumors in rats were treated locally via intracranial implantation of a poly[bis(p-carboxyphenoxy propane) sebacic acid] (pCPP:SA) 20/80 molar ratio wafers loaded with mitoxantrone. The studies showed that increasing the concentration of mitoxantrone lead to an increase median day survival.\textsuperscript{41} Similar results of a possible increase in patients’ survival and increase in the number of long term survivors were seen
in human patients when mitoxantrone was delivered locally via an Ommaya Reservoir following radiation. These studies established that mitoxantrone delivered locally has promise in the treatment of malignant brain cancer.

**Glioma Models**

In order to evaluate the efficacy of chemotherapy in the treatment of high mortality cancers such as malignant gliomas; a valid animal model is needed to isolate promising therapeutic therapies. Since the mid-1970’s, the rat model has been used extensively, and has become the most widely used animal model. There have been four strategies employed in generating gliomas in animals: 1) chemical mutagen-induced models 2) xeno- and allograft transplantation-induced models 3) germline genetic modification-induced models and 4) somatic genetic modification-induced models.

The most common methods of generating gliomas used for preclinical trials are xeno- and allograft transplantation. Human or rat glioma cell-lines are injected subcutaneously in the flank or directly into the brain of rats to generate gliomas at relatively predictable rates that develop with high incidence. There are an abundant number of different animal models used in neuro-oncolgy. One of the oldest transplantable cell-lines is Rat Glioma 2 (RG-2). This tumor line was produced by IV administration of a single dose of 50 mg/kg of N-ethyl-N-nitrosourea (ENU) to pregnant CD Fischer rats on the 20\textsuperscript{th} day of gestation. The subsequent tumor was cloned and termed RG-2. RG-2 cells have been shown to grow very well in infinite cell culture and have a mean doubling time of 20 hours. RG-2 tumors cells were used to study the efficacy of various mitoxantrone and mitoxantrone loaded mesospheres treatment modalities at varying doses via \textit{in vitro} evaluations.
**Microsphere Synthesis**

**Steric Stabilization Process**

Casein microspheres and gelatin mesospheres were prepared using steric stabilization process used in the production albumin microspheres. This process consists (Figure 2.4) of (A) dispersing a small aqueous protein solution (dispersed phase) in an immiscible liquid (continuous phase) with the aid of a high speed paddle mixer (B), followed by crosslinking (C), centrifugation, and drying (D). Temperatures above the gelation temperature were used in the synthesis of gelatin mesospheres.

To develop a useful drug delivery system, the particle size and the crosslink density of the formed particles must be carefully controlled. The particle size controls the diffusion characteristics, release rates, and payload of the encapsulated drug. Small particles may exhibit poor loading efficiency; may migrate from the injection site; or may exhibit undesirably rapid release rates. On the other hand, large particles may not easily pass through a syringe needle.

The rate of release and degradation is greatly dependent upon the degree of crosslinking. Higher crosslink densities have been shown to exhibit a slower release. This may be contributed to the swelling of the crosslinked network.
Figure 2.4 Processing conditions for the synthesis of micro/mesospheres
The greater the crosslink density, the slower the infusion of water into the protein matrix, leading to reduced release rates and enzymatic degradation. By varying the crosslinker concentrations, the rate of release and degradation can be tailored to provide prolonged sustained release profiles.

The particle size may be controlled by adjusting the concentrations of the dispersed and continuous phases, the crosslink density, and the stirring rate. By controlling these factors, the desired particle size may be achieved.

A recent evaluation of the effects of processing conditions on the production of smooth spherical mitoxantrone loaded and unloaded albumin microspheres in the size range 4–30 µm has been completed. The effects on process conditions, albumin (BSA) concentration, cellulose acetate butyrate (CAB) stabilizer concentration, glutaraldehyde (GTA) crosslinker concentration, and dispersed to continuous phase volume (D/C) ratio, on particle size of microspheres were investigated. The data suggests that the particle size of mitoxantrone loaded and unloaded microspheres increased as protein concentration was increased or the stabilizer concentration was decreased. There was no evidence to suggest that the D/C ratio and crosslinker concentration had an effect on particle size. Drug loading of mitoxantrone was also shown to achieve loading efficiencies greater than 80%, regardless of particle size and crosslinker concentration.

Importance for the utilization of Genipin

Genipin is isolated from a parent compound, geniposide, which is obtained from gardenia (Gardenia jasminoides ELLIS) fruit. Genipin has been produced by enzymatic hydrolysis using β-geniposide or synthetically by the Buchi et al method. Its major function is to react with proteins to form a blue pigment, which was initially used
to produce food dyes. Recently, it has been used as a biological tissue fixative, as well as a crosslinker for biological patches, in the immobilization of enzymes and the preparation of gelatin microparticles. It has also been used in the preparation of polysaccharide microspheres. In traditional Chinese medicine, genipin has been used to treat inflammatory and hepatic diseases, as well as jaundice. Finally, it has been reported to have neuritogenic effects on cultured neuronal cells. Various crosslinking agents such as formaldehyde, glutaraldehyde, dialdehyde, dialdehyde starch, and epoxy compounds have been employed in the chemical modification of biological tissue. The chemical structures of glutaraldehyde and genipin are illustrated in Figure 2.5.

![Chemical structures of glutaraldehyde and genipin](image)

Figure 2.5 Chemical structures of glutaraldehyde and genipin

One of the major drawbacks in the utilization of these synthetic crosslinking agents, is the increased cytotoxicity to surrounding tissue. In order to develop a more biocompatible crosslinking agent, a naturally occurring reagent, genipin, has been investigated.

Several studies have been conducted on the stability of genipin fixed tissue. These studies have reported that genipin reacts primarily with the following amino acid groups:
lysine; hydroxylysine; argine; glutathione; and cysteine. Although the reaction mechanism is not fully understood, it has been suggested that it proceeds by nucleophilic attack from a primary amine on the olefinic carbon of genipin, which is followed by opening of the dihydropyran ring. The resulting aldehyde group is attacked by the secondary amine group forming a genipin-amino group. Figure 2.6 is a schematic representation of the reaction of genipin with collagen, which may form intramolecular or intermolecular crosslinks within the collagen fibers. This reaction mechanism shows that for every two moles of primary amine functional groups, it takes two moles of genipin to achieve a molecular crosslink. Although genipin was shown to have similar degrees of crosslinking (denaturation temperature after fixation), and mechanical strengths to glutaraldehyde fixed tissue, the rate of reaction was significantly lower than glutaraldehyde. Finally, the effects of pH, temperature, and concentration were investigated in controlling genipin fixation conditions.

The pH showed an increase in reactivity of genipin with pH from high to low: neutral pH (7.4 or 8.4) > basic pH (10.4) > acidic pH (4.0), whereas, temperature and concentration had little or no effect on reactivity.

Clinically, the most common crosslinking agent used in the biological fixation of tissue is glutaraldehyde. It has limited biocompatibility due to the development of calcification, lack of adaptability to the host tissue, and cytotoxic effects in vivo. As stated above, genipin was evaluated to eliminate the problems associated with glutaraldehyde. In order to compare the biocompatibility of genipin to glutaraldehyde, the cytotoxicity was also evaluated. Genipin was determined to be approximately 5,000~10,000 times less cytotoxic than glutaraldehyde.
Recently, it has been reported that genipin has been used in the crosslinking of chitosan microspheres. Chitosan microspheres were prepared by a water and oil suspension, and a spray dry method. The water and oil suspension was suspended by a mechanical mixer and crosslinked by the addition of genipin.

Particle size was reported to vary between 100 to 200 um with increasing chitosan concentration and decreasing the stirring rate, whereas, the particle size for the spray dried method was less than 10 um with size decreasing with increasing air flow-rate, and increasing viscosity of chitosan solution.
Biodegradable Protein Matrixes

Casein

Much of the reported use of casein in the synthesis of microspheres has been limited to research conducted by Dr Goldberg and/or Dr Jayakrishnan, who was a Post Doctorial Fellow under Dr Goldberg. Casein microspheres have been loaded with various chemotherapy agents, doxorubicin, 5-fluorouracil, and mitoxantrone. The efficacy of intratumoral injections of these various chemotherapy drug loaded casein microspheres have been evaluated in a Sp1017 mammary carcinoma and a Lewis lung carcinoma. In these investigations, drug-loaded casein microspheres were reported to reduce tumor volume and increase survival. Casein microspheres have also been loaded with progesterone, a steroid used to control fertility, and theophylline used in the treatment of acute and chronic bronchial asthma.

Casein proteins are made up of a family of phosphorylated proteins (αs1-, αs2-, β- and κ-caseins) and are the major constituent in bovine milk. Casein exists in milk as an aggregated form known as casein micelles. These micelles are responsible for the transportation of high concentrations of minerals, calcium and phosphate. These concentrations would exceed the saturation limit in the absence of casein and cause the pathological calcification of the mammary glands. Figure 2.7 is a schematic representation of the self-association of casein.

There are several rationales for the use of casein in the development of a drug delivery system. Casein is a natural, amphiphilic protein, whose main biological function is to transport nutrients. It has enhanced biocompatibility and the ability to be biodegradable. Finally, there is a relatively inexpensive, abundant supply of this protein.
Gelatin

Gelatin has been widely used in the production of controlled release system in the form of gels,\textsuperscript{144} particles,\textsuperscript{145} microspheres,\textsuperscript{101, 103-105, 146} and nanospheres.\textsuperscript{147-150}

Gelatin is a natural polymer derived from thermal denaturation or physical and chemical degradation of type I collagen.\textsuperscript{151} Type I collagen is a tropocollagen molecule; a triple helix of three polypeptide chains, which is predominately made from bovine skin, tendon, and bone, as seen in Figure 2.8. Two of the three polypeptide chains are identical with 1056 amino acid residues, and the third has 1038 residues. Its primary structure is predominately glycine residues in a Gly-X-Y triplet. X and Y are frequently made up of proline or hydroxyproline amino acid residues. Inter-chain crosslinking reacts through lysine and hydroxylysine residues.\textsuperscript{152}
There are several rationales for the use of gelatin. It is a natural polymer which allows for the development of biodegradable drug delivery systems with enhanced biocompatibility. It is relatively inexpensive, can be obtained pyrogen-free, and has putative bioadhesive properties. One of the major limitations for the use of gelatin as a sustained release system is its rapid dissolution in an aqueous environment, leading to uncontrolled drug release. However, this limitation can be overcome due to the large number of pendant functional groups. Various chemical crosslinking reagents such as genipin, dialdehydes, diisocyanates, carbodiimides, polyepoxy compounds, and acyl azid methods can be used to form an insoluble network, which allow controlled delivery of therapeutic drugs.

As stated earlier, glutaraldehyde is a commonly used crosslinker for tissue fixatives. Glutaraldehyde reacts with the $\alpha$-amino groups of the lysine molecules by
Schiff base reactions. Glutaraldehyde is known to bind to the -OH or -NH$_2$. Gelatin does not contain any free –OH groups, therefore, lysine amino acid residues are the only available -NH$_2$ sites for binding. Gelatin chains contain ~30 lysine molecule per 1000 amino acid residues. At room temperature (20$^\circ$C), a gelatin molecule is likely to have ~2000 amino acid residues, hence, ~60 lysine molecules will be present per gelatin chain.$^{154}$

Figure 2.9 is a schematic representation of the possible reactions between gelatin and glutaraldehyde.$^{155}$ Since two moles of genipin have been shown to achieve the same chemical crosslinks as one mole of glutaraldehyde, genipin may be a valid chemical crosslinker in the formation of gelatin mesospheres, and may afford better size control, drug loading, release rates, and biocompatibility

**Gelatin/Carboxymethyl Cellulose Blends**

Past research has shown that the addition of an anionic polysaccharide, polyglutamic acid, to protein microspheres improved the loading and release properties of a cationic drug, due to electrostatic interactions.$^{17,156}$ Based on this research, it was postulated that the addition of carboxymethyl cellulose (CMC), an anionic polysaccharide, would achieve similar results when blended with gelatin.

CMC is derived by the substitution of the hydroxyl groups on the anhydroglucose units which make up cellulose with carboxymethyl groups. This process proceeds by reacting cellulose with aqueous sodium hydroxide followed by the addition of sodium chloroacetate. This reaction results in the formation of a sodium salt, as seen in Figure 2.10. The maximum average degree of substitution per anhydroglucose unit is 3.0.$^{157}$
Figure 2.9 Chemical reactions between gelatin and glutaraldehyde
Goals of this Research

In view of the systemic toxicity and limited efficacy of conventional chemotherapy, new treatment modalities are needed. Prior research from our laboratory has shown that direct IT injection of drug loaded protein micro/mesospheres exhibited high drug concentration at the tumor site with reduced systemic toxicity. The overall goal of this research was to develop new localized IT treatment modalities. These innovations were accomplished via development of optimized synthesis methods in the preparation of novel protein micro/mesosphere compositions and mitoxantrone loaded gelatin mesosphere compositions for use in localized IT chemotherapies.
CHAPTER 3
PROTEIN MICRO/MESOSPHERES SYNTHESIS & CHARACTERIZATION

Introduction

The purpose of this research was to establish processing conditions to synthesize new modes of delivering chemotherapy agents for localized IT therapies. Smooth, spherical casein microspheres (CMS), gelatin mesospheres (GMS), and gelatin/carboxymethyl cellulose mesospheres (GCMS) with mean dry particle sizes of 30-5 um, 2 um, and 2 um, respectively, were synthesized using a steric stabilization process. The effects of processing conditions, stirring rate, and crosslinker concentration were investigated in the synthesis of CMS. All other mesosphere preparations investigated the effects of varying the crosslinker, glutaraldehyde and genipin, and crosslinker concentration in the synthesis of GMS and the GCMS. The incorporation of genipin, a novel, natural chemical crosslinker, may enhance drug loading and afford better control of drug release. GCMS were synthesized with varying concentrations of carboxymethyl cellulose, in order to potentially enhance the drug release profile of a cationic drug.

Materials and Methods

Materials

Casein and gelatin were purchased from Sigma Chemical Company. Casein, isolated from bovine milk, was used in the synthesis of casein microspheres. Casein is made up of several subunits, which vary in molecular weight, isoelectric point, and level of phosphorylation. The approximate casein composition is $\alpha$-s1 (12-15 g/ml); $\alpha$-s2 (3-4...
g/ml); β (9-11 g/ml); and κ (2-4 g/ml), molecular weight of subunits 22-23.7, 25, 24, and 19 kDa, respectively. Gelatin ~ 225 bloom isolated from calf skin (calculated molecular weigh 1.2x10^5 g/mol) was used in the synthesis of gelatin mesospheres and gelatin films. Sodium carboxymethyl cellulose, Type 7LF (CMC) (reported molecular weight 9.0x10^4 g/mol) was donated by Aqualon. CMC was used in the preparation of blended gelatin/CMC mesospheres. Cellulose acetate butyrate (CAB), 17 % butyryl content, was purchased from Acros Organics. CAB was used as a stabilization agent in the synthesis of protein micro/mesospheres.

Aqueous glutaraldehyde solutions 25 w/w % Type II was purchased from Sigma Chemical Company. Genipin powder was purchased from Challenge Bioproducts Co. Ltd. Glutaraldehyde and genipin were used to covalently crosslink the protein micro/mesospheres.

Deionized Type I and Type II water were prepared using a Barnstead NANOpure™ Ultrapure Water System. The resistivity of the deionized water (ultrapure water) was at least 17.4 MΩ-cm. Methanol HPLC grade was purchased from Fisher Scientific and used as the mobile phase for particle size determination. All other solvents, reagents, and salts were purchased from Fisher Scientific or Acros Organics and were Certified A.C.S. grade unless otherwise specified.

**Casein Microsphere Synthesis**

Casein microspheres (CMS) were synthesized using a steric stabilization process. The suspension was formed by the addition of 3 ml of 15 w/v % casein solution in 47 ml of a 3 w/v % cellulose acetate butyrate (CAB) /1-2 dichloroethane (DCE) and using a high speed mechanical-stirrer with a two inch, two-blade propeller, as demonstrated in Figure
2.4. The stirring rate was maintained for 20 minutes at varying rpms after which the appropriate crosslinker concentration was added to crosslink the casein (Note: 1 w/w % GTA corresponds to 0.810 mmol GTA/mmol lysine per casein molecule). The stirring rate was reduced to 600 rpm and the reaction was continued for two hours. After two hours, 50 ml of acetone was added and the reaction was continued for another hour. The microspheres were collected by centrifugation at 3000 rpm and washed in a series of four acetone resuspensions and centrifugations. The mesospheres were air dried. The specific processing conditions for casein microsphere synthesis are given in Table 3.1.

Table 3.1 Experimental conditions used in the synthesis of casein microspheres

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Experimental Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein w/v %</td>
<td>CAB w/v %</td>
</tr>
<tr>
<td>Stirring Rate w/w %</td>
<td>GTA w/w %</td>
</tr>
<tr>
<td>15.0</td>
<td>3.0</td>
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<tr>
<td>15.0</td>
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<td>3.0</td>
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<tr>
<td>15.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**Solution preparation**

**Casein.** Casein is soluble in water under basic conditions. Thus, casein solutions were prepared in 0.5 M NaOH. Also, casein solution foamed significantly when agitated making the conventional technique of solution preparation in volumetric flasks less than
ideal. Casein solutions were prepared in a 50 ml conical as close to the desired concentration as possible. The true concentration was measured gravimetrically, and the density of the solution was measured.

Approximately 1 ml of the casein solutions were dried at 130°C on a Mettler LJ16 Moisture Analyzer. Using the dry weight and density the weight per volume percent was determined. Concentration was adjusted until true concentration was achieved within 0.5%.

**Glutaraldehyde.** Glutaraldehyde solutions were prepared by vacuum distilling 25% aqueous glutaraldehyde solution and recovering the glutaraldehyde distillate. The distillate was then dissolved in DCE to a final concentration of 40 mg/ml.

**Cellulose Acetate Butyrate.** Cellulose acetate butyrate solutions were prepared by dissolving solid cellulose acetate butyrate in DCE to a final concentration of 3.0 w/v%.

**Particle sizing**

**Scanning Electron Microscopy.** Scanning Electron Microscopy (SEM) was used to qualitatively examine the dry particle size of the synthesized mesospheres. Dry mesospheres were mounted on double-sided tape on aluminum SEM stubs. The stubs were then coated with a gold/palladium alloy for 5 minutes using a Technix Hummer V sputter Coater. The samples were analyzed using a Jeol SEM 6400 scanning electron microscope at 5 KeV accelerating voltage; a condenser lens setting of 8 to 10; and a working distance of 15mm. This analysis was conducted with the assistance of Paul Martin.

**Coulter® LS™ 230 Particle Size Analyzer.** The dry particle size of the gelatin mesospheres was measured quantitatively using a Coulter® LS™ 230 particle size analyzer with the small volume module. A 1% mesosphere suspension was prepared in 5
ml of HPLC grade Methanol. Minimal particle swelling was observed in methanol. The suspension was sonicated to ensure complete dispersion and then added drop wise until the obstruction read, 12 to 15. Particle size data were analyzed using Coulter® LS™ software.

**Statistical analysis**

The effects of stirring rate and crosslink concentration on particle size of casein microspheres were analyzed using a two way ANOVA. Pairwise multiple comparisons (Tukey Test) were used to evaluate the interactions between groups. The statistical calculations were carried out using the Sigma Stat® software package.

**Crosslinked Gelatin Film Synthesis**

Crosslinked gelatin films were synthesized to compare the modulus, tensile strength, and percent swellability with varying crosslinkers, GEN and GTA, and crosslinker concentrations. A stock solution of 60 mg/ml GEN in isopropanol was prepared, and three crosslinker concentrations were used, 2.3 (C1); 11.3 (C2); and 20.3 (C3) w/w % [20; 100; 200 %MEQ, respectively]. Also, a 25% aqueous glutaraldehyde solution was used, and three crosslinker concentrations were used, 2.5 (C1); 4.5 (C2) and 6.5 (C3) w/w % [100, 200, and 300 %MEQ, respectively]. Molar equivalents were approximated using \( \text{Mw}_{\text{gelatin}}=1.2 \times 10^5 \text{ g/mol} \) (calculated using GPC/MALs) and an average molecular weight of repeat unit \( \text{M}_{\text{rp}}=65.3 \text{ g/mol} \). Note: 4.5 times the concentration of GEN is needed for a given concentration of glutaraldehyde to obtain comparable crosslinker concentrations, since two moles of genipin is needed to achieve the same chemical crosslink as one mole of GTA.

The very rapid rate of reaction of GTA in comparison to the slow rate of reaction of GEN greatly hindered the development of comparable protocols. GEN crosslinked
gelatin films were prepared by mixing appropriate concentrations of genipin with heated (40°C) 10 w/v % gelatin. After the addition of GEN, thorough mixing was insured by mixing the sample on rotating table for ten minutes followed by ten minutes in a 40°C oven to liquefy. Then 8 ml of solution was transferred into a 3”x3”x2 mm glass plate mold and allowed to react overnight at room temperature. Crosslinked films were removed from the mold and washed by a series of four 1 hr phosphate buffered saline (PBS) washes. The supernatant was analyzed on a Shimadzu UV-2401PC (UV-VIS) at 254 nm against a matched matrix background to insure complete removal of free un-reacted GEN. Due to previously stated problems with using GTA as a crosslinker, this protocol was invalid for the synthesis of GTA crosslinked films. GTA crosslinked films were prepared by casting 8 ml of 10 w/v % gelatin solutions into a 3”x3”x1 mm and allowed to dry. Four ml of the appropriate GTA concentration was evenly spread over the surface until complete wetting of the surface was achieved and films were transferred into a 40°C oven and allowed to react for 1 hr. Crosslinked films were removed from the molds and hydrated in phosphate buffered saline (PBS).

Solution preparation

Gelatin. Gelatin films were synthesized from 10 w/w % gelatin dissolved in ultrapure water. Gelatin is only soluble in ultrapure water at elevated temperatures. Thus, gelatin solutions were prepared in 35°C ultrapure water. Due to foaming with agitation, gelatin solutions were prepared in a 50 ml conical as close to the desired concentration as possible. The true concentration was measured gravimetrically, and the density of the solution was measured.

Approximately 1 ml of the gelatin solution was dried at 130°C on a Mettler LJ16 Moisture Analyzer. Using the dry weight and density, the weight per volume percent was
determined. Concentration was adjusted until true concentration was achieved within 0.5%.

**Phosphate buffered saline.** Isotonic phosphate buffered saline (PBS) was prepared by mixing 50 mM sodium phosphate monobasic solution with a 50 mM sodium phosphate dibasic solution until a desired pH of 7.4 was reached. Osmolarity of the solutions was adjusted to 300 mOsm with the addition of sodium chloride (NaCl). Appropriate amounts of NaCl was determined by the preparation of a calibration plot by varying the concentrations (0 to 10 mg/ml) of NaCl in the phosphate buffer. The osmolarity of the standards was measured using Precision Instruments u Osmette™. The resulting PBS solution was sterile filtered through a 0.22 um filter.

**Modulus and tensile strength measurements**

Tensile specimens were cut from hydrated crosslinked gelatin films using ASTM D638-M3 type L dog bone die. Sample thicknesses varied with crosslinker concentrations and were determined using digital calipers. Tensile measurements were made according to ASTM D412-97 on an Instron model 1122 equipped with TestWorks 4.02 software for analysis. Strain measurements were based on crosshead displacement of 2 in/min.

**Swelling measurements**

Dry crosslinked films were weighed to determine dry weight, \( W_{\text{dry}} \), and hydrated in PBS for 24 hours and reweighed to determine hydrated weight, \( W_{\text{hydrated}} \). Percent swelling (\%Swell) was calculated using the following formula:

\[
\% \text{Swell} = \left( 100 \left( \frac{W_{\text{hydrated}} - W_{\text{dry}}}{W_{\text{dry}}} \right) \right)
\]
Statistical analysis

The effects of crosslinker and crosslink concentration on modulus, tensile strength, and percent swellability were analyzed using a two way ANOVA. Pairwise multiple comparisons (Tukey Test) were used to evaluate the interactions between groups. The statistical calculations were carried out using the Sigma Stat® software package.

Gelatin Mesosphere Synthesis

Gelatin mesospheres were synthesized using a steric stabilization process, as demonstrated in Figure 2.4. Two different crosslinking agents, genipin (GEN) and glutaraldehyde (GTA) were used in this study. A stock solution of 60 mg/ml GEN (Challenge Bioproducts Co., Ltd.) in dry acetone was prepared, and four crosslinker concentration were used, 2.3 (C1); 11.3 (C2); 20.3 (C3) and 29.3 (C4) w/w % [20, 100, 200, and 300 %MEQ, respectively]. Also, a stock solution of 40 mg/ml GTA in 1-2 dichloroethane (DCE) was prepared, and four crosslinker concentration were used, 0.5 (C1); 2.5 (C2); 4.5 (C3) and 6.5 (C4) w/w % [20, 100, 200, and 300 % MEQ, respectively]. Mol equivalents were approximated using \( M_w = 1.2 \times 10^5 \) g/mol (calculated using GPC/MALS) and an average molecular weight of repeat unit \( M_{rp} = 65.3 \) g/mol. Note: 4.5 times the concentration of GEN is needed for a given concentration of glutaraldehyde to obtained comparable crosslink densities, since two moles of genipin is needed to achieve the same chemical crosslink as one mole of GTA.

Suspension was formed by the addition of 3 ml of a heated 10 w/v% gelatin solution in 47 ml of a heated 3 w/v% cellulose acetate butyrate (CAB) /1-2 dichloroethane (DCE) and using a high speed mechanical-stirrer with a two inch, two-blade propeller. The gelatin solution was heated for 1 hour at 40°C. The mixing CAB solution was heated using a water bath at 40°C. The heat was removed 5 minutes after
the addition of the gelatin and the suspension was allowed to return to room temperature. The stirring rate was maintained for 20 minutes at 1500 rpm after which the appropriate crosslinker and crosslinker concentration was added to crosslink the gelatin. The stirring rate was reduced to 600 rpm and the reaction was continued for two hours. After two hours, 50 ml of acetone was added (if needed to replace evaporated DCE) and the reaction was continued for another hour. The mesospheres were collected by centrifugation at 3000 rpm and washed in a series of four acetone resuspensions and centrifugations. The mesospheres were then air dried. Processing conditions are listed in Table 3.2.

Table 3.2 Experimental conditions used in the synthesis of gelatin mesospheres

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin w/v %</td>
<td>CAB w/v %</td>
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<tr>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
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<tr>
<td>10.0</td>
<td>3.0</td>
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</tbody>
</table>

**Gelatin/CMC Mesosphere Synthesis**

Gelatin mesospheres containing various concentrations of carboxymethyl cellulose (CMC), were synthesized using the same techniques used in the synthesis of unloaded gelatin mesospheres, as demonstrated in Figure 2.4. The only exception was that the 10 w/v % gelatin solution contained various concentrations of medium molecular weight CMC, (0.0, 0.5, 1.0, and 1.5 w/w %). The same crosslinking agents, GEN and GTA were used in this study. A stock solution of 60 mg/ml GEN in acetone was prepared, and one
crosslinker concentration was used, 20.3 (C1) w/w % [200 % MEQ]. Also, a stock solution of 40 mg/ml GTA in 1-2 dichloroethane was prepared, and one crosslinker concentration was used, 4.5 (C1) w/w % [200 % MEQ].

Gelatin/CMC mesospheres (GCMS) were produced using a steric stabilization process. Experimental conditions are listed in Table 3.3. Suspension was formed by adding 3 ml of a heated 10 w/v % gelatin solution containing appropriate amounts of CMC in 47 ml of a heated 3 w/v % cellulose acetate butyrate (CAB) /1-2 dichloroethane (DCE) and using a high speed mechanical-stirrer with a two inch, two-blade propeller. The gelatin/CMC solution was heated for 1 hr at 40°C. The mixing CAB solution was heated using a water bath at 40°C. The heat was removed 5 minutes after the addition of the gelatin and the suspension was allowed to return to room temperature. The stirring rate was maintained for 20 minutes at 1500 rpm after which the appropriate crosslinker and crosslinker concentration were added to crosslink the gelatin. The stirring rate was reduced to 600 rpm and the reaction was continued for two hours. After two hours, 50 ml of acetone was added (if needed to replace evaporated DCE) and the reaction will be continued for another hour. The GCMS was collected by centrifugation at 3000 rpm and washed in a series of four acetone resuspensions and centrifugations. The mesospheres were air dried.
Table 3.3 Experimental conditions in the synthesis of GCMS

<table>
<thead>
<tr>
<th>Gelatin w/v %</th>
<th>CMC w/w %</th>
<th>CAB w/v %</th>
<th>GTA w/w %</th>
<th>GEN w/w %</th>
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<tr>
<td>10.0</td>
<td>0.0</td>
<td>3.0</td>
<td>4.5</td>
<td>20.3</td>
</tr>
<tr>
<td>10.0</td>
<td>0.5</td>
<td>3.0</td>
<td>4.5</td>
<td>20.3</td>
</tr>
<tr>
<td>10.0</td>
<td>1.0</td>
<td>3.0</td>
<td>4.5</td>
<td>20.3</td>
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<td>10.0</td>
<td>1.5</td>
<td>3.0</td>
<td>4.5</td>
<td>20.3</td>
</tr>
</tbody>
</table>

Particle sizing

**Scanning Electron Microscopy.** SEM was used to qualitatively examine the dry particle size of the synthesized mesospheres. Dry mesospheres were mounted on double-sided tape on aluminum SEM stubs. The stubs were then coated with a gold/palladium alloy for 5 minutes using a Technix Hummer V sputter Coater. The samples were analyzed using a Jeol SEM 6400 scanning electron microscope at 2.5 - 5 KeV accelerating voltage; a condenser lens setting of 8 to 10; and a working distance of 15mm. This analysis was conducted with the assistance of Paul Martin.

**Coulter® LS™ 230 Particle Size Analyzer.** The dry particle size of the gelatin mesospheres were measured quantitatively using a Coulter® LS™ 230 particle size analyzer with the small volume module. A 1% mesosphere suspension was prepared in 5 ml of HPLC grade Methanol. Note: Under the restraints of these evaluations, minimal or no swelling was observed. The suspension was sonicated to ensure complete dispersion and then added drop wise until the obstruction read, 12 to 15. Data were analyzed using Coulter® LS™ 32 software.
Solution preparation

**Gelatin.** Gelatin is soluble in water at elevated temperatures. Thus, gelatin solutions were prepared in 35°C ultrapure water. Also, gelatin solution foamed significantly when agitated making the conventional technique of solution preparation in volumetric flasks less than ideal. Gelatin solutions were prepared in a 50 ml conical as close to the desired concentration as possible. The true concentration was measured gravimetrically, and the density of the solution was measured.

Approximately one ml of the gelatin solutions were dried at 130°C on a Mettler LJ16 Moisture Analyzer. Using the dry weight and the density, the weight per volume percent was determined. Concentration was adjusted until true concentration was achieved within 0.5%.

**Gelatin/CMC.** Gelatin solutions were prepared using the same techniques described in the gelatin solution preparation. Appropriate amounts of medium molecular weight, CMC were added to a heated (40°C) 10 w/v% gelatin solution and dissolved.

**Glutaraldehyde.** Glutaraldehyde solutions were prepared by vacuum distilling 25% aqueous glutaraldehyde solution and recovering the glutaraldehyde distillate. The distillate was then dissolved in DCE to a final concentration of 40 mg/ml.

**Genipin.** Genipin solutions were prepared by dissolving solid genipin in dry acetone to a final concentration of 60 mg/ml.

**Cellulose Acetate Butyrate.** Cellulose acetate butyrate solutions were prepared by dissolving solid cellulose acetate butyrate in DCE to a final concentration of 3.0 w/v%.

Statistical analysis

The effects of crosslinker and crosslink concentration on particle size of gelatin mesospheres and the effects of crosslinker and CMC concentration on particle size of
gelatin/CMC mesospheres were analyzed using a two way ANOVA. Pairwise multiple comparisons (Tukey Test) were used to evaluate the interactions between groups. The statistical calculations were carried out using the Sigma Stat® software package.

**Results and Discussion**

**Casein Microspheres**

Casein microspheres were synthesized using steric stabilization process. The effects of processing conditions; CAB (steric hindrance agent) concentration, protein concentration, crosslinker concentration, D/C (dispersed to continuous phase) volume ratio, crosslinking reaction time, crosslinking stirring rate, and initial stirring rate, were examined in some detail in the synthesis albumin microspheres. Building on the information obtained from previous albumin microsphere studies, several pilot studies were designed in order to optimize the processing condition to achieve smooth, spherical microspheres.

![Figure 3.1 1000x SEM image of optimized processing condition for the synthesis of casein microspheres.](image)
Pilot studies evaluated changing the concentration of casein (10, 15, and 20 w/v %), d/c ratio (6 and 12 %), and CAB (0.5, 1, 2, 3 w/v %), and the stirring rate (600, 800, 1000, 1250, 1500, and 1800 rpm) on particle size and morphology. The average particle size and morphology of the casein microspheres were qualitatively observed with SEM. After many unsuccessful combinations, it was determined that 15 w/v% casein dispersed in 3 w/v% CAB at 1500 rpms would produce smooth spherical microspheres (Figure 3.1).

One major problem which had to be addressed in the pilot studies was the synthesis of casein microspheres without precipitants forming on the surface of the microspheres (Figure 3.2). These precipitants resembled hair follicles, and therefore were termed “the hairy ball effect”. Further evaluations concluded that the casein was precipitating out of solution due to possible exposure to the atmosphere.

Figure 3.2 SEM image, 3000x, of precipitants on the surface of casein microspheres
To achieve smooth, spherical microspheres, casein solutions were prepared in small volumes to reduce exposure to the atmosphere. After the completion of the initial pilot studies, a 3x3 factorial design study was designed to evaluate the effects of crosslinker concentration (1, 2.5 and 4 w/w % GTA) [80, 200, and 320 % MEQ] and stirring rate (1000, 1250, and 1500 rpm) in the synthesis of casein microspheres. Experimental conditions are listed in Table 3.4.

Table 3.4 Mean particle size of casein microspheres

<table>
<thead>
<tr>
<th>Casein Microspheres</th>
<th>Stirring Rate</th>
<th>GTA Concentration</th>
<th>1000 rpm</th>
<th>1250 rpm</th>
<th>1500 rpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Particle Size</td>
<td>Stirring Rate</td>
<td>GTA Concentration</td>
<td>1.0 w/w %</td>
<td>2.5 w/w %</td>
<td>4.0 w/w %</td>
</tr>
<tr>
<td></td>
<td>1000 rpm</td>
<td>1.0 w/w %</td>
<td>29.1</td>
<td>27.8</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>1250 rpm</td>
<td>1.0 w/w %</td>
<td>16.4</td>
<td>22.5</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>1500 rpm</td>
<td>1.0 w/w %</td>
<td>25.3</td>
<td>13.2</td>
<td>4.5</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td>1.0 w/w %</td>
<td>6.3</td>
<td>13.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 w/w %</td>
<td>10.4</td>
<td>4.5</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0 w/w %</td>
<td></td>
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</table>

The average particle size of the casein microspheres was qualitatively observed with SEM and quantitatively measured with a Coulter® LS™ 230 particle size analyzer. The particle size data are tabulated in Table 3.4. A two-way ANOVA and Tukey multiple comparison test were used to compare the effects of crosslinker concentration and stirring rate on particle size. The analysis showed that there was a significant
difference with varying stirring rate ($p=0.019$) and crosslink density ($p<0.001$) on particle size. Increasing stirring rate and GTA concentration lead to a decrease in particle size as shown in Figure 3.3. Pairwise comparisons showed that there was a significant difference ($p<0.5$) between stirring rates 1000 rpm vs. 1500 rpm and 1000 vs. 1250 rpm, and crosslink densities 1.0 wt% vs. 4.0 wt% and 2.5 wt% vs. 4.0 wt%. Figure 3.4 is the plots of volume % vs. particle diameter. From these plots, the particle size distribution ranged from 0.1 – 100 um with a shift from one distribution for low stirring rates and crosslinker concentrations to two distributions at higher stirring rates and crosslinker concentrations. Figure 3.5 is corresponding images from each of the above defined conditions. From these images, the morphology of the microspheres was shown to vary from a smooth spherical to a rough pitted microsphere with decreasing crosslink concentrations and stirring rates.

![Figure 3.3](image_url)  
*Figure 3.3 Graphical representation of particle size with changing crosslinker concentration and stirring rate*
I. 

Figure 3.4 Particle size distributions for casein microspheres with varying stirring rate (I. 1000, II. 1250 and III. 1500 rpm) and crosslinker concentration.
Figure 3.5 3000x SEM images A) 1000rpm 1\%GTA B) 1250rpm 1\%GTA C) 1500rpm 1\%GTA D) 1000rpm 2.5\%GTA E) 1250rpm 2.5\%GTA F) 1500rpm 2.5\%GTA G) 1000rpm 4\%GTA H) 1250 rpm 4\%GTA I) 1500rpm 4\%GTA
Crosslinked Gelatin Films

Crosslinked gelatin films were synthesized to evaluate the validity of using GEN as a chemical crosslinker. GEN and GTA crosslinked films were synthesized and the modulus, tensile strength, and percent swelling were characterized. This study was designed as a 2x3 factorial design in order to evaluate the effects of varying crosslinker and crosslinker concentrations. Due to constraints imposed by the various crosslinkers, only two of the three crosslinker concentrations were applicable for comparison. Crosslinker concentrations utilized in this study were comparable to the concentrations used in the gelatin mesosphere synthesis.

Three crosslinker concentrations of GEN were evaluated, 2.3 (C1); 11.3 (C2); and 20.3 (C3) w/w % [20, 100, and 200 % MEQ]. After all the free genipin was removed via PBS washes, GEN crosslinked gelatin films were fully hydrated in PBS and the modulus, tensile strength, and percent swellability were determined, Table 3.5.

GEN proved to be a valid chemical crosslinker. The modulus, tensile strength, and the percent swellability of the hydrated films were determined to range between 12.8 to 147.0 kPa; 100.0 to 297.5 to 205.0 kPa; and 759.0 to 244.0 % with increasing crosslinker concentration. Also as reported, crosslinked films underwent a colorimetric change to a deep blue color, with the progression of the GEN crosslinking reaction, Figure 3.6. The statistical analysis suggested the concentration of GEN had a statistically significant effect on modulus, tensile strength, and percent swellability (p<0.001). The pair-wise multiple comparison tests (Tukey Test) showed that increasing the GEN concentration significantly increases the modulus and tensile strength (at C1 to C2 and C1 to C3), while decreasing the percent swellability (p<0.05).
Table 3.5 Modulus and Percent Swellability data for GEN crosslinked films

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Modulus</th>
<th>Tensile Strength</th>
<th>Percent Swelling</th>
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<tbody>
<tr>
<td></td>
<td>Gelatin w/v%</td>
<td>GEN w/w%</td>
<td>Average</td>
</tr>
<tr>
<td>10.0 w/v%</td>
<td>2.3 (C1)</td>
<td>12.8 kPa</td>
<td>1.3 kPa</td>
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<tr>
<td>10.0 w/v%</td>
<td>11.3 (C2)</td>
<td>94.0 kPa</td>
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<tr>
<td>10.0 w/v%</td>
<td>20.3 (C3)</td>
<td>147.0 kPa</td>
<td>14.3 kPa</td>
</tr>
</tbody>
</table>

Three GTA crosslinker concentrations were evaluated, 2.5 (C2); 4.5 (C3) and 6.5 (C4) w/w % [100, 200, and 300 %MEQ]. After GTA crosslinked films were prepared, crosslinked gelatin films were fully hydrated in PBS and the modulus, tensile strength, and the percent swellability were determined, Table 3.6.

Chemical crosslinking of gelatin films was achieved with GTA. The modulus, tensile strength, and the percent swellability of the hydrated films were determined to range between 7.4 to 133.2 kPa; 40.2 to 199.9 kPa; and 851.7 to 432.0 % with increasing crosslinker concentration. The statistical analysis suggested the concentration of GTA
had a statistically significant effect on modulus, tensile strength, and percent swellability (p<0.001). The pair-wise multiple comparison tests (Tukey Test) showed that increasing the GTA concentration significantly increases the modulus and tensile strength, while decreasing the percent swellability (p<0.05).

Two way ANOVA statistical analysis tests suggested that there was a statistical difference of varying the crosslinker and crosslinker concentration on the modulus, tensile strength, and percent swellability (p<0.001). The pair-wise multiple comparison tests (Tukey Test) showed that for a given crosslinker concentration, GEN achieved a greater degree of chemical crosslinks, which is evident in the overall higher modulus and tensile strength and lowered the percent swellability (p<0.05). This is graphically represented in Figure 3.7 - Figure 3.9.

Table 3.6 Modulus and Percent Swellability data for GTA crosslinked films

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Modulus</th>
<th>Tensile Strength</th>
<th>Percent Swelling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Stdev</td>
<td>Average</td>
</tr>
<tr>
<td>Gelatin w/v%</td>
<td>GTA w/w%</td>
<td>kPa</td>
<td>kPa</td>
</tr>
<tr>
<td>10.0</td>
<td>2.5 (C2)</td>
<td>7.4</td>
<td>2.4</td>
</tr>
<tr>
<td>10.0</td>
<td>4.5 (C3)</td>
<td>34.1</td>
<td>5.3</td>
</tr>
<tr>
<td>10.0</td>
<td>6.5 (C4)</td>
<td>133.2</td>
<td>30.0</td>
</tr>
</tbody>
</table>
Figure 3.7 Stress vs. strain plots for varying crosslinker: I. GEN and II. GTA
Figure 3.8 Graphical representation of the I. modulus and the II. tensile strength with varying crosslinkers and crosslinker concentrations.
Gelatin mesospheres were synthesized using steric stabilization process. The effects of processing conditions; CAB (steric hindrance agent) concentration, protein concentration, crosslinker concentration, D/C (dispersed to continuous phase) volume ratio, crosslinking reaction time, crosslinking stir rate, and initial stirring rate, were examined in some detail in the synthesis of casein and albumin microspheres. Building on the information obtained from the previous studies, processing conditions for obtaining gelatin mesospheres with a mean particle size in the range of 1 to 10 μm were formulated and are described.

The processing conditions for the synthesis of gelatin mesospheres were based largely on the conditions established in the synthesis of casein microspheres. One of the
major difficulties in synthesizing gelatin mesospheres is that gelatin is a gel at room
temperature; therefore processing temperatures had to be evaluated. There are reported
suspension methods for the synthesis of gelatin microspheres by dispersing an aqueous
gelatin solution in oil e.g. mineral oil, at some predefined temperature above gelation.
Suspension was cooled below the gelation temperature and particles were collected.
After collection, particles were crosslinked in a second crosslinking step. By using
mineral oil as the continuous phase, evaporation of continuous phase was not a problem.

The steric stabilization process, which has been developed in our research
laboratory, uses cellulose acetate butyrate dissolved in DCE as the continuous phase.
DCE has a boiling point of 84°C and the evaporation of DCE during the microsphere
synthesis in not uncommon. This has always been a concern in the past due to the shear
induced evaporation. With the addition of heat to the process, great care is needed to
prevent accelerated evaporation of the solvent, which would change the overall
concentration of the continuous phase and hinder the production of uniform mesospheres.
To address this problem, initial pilot studies were evaluated mixing temperatures (25, 40,
and 45°C) and times of heating (0.0, 5.0, 10.0, and 15.0 min) to limit the overall
evaporation. No heating during the formation of the dispersion lead to the formation of
large particle size distribution (Figure 3.10) and long heating times and high temperatures
promoted solvent evaporation. It was determined that the optimal conditions would be to
heat the CAB solution at 40°C for 5 minutes followed by the addition of 10 w/v% gelatin
solution. Heating was continued for an additional 5 minutes and dispersion was allowed
to return to room temperature.
After the processing conditions were optimized, the evaluations changed to studying the effects of using a novel crosslinking agent, genipin, which is totally natural and may promote enhanced drug loading and release. In order to examine the effects of varying crosslinker, genipin and glutaraldehyde, and crosslinker concentration on particle size, a 2x4 factorial design was used. Experimental conditions are listed in Table 3.1.

The average particle size of the mesospheres was qualitatively observed with SEM and quantitatively measured with a Coulter® LS™ 230 particle size analyzer. The particle size data are tabulated in Table 3.7 and graphed in Figure 3.11. This can be further correlated with the particle size distributions plots, Figure 3.12. A two-way ANOVA was used to compare the effects of crosslinker and crosslinker concentration on particle size. The analysis showed that there was no statistical difference in particle size, 2 um, with varying crosslinker (p=0.237) and crosslinker concentration (p=0.788).

Figure 3.13 is SEM micrographs of GEN and GTA crosslinked mesospheres that were synthesized. From these images, there appears to be no evidence to suggest that varying
the crosslinker and the crosslinker concentration had an effect on surface roughness or shape of the mesospheres. All the conditions, appear to produce smooth; spherical mesospheres with similar size distributions of 0.04 – 20 um.

Table 3.7 Gelatin mesosphere synthesis conditions

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Concentration Levels</td>
<td>Mean Particle Size</td>
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<tr>
<td>Gelatin w/v %</td>
<td>CAB w/v %</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Gelatin w/v %</th>
<th>CAB w/v %</th>
<th>GTA w/w %</th>
<th>Levels</th>
<th>Mean Particle Size</th>
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</thead>
<tbody>
<tr>
<td>10.0</td>
<td>3.0</td>
<td>0.5</td>
<td>1</td>
<td>2.6 +/- 0.9</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
<td>2.5</td>
<td>2</td>
<td>2.1 +/- 0.3</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
<td>4.5</td>
<td>3</td>
<td>2.1 +/- 0.4</td>
</tr>
<tr>
<td>10.0</td>
<td>3.0</td>
<td>6.5</td>
<td>4</td>
<td>2.3 +/- 0.6</td>
</tr>
</tbody>
</table>

Figure 3.11 Graphical representation of particle size with changing crosslinker and crosslinker concentration
I.

Figure 3.12 Particle size distributions for gelatin mesospheres with varying crosslinker: I. GEN and II. GTA

II.
Figure 3.13 SEM micrographs 3000x of I. GEN and II. GTA crosslinked gelatin mesospheres at varying concentration: A) C1; B) C2; C) C3; D) C4 wt%
Synthesis of Gelatin/CMC Mesospheres

The synthesis of gelatin mesospheres blended with an ionic polysaccharide, CMC, was evaluated as a possible means of enhancing the drug loading, which decreased the mass of mesospheres needed to achieve a desired therapeutic dose. The principle for this rationale is contributed to the cationic nature of the drug; therefore increasing the anionic charge of the mesospheres that may create a higher affinity for the drug. The processing conditions for the synthesis of gelatin/CMC mesospheres (GCMS) were unchanged. Appropriate concentrations of CMC were blended with the gelatin before processing.

The average particle size for GCMS was qualitatively observed with SEM and quantitatively measured with a Coulter® LS™ 230 particle size analyzer. The particle size data are tabulated in Table 3.8 and graphed in Figure 3.14. The statistical analysis software reported that there was a statistical difference on particle size with varying concentration of CMC (p=0.004) and no statistical difference on particle size with changing crosslinker (p=0.647). Also, there was no statistically significant interaction (p=0.270) between the crosslinker and CAB concentration. The pair-wise multiple comparison tests reported that increasing of CMC significantly increased the mean particle size of the mesospheres (2 to 4 um) (p<0.05). This can be further correlated with the particle size distributions plots, Figure 3.15. The SEM images, Figure 3.16 demonstrates that under the above defined conditions, smooth spherical mesospheres can be synthesized with increasing concentrations of CMC.
Table 3.8  GCMS experimental conditions

<table>
<thead>
<tr>
<th>Concentration Conditions</th>
<th>Levels</th>
<th>Concentration</th>
<th>Levels</th>
<th>Gelatin</th>
<th>CMC</th>
<th>CAB</th>
<th>GEN</th>
<th>Mean Particle Size</th>
<th>um</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin CMC CAB GEN</td>
<td>w/v %</td>
<td>w/w %</td>
<td>w/v %</td>
<td>w/w %</td>
<td>10.0</td>
<td>0.0</td>
<td>3.0</td>
<td>20.3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>w/v %</td>
<td>w/w %</td>
<td>w/v %</td>
<td>w/w %</td>
<td>10.0</td>
<td>0.5</td>
<td>3.0</td>
<td>20.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>w/v %</td>
<td>w/w %</td>
<td>w/v %</td>
<td>w/w %</td>
<td>10.0</td>
<td>1.0</td>
<td>3.0</td>
<td>20.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>w/v %</td>
<td>w/w %</td>
<td>w/v %</td>
<td>w/w %</td>
<td>10.0</td>
<td>1.5</td>
<td>3.0</td>
<td>20.3</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 3.14  Graphical representation of particle size with changing crosslinker and crosslinker concentration
Figure 3.15  Particle size distributions for GCMS with varying crosslinker (I. GEN and II. GTA) and crosslinker concentration
Figure 3.16 SEM micrographs 3000x of I. GTA and II. GEN crosslinked gelatin mesospheres at varying concentration of CMC: A) 0.0; B) 5.0; C) 9.0; D) 13.0 w/w%
Summary of Research

Casein microspheres

Casein microspheres were synthesized using a steric stabilization process, which was adapted from conditions reported in the synthesis of albumin microspheres \(^4\) and casein microspheres.\(^{18}\) Processing conditions were established to produce smooth spherical particles, with mean particle sizes ranging from 30-5 \(\mu\text{m}\).

Past studies \(^4\) have shown that increasing the stirring rate would decrease the mean particle size, due to increased shear. A change in particle size with decreasing crosslinker concentration was not evident. The change in particle size with changing crosslinker concentrations may be due to the inability of the crosslinker to effectively react with the large number of small particles at low crosslinker concentrations, which would result in the formation of fewer, but larger particles. This may explain the shift to binodal particle size distribution at higher crosslinker concentrations. The auto polymerization of GTA at high pH\(^{161}\) may also be a contributing factor by reducing the number of GTA molecules available to react with the free amines on the casein microspheres.

Crosslinked gelatin films

Crosslinked gelatin films were successfully synthesized with varying crosslinkers, GEN and GTA. Under the processing conditions that were established for the synthesis of gelatin films, GEN has been shown not only to be a valid chemical crosslinker, but also a superior alternative to GTA. This may be contributed to the slow reactivity of GEN, which allowed for complete mixing; therefore a higher degree of chemical crosslinking was achieved.
**Gelatin mesospheres**

Gelatin mesospheres were synthesized using a steric stabilization process, which was adapted from conditions reported in the synthesis of albumin microspheres and casein microspheres. Processing conditions were established to produce smooth, spherical particles, with a mean dry particle size of 2 µm, with a particle size distribution of 0.04 – 20 µm. Investigative studies on processing conditions showed that varying the crosslinker and the crosslinker concentration had little or no effect on particle morphology and no effect on dry particle size or particle size distribution.

**Gelatin/CMC mesospheres**

Gelatin/CMC mesospheres were synthesized using a steric stabilization process, which was adapted from conditions used in the synthesis of gelatin mesospheres. Smooth, spherical particles, with a mean dry particle size range of 2 – 4 µm were synthesized. Increasing the amount of CMC increased the total concentration of the aqueous phase for a given concentration of continuous phase, therefore an increase in particle size was observed, with no change in particle morphology. Varying the crosslinker had no effect on particle size and particle morphology.
CHAPTER 4
EVALUATIONS OF DRUG LOADED PROTEIN MESOSPHERES

Introduction

The forgoing research investigates the validity of \textit{in situ} gelatin mesosphere compositions and various other modalities in the treatment of high mortalities cancers via \textit{in vitro} rat glioma 2 evaluations and \textit{in vivo} 16/C MMAC evaluations.

The efficacy of using MXN loaded mesospheres for localized IT therapies was evaluated through \textit{in vitro} treatments of rat glioma cells (RG-2). Various treatment modalities were investigated: 1) MXN only, 2) MXN plus gelatin, 3) MXN-GMS with varying crosslinkers (genipin and glutaraldehyde) and crosslinker concentrations, 4) MXN-CMS, 5) Free MXN plus MXN-GMS with varying crosslinkers (genipin and glutaraldehyde) and crosslinker concentrations, and 6) controls (no treatment and/or MXN).

\textit{In vivo} studies evaluated the validity of localized IT therapies using MXN loaded gelatin mesospheres compositions in the treatment of 16/C MMAC. The MXN-GMS research was performed in conjunction with Shema Freeman and Amanda York using previously established methodologies.\textsuperscript{4, 5}

Material and Methods

Materials

Gelatin was purchased from Sigma Chemical Company. Gelatin ~ 225 bloom isolated from calf skin (calculated molecular weigh $1.2 \times 10^5$ g/mol) was used in the synthesis of MXN-GMS and MXN-GCMS. Sodium carboxymethyl cellulose, Type 7LF
(CMC) (reported molecular weight $9.0\times10^4$ g/mol) was donated by Aqualon. CMC was used in the preparation of MXN-GCMS. Mitoxantrone was donated by Lederle Laboratories. MXN reported purity was 82.75% and was used without any further purification. Cellulose acetate butyrate (CAB), 17% butyryl content, was purchased from Acros Organics. CAB was used as a stabilization agent in the synthesis of MXN loaded gelatin mesosphere compositions.

Aqueous glutaraldehyde solutions (25 w/w % Type II) were purchased from Sigma Chemical Company. Genipin powder was purchased from Challenge Bioproducts Co. Ltd. Glutaraldehyde and genipin was used in the synthesis of MXN loaded gelatin mesosphere compositions as a chemical crosslinker.

Enzymes and salts used in the preparation of digestion buffer were purchased from Sigma Chemical Company: Papaya latex papain (22 U/mg solid), bacterial protease Type VIII (11 U/mg solid), and pepsin (4550 U/mg solid, ethylenediamine tetracetic acid (EDTA) disodium salt: dehydrate, (L-cysteine hydrochloride hydrate, and trichloroacetic acid.

The following items were used in culturing of RG-2 cells: Dulbecco’s Modified Eagle Medium, fetal bovine serum, and penicillin streptomycin were purchased from Gibco™ and trypsin was purchased from Cellgro®.

Deionized Type I and Type II water were prepared using a Barnstead NANOpure™ Ultrapure Water System. The resistivity of the deionized water (ultrapure water) used was at least 17.4 MΩ•cm.

Methanol HPLC grade was purchased from Fisher Scientific, and used as the mobile phase for particle size determination. All other solvents, reagents, and salts were
purchased from Fisher Scientific or Acros Organics and were Certified A. C. S. grade unless otherwise specified.

**Synthesis of In Situ Loaded MXN Gelatin Mesosphere Compositions**

**In situ loaded MXN gelatin mesospheres**

*In situ* loaded MXN gelatin mesospheres (MXN-GMS) were synthesized using the same techniques as the unloaded gelatin mesospheres except that the 10 w/v % gelatin solution consisted of 15 w/w % MXN. The same crosslinking agents, GEN and GTA were used in this study. A stock solution of 60 mg/ml GEN in dry acetone was prepared, and two crosslinker concentrations were used, 2.3 (C1) and 20.3 (C2) w/w % (20 and 200 % MEQ). Also, a stock solutions of 40 mg/ml GTA in 1-2 dichloroethane was prepared, and two crosslinker concentrations were used, 0.5 (C1) and 4.5 (C2) w/w % (20 and 200 % MEQ).

MXN-GMS were produced using a steric stabilization process. Suspension was formed by adding 3 ml of a heated 10 w/v % gelatin/15 w/w % MXN solution in 47 ml of a heated 3 w/v % cellulose acetate butyrate (CAB) /1-2 dichloroethane (DCE) and using a high speed mechanical-stirrer with a two inch, two-blade propeller. The gelatin solution was heated for 1 hr at 40°C. The CAB solution was heated for 5 minutes while stirring using a water bath at 40°C. The heat was removed 5 minutes after the addition of the gelatin and the suspension was allowed to return to room temperature. The stirring rate was maintained for 20 minutes at 1500 rpm after which the appropriate crosslinker and crosslinker concentration were added to crosslink the gelatin. The stirring rate was reduced to 600 rpm and the reaction was continued for two hours. After two hours, 50 ml of acetone was added (if needed to replace evaporated DCE) and the reaction was continued for another hour. The MXN-GMS was collected by centrifugation at 3000 rpm.
and washed in a series of four acetone resuspensions and centrifugations. The MXN-loaded mesospheres were air dried. Processing conditions are listed in Table 4.1.

Table 4.1 Experimental conditions used in the synthesis of MXN-GMS

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Gelatin</th>
<th>MXN</th>
<th>CAB</th>
<th>GTA</th>
<th>GEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>w/v %</td>
<td>w/w %</td>
<td>w/v %</td>
<td>w/w %</td>
<td>w/w %</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>15.0</td>
<td>3.0</td>
<td>0.5 (C1)</td>
<td>2.3 (C1)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>15.0</td>
<td>3.0</td>
<td>4.5 (C2)</td>
<td>20.3 (C2)</td>
</tr>
</tbody>
</table>

In order to examine the effects of varying crosslinker and crosslinker concentration on MXN-GMS’s particle size, particle morphology, particle percent swellability, MXN loading, and MXN release, a 2x2 factorial design was used.

**In situ MXN Loaded gelatin/CMC mesospheres**

In situ loaded MXN-GCMS were synthesized using the same steric stabilization process described in the synthesis of unloaded GCMS. The only addition was the 15 w/w % MXN to the gelatin/CMC solution. In order to conserve drug, optimized conditions of 13.0 w/w % CMC and 4.5 w/w % GTA (200 % MEQ) were used.

Gelatin/CMC mesospheres (GCMS) were produced using a steric stabilization process. Suspension was formed by adding 3 ml of a heated 10 w/v % gelatin solution containing 1.5 w/w % CMC in 47 ml of a heated 3 w/v % cellulose acetate butyrate (CAB)/1-2 dichloroethane (DCE) and using a high speed mechanical-stirrer with a two inch, two-blade propeller. The gelatin/CMC solution was heated for 1 hr at 40°C. The CAB solution was heated for 5 minutes, while stirring using a water bath at 40°C. The heat was removed 5 minutes after the addition of the gelatin and the suspension was allowed to return to room temperature. The stirring rate was maintained for 20 minutes at
1500 rpm after which the 4.5 w/w % GTA (200 % MEQ) was added to crosslink the gelatin. The stirring rate was reduced to 600 rpm and the reaction was continued for two hours. After two hours, 50 ml of acetone was added (if needed to replace evaporated DCE) and the reaction was continued for another hour. The GCMS was collected by centrifugation at 3000 rpm and washed in a series of four acetone resuspensions and centrifugations. The MXN-GCMS were air dried.

**Particle sizing**

The particle size of the gelatin mesosphere compositions was qualitatively measured using the Coulter® LS™ 230 particle size analyzer with the small volume module. A 1 % mesosphere suspension was prepared in 5 ml of Methanol. The suspension was sonicated to ensure complete dispersion and then added drop wise until the obstruction read approximately 12 to 15. Data was analyzed using Coulter® LS™ 32 software.

SEM was used to qualitatively observe the particle size and morphology of the synthesized mesospheres. Dry mesospheres were mounted on double-sided tape on aluminum SEM stubs. The stubs were then coated with a gold/palladium alloy for 2.5 to 5.0 minutes using a Technix Hummer V sputter Coater. The samples were analyzed using a Jeol SEM 6400 scanning electron microscope at 5 KeV accelerating voltage; a condenser lens setting of 8 to 10; and a working distance of 15mm.

**Swelling study**

Optical microscopy was used to qualitatively monitor the change in particle size with degree of hydration. Dry MXN loaded mesospheres were sprinkled on a glass slide and hydrated via the addition of a drop of phosphate buffered saline. Digital images were captured at every 30 seconds for the first 10 minutes followed by every 5 minute intervals
for the next 10 minutes then at the 30 minute time point. These images were obtained using a Zeiss Axioskop 2 optical microscope equipped with SPOT™ digital camera.

The average particle size for each time point was quantitatively determined by measuring the diameter of 40 random particles using SPOT™ and Image Pro Plus™ image analysis software.

**Digestion study**

MXN-GMS and MXN-GCMS were digested, and analyzed for MXN content. They were digested by incubating 5 mg of MXN-loaded mesospheres in 10 ml of digestion buffer at 37 °C for 72 hrs. The digestion buffer was prepared by dissolving 720 mg ethylenediamine tetracetic acid (EDTA) disodium salt: dehydrate, 80 mg L-cysteine hydrochloride hydrate, 50 mg papaya latex papain, and 50 mg bacterial protease Type VIII in a 100 ml 0.1 M phosphate buffer solution (pH 7.0). Digestion was confirmed by optical microscopy. The protein was precipitated by addition of an equal volume of a 10 w/v % trichloroacetic acid (TCA) solution. The suspension was centrifuged at 3000 rpm for 10 minutes and the supernatant was analyzed on a Shimadzu UV-2401PC (UV-VIS) at 610 nm against a matched matrix background. Standard MXN solutions and gelatin solutions were incubated under the same conditions then precipitated with TCA to account for drug degradation or loss in this process.4

**In vitro release study**

Two mg MXN-GMS were transferred into 2 ml Ultrafree-CL Centrifugal Filter Devices (low binding Duraproe PVDF membrane 0.1 um) Fisher Cat # UFC40VV25 and incubated for in 1 ml phosphate buffered saline (PBS). At predetermined time points (every hr for the first 8 hrs, then every 24 hrs for 10 days), 1ml aliquots were obtained and replaced with 1ml of PBS in order to retain initial volume. Aliquots collected from
the in vitro release studies were analyzed by Shimadzu UV-2401PC UV-Visible Spectrophotometer at 610 nm against a matched matrix background to determine the release profiles as a function of crosslinker and crosslinker concentration. Experimental conditions are listed in Table 4.1.

**Solution preparation**

**MXN gelatin.** Gelatin is soluble in water at elevated temperatures. Thus, gelatin solutions were prepared in 35°C nanopure water. Also, gelatin solution foamed significantly when agitated, therefore making the conventional technique of solution preparation in volumetric flasks less than ideal. Gelatin solutions were prepared in a 50 ml conical as close to the desired concentration as possible. The true concentration was measured gravimetrically, and the density of the solution was measured.

Approximately 1 ml of the gelatin solutions were dried at 130°C on a Mettler LJ16 Moisture Analyzer. Using the dry weight and density, the weight per volume percent was determined. Concentration was adjusted until true concentration was achieved within 0.5%.

After the preparation of the gelatin solution, 30 ml of the gelatin solution was transferred to a 50 ml centrifuge tube and heated to 40 °C. The gelatin solution was removed from the oven and 15.0 w/w% MXN was added. MXN gelatin solution was then placed on a rotating table to insure complete mixing.

**MXN gelatin/CMC.** In situ loaded MXN Gelatin/CMC solutions were prepared using the same techniques described in the in situ loaded MXN gelatin solution preparation. Appropriate amounts of medium molecular weight CMC were added to a heated (40°C) 10 w/v % gelatin solution and dissolved.
**Glutaraldehyde.** Glutaraldehyde solutions were prepared by vacuum distilling 25% aqueous glutaraldehyde solution and recovering the glutaraldehyde distillate. The distillate was then dissolved in DCE to a final concentration of 40 mg/ml.

**Genipin.** Genipin solutions were prepared by dissolving solid genipin in dry acetone to a final concentration of 60 mg/ml.

**Cellulose acetate butyrate.** Cellulose acetate butyrate solutions were prepared by dissolving solid cellulose acetate butyrate in DCE to a final concentration of 3.0 w/v %.

**Phosphate buffered saline.** Isotonic phosphate buffered saline was prepared by mixing 50 mM sodium phosphate monobasic solution with a 50 mM sodium phosphate dibasic solution until a desired pH of 7.4 was reached. Osmolarity of the solutions was adjusted to 300 mOsm with the addition of sodium chloride (NaCl). Appropriate amounts of NaCl was determine by the preparation of a calibration plot by varying the concentrations (0 to 10 mg/ml) of NaCl in the phosphate buffer. The osmolarity of the standards was measured using Precision Instruments u Osmette™. The resulting PBS solution was sterile filtered through a 0.22 um filter.

**RG-2 Cell Cultures**

After achieving confluence, RG-2 cells, donated by Dr. Black, were harvested. The culture media was vacuumed from cell culture flask and replaced with 10 ml of 10:1 PBS/trypsin. Culture flasks were incubated (37°C, 8% CO₂) for 5 minutes for trypsinization. The cell suspension was aspirated and transferred to 50 ml conicals containing equal volume of Dubelco’s Minimal Essential Media (DMEM) with 10% fetal bovine serum and 0.1% penicillin/streptomycin (complete media) and centrifuged (4x g, 5 minutes). The supernatant was removed and cells were triturated in 5 ml of complete media. The concentration of viable cells (cell number/ml) was determined by vital
staining with trypan blue and counted manually using a hemocytometer. The cell concentration was adjusted to $3 \times 10^4$ cells/ml, and 75 ul of the cell suspension was seeded in a 96 well plate. Cell cultures were maintained in an incubator (37°C, 8% CO$_2$) for 24 hrs prior to treatment (Day 0) to allow for adequate cellular attachment. Following this period, 75 ul of treatment solution (prepared in DMEM) was added to each well. Treatment periods ranged from 1 to 4 days. These in vitro cell cultures were conducted with the assistance of Josh Stopek, Amanda York, and Barry Flanary.

**Treatments**

Various in vitro evaluations were designed to study the efficacy of using free MXN and bound MXN delivery systems in the treatment of RG-2 glioma cells. These studies examined the cytotoxic effects of varying the dose, (25.0, 12.5, and 0.5 ppm), of MXN, as well as the way the MXN was delivered (free or bound). There were twelve different treatment groups excluding respective controls: 1) MXN only, 2) MXN and gelatin, 3) MXN loaded GTA C1 crosslinked gelatin mesospheres, 4) MXN loaded GTA C2 crosslinked gelatin mesospheres, 5) MXN loaded GEN C1 crosslinked gelatin mesospheres, 6) MXN loaded GEN C2 crosslinked gelatin mesospheres, 7) MXN-GCMS, 8) Free MXN plus MXN loaded GTA C1 crosslinked gelatin mesospheres, 9) Free MXN plus MXN loaded GTA C2 crosslinked gelatin mesospheres, 10) Free MXN plus MXN loaded GEN C1 crosslinked gelatin mesospheres, 11) Free MXN plus MXN loaded GEN C2 crosslinked gelatin mesospheres, 12) Controls (non-treated and non-MXN modalities).

All treatment groups were prepared in DMEM and non-mesosphere treatment groups were sterilized using 0.22um syringe filter. For each treatment group, 100 ul/ml stock solutions were prepared and diluted to desired concentrations. The amount of
mesospheres and gelatin needed for each study was determined based on the percent loading of MXN. For the Free MXN plus MXN loaded mesosphere study, 25 % MXN was added to 75 % MXN loaded mesospheres to make up the total desired doses.

**RG-2 Cellular Viability/Proliferation**

Treatment of cytotoxicity was observed by monitoring the cell proliferation and morphology using colorimetric assay (MTT based) and bright field optical microscopy, respectively. After appropriate treatment period, 15 ul of MTT labeling reagent (final concentration 0.5 mg/ml) was added to each cell culture well. The cell culture plate was incubated for 4 hrs in a humidified atmosphere (37 °C, 5 % CO₂), and 100 ul of solubilization solution was added to each cell culture well, and incubated over night in a humidified atmosphere. Spectrophotometrical absorbance of the samples and references were measured at 550 and 655 nm, using a microtiter plate (ELISA) reader (BIORAD). Bright field optical microscopy was performed daily using inverted microscopy equipped with a digital camera. These *in vitro* evaluations were conducted with the assistance of Josh Stopek and Amanda York.

**Results and Discussion**

Results from our initial studies have shown exciting preclinical results and indicated IT mitoxantrone, especially in micro/mesosphere preparations, may significantly improve survival with decreased systemic toxicity. Techniques in the synthesis of MXN-BSA and the effects of processing conditions on particle size, drug loading and release, and *in vivo* safety and efficacy were evaluated. These evaluations were co investigated with Drs. Almond and Hadba and have been reported.⁴ ⁵
In situ mitoxantrone loaded albumin microspheres (MXN-BSA) were prepared using a steric stabilization process, where by a 20 w/v % bovine serum albumin solution containing 15 w/w % MXN was dispersed in a 2.75 % CAB solution using similar methods described in Figure 2.4. The concentration of GTA was varied (4.0, 8.0, and 12.0 w/w %) [100; 300; and 500 % MEQ] and the effects on particle size and morphology, and drug loading and release were evaluated.

The dry particle size was determined using SEM and a particle size analyzer. The dry particle morphology was evaluated under SEM. The percent MXN loading was determined by enzymatically degrading the microspheres and analyzing the drug concentration. The percent release was evaluated by incubated MXN-BSA in an infinite sink of PBS and monitoring the change MXN concentration with time.

Smooth spherical 15 um MXN-BSA containing 14 w/w % MXN were successfully synthesized. The percent in vitro release was shown to improve (55 to 85 to 95 %) with increasing GTA concentrations.

Preclinical in vivo studies evaluated the validity of localized IT therapies using MXN-BSA compositions in the treatment of 16/C murine mammary adenocarcinoma (MMAC). C3H/HeJ mice were injected subcutaneously into the flank of the mouse. Once the tumor reached a size of 10 mm in its largest dimension approximately 10 to 14 days post-inoculation, the mice was randomly assigned a treatment group. Treatment groups consisted of varying dose regiments of 8 mg/kg to 48 mg/kg of free MXN and MXN-BSA were administered IT via a 100 ul injections through a 25 Ga needle. To insure complete perfusion of the tumor, 5 injections (4 injections around the perimeter
and 1 injection in the center of the tumor) of 20 ul injections for a total of 100 ul were administered. Tumor size and body was monitored daily.

These studies suggested that these preparations could be safely administered IT up to 48 mg/kg doses, in addition there was no evidence higher doses resulted in improved longer term survival when compared to 32 mg/kg dose. Conversely, the maximum tolerated IT MXN dose was 12 mg/kg. Together, these results indicated microsphere bound drug may be safely administered IT at high doses exerting antitumor effects for extended periods of time.

The success observed using albumin microspheres has sparked new interests in evaluating other protein compositions, which may further enhance or complement the favorable properties of albumin compositions. In contrast, gelatin has been shown to degrade more readily with faster drug release. Gelatin mesospheres may offer drug loading and release properties not afforded by albumin preparations. The following details the accomplishments of this research focused on the novel MXN loaded gelatin mesospheres.

**In situ MXN Loaded Gelatin Mesospheres**

The average particle size for MXN-GMS was qualitatively observed with SEM and quantitatively measured with a Coulter® LS™ 230 particle size analyzer. The particle size data are tabulated in **Table 4.2** and are graphed in **Figure 4.1**. The particle size distributions are graphed in **Figure 4.2**. The statistical analysis software reported that there is no statistical difference on particle size with changing crosslinker (p=0.060) or crosslinker concentration (p=0.941). Also, there was no statistically significant interaction (p=0.185) between the crosslinker and crosslinker concentration. The mean particle size was 2 um and the particle size distribution ranged from 0.04 – 20 um.
Figure 4.3 is SEM micrographs of GEN and GTA crosslinked in MXN loaded mesospheres that were synthesized. From these images, there appears to be no evidence to suggest that varying the crosslinker and the crosslinker concentration had an effect on particle morphology. All the conditions, appear to produce smooth; spherical mesospheres with similar size distributions. Also, the addition of MXN had no significant effects on particle size, when compared to unloaded mesospheres (p=0.980).

After the completion of MXN-GMS, MXN-GCMS were synthesized using optimized conditions of 13 w/w % CMC and 4.5 w/w % GTA, which was determined from preliminary GCMS studies. The average particle size for MXN-GCMS was qualitatively observed with SEM and quantitatively measured with a Coulter® LS™ 230 particle size analyzer. The mean dry particle size was 2 um with a particle size distribution of 0.04 – 20 um. The particle morphology (Figure 4.4) varied with increasing concentration of CMC.

Table 4.2 Particle size data for varying crosslinkers and crosslinker concentrations in the synthesis of MXN-GMS

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
</tr>
<tr>
<td>Protein/Drug</td>
<td>Crosslinker</td>
</tr>
<tr>
<td>Gelatin w/v%</td>
<td>MXN w/w</td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
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<td>10.0</td>
<td>15</td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
</tr>
</tbody>
</table>
Figure 4.1 Graphical representation of the particle size with varying crosslinkers and crosslinker concentrations in the synthesis of MXN-GMS

Figure 4.2 Particle size distributions with varying crosslinkers and crosslinker concentrations in the synthesis of MXN-GMS
Figure 4.3 SEM micrographs 3000x of MXN-GMS with varying crosslinkers and crosslinker concentrations: A) GEN C1; B) GEN C2; C) GTA C1; and D) GTA C2 w/w %

Figure 4.4 SEM micrographs 3000x of MXN-GCMS varying concentrations of CMC: A) 0.0 % CMC and B) 13.0 % CMC

These variations were not observed in the synthesis of GCMS. The morphology of MXN-GCMS containing 13.0 w/w % CMC resembles coarsening, which may have
resulted by spinodal phase separation, as previously reported.\textsuperscript{4} The phase separation may have resulted from the charge cancellation from the ionic interactions of the anionic polysaccharide and the cationic drug.

**Swelling Studies**

In the past, particle size and morphology of micro/mesospheres were investigated using SEM in their dry state. Using optical microscopy and image analysis software, MXN loaded gelatin mesosphere compositions were investigated in their hydrated state and the percent swellability of was determined. Fully hydrated mesospheres were observed within the first minute of this study, as seen in Figure 4.5 and 4.6, and the percent change in particle size, surface area, and volume are listed in Table 4.3.

<table>
<thead>
<tr>
<th>Crosslinker</th>
<th>Conc.</th>
<th>Particle size</th>
<th>Surface Area</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN C1</td>
<td>750</td>
<td>4,226</td>
<td>61,385</td>
<td></td>
</tr>
<tr>
<td>GEN C2</td>
<td>504</td>
<td>2,029</td>
<td>21,984</td>
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<tr>
<td>GTA C1</td>
<td>773</td>
<td>3,709</td>
<td>66,485</td>
<td></td>
</tr>
<tr>
<td>GTA C2</td>
<td>509</td>
<td>1,785</td>
<td>22,470</td>
<td></td>
</tr>
</tbody>
</table>

Two way ANOVA statistical analysis tests suggested that there was no statistical difference of varying the crosslinker, but there was a statistical difference of varying crosslinker concentration on the percent swellability and the percent change in surface area and in volume (p<0.001). The pair-wise multiple comparison tests (Tukey Test) showed that increasing the crosslinker concentration decreased the percent swellability (750 to 500 %) and the percent change in surface area (4,000 to 2000 %) and in volume (60,000 to 20,000 %) [p<0.05]. The change in particle size with varying crosslinkers and crosslinker concentrations is graphically represented in Figure 4.7.
Figure 4.5 Optical images of hydrated MXN-GMS crosslinked with A) GEN C1 and B) GEN C2
Figure 4.6 Optical image of MXN-GMS crosslinked with A) GTA C1 and B) GTA C2
Figure 4.7 Graphical representation of the change in MXN-GMS’s particle size with hydration at varying crosslinker (I. GEN and II. GTA) and crosslinker concentrations
MXN Loaded Mesosphere’s Loading and Release

*In situ* and post loading are two methods of producing drug loaded mesospheres. Post loading is achieved by soaking the unloaded mesospheres in a concentrated drug solution, whereby loading is achieved. *In situ* loading is achieved by incorporating the drug in an aqueous protein solution, which is then used in the mesospheres synthesis. Recent studies have shown higher MXN loading efficiencies were achieved using *in situ* loading techniques. Therefore, *in situ* loading techniques were used in the production of MXN loaded gelatin mesosphere compositions.

After the successful synthesis of MXN-GMS, the percent loading was determined. Mesospheres were digested and aliquots were analyzed to determine the drug concentration. The percent loading is tabulated in Table 4.4.

Table 4.4  MXN-GMS’s percent MXN loading and MXN release

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Concentration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gelatin</td>
<td>MXN</td>
</tr>
<tr>
<td></td>
<td>w/v %</td>
<td>w/w %</td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
<td>GEN (C1)</td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
<td>GEN (C2)</td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
<td>GTA (C1)</td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
<td>GTA (C2)</td>
</tr>
</tbody>
</table>

A two-way ANOVA was used to compare the effects of crosslinker and crosslinker concentration on drug loading. The analysis showed that there was statistical difference (p=0.004) on varying crosslinker on percent drug loading. Pairwise comparisons showed that GEN crosslinked mesospheres achieved higher drug loading (12.8 w/w %) than GTA
crosslinked controls (11.5 w/w %) (p<0.05). Figure 4.8 is a graphical representation of the loading efficiencies with varying crosslinker and crosslinker concentration.

*In vitro* release studies were postulated to determine the diffusivity of the drug out of the mesospheres. *In situ* MXN-GMS were incubated in PBS and aliquots were taken at predetermined time points (every hour for the first eight hours then every 24 hrs for 10 days), Figure 4.9. MXN release was limited to the first 4 hrs, after which no change in percent release was observed. The overall percent release was listed in Table 4.5. A two-way ANOVA was used to compare the effects of crosslinker and crosslinker concentration on percent release.

![Figure 4.8 Percent loading of MXN for varying crosslinker (GEN and GTA) and crosslinker concentrations](image-url)
The analysis showed that there was a statistical difference (p=0.037) on varying crosslinker concentration on percent release. Pairwise comparisons showed that increasing crosslinker concentration reduced the overall release (78 to 65%; 65 to 50%) of MXN (p<0.05). No statistical difference (p=0.360) was observed with varying crosslinker.

After the completion of the GCMS study, in situ loaded MXN-GCMS were synthesized using optimized conditions, listed in Table 4.5. The same digestion and in vitro release studies used in the MXN-GMS were used to determine the percent MXN
loading and release. A one-way ANOVA was used to compare the effect of varying the CMC concentration on drug loading and release. The analysis showed that there was a statistically significant effect (p<0.001) of the CMC concentration on percent drug loading and release. Pair-wise multiple comparison tests showed increasing CMC concentrations increased the overall percent release from 50 to 78 %, while decreasing the percent drug loading from 12 to 8 % (p<0.05). The decrease in drug loading may be attributed to incomplete digestion. The release profiles are depicted in Figure 4.10.

From these curves, MXN-GCMS containing 0.0 w/w% CMC released up to 4 hrs, whereas, MXN-GCMS containing 13.0 w/w% CMC released for 240 hrs.

The binding affinity of the drug to the PVDF membrane was also determined. Release study of free MXN solutions was shown to only achieve 92 % release. Therefore, overall percent release of the MXN-GMS and MXN-GCMS may be slightly higher.

Table 4.5  MXN-GCMS percent MXN loading and release

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration</strong></td>
<td><strong>Results</strong></td>
</tr>
<tr>
<td>Gelatin w/v %</td>
<td>CMC w/w %</td>
</tr>
<tr>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td>10.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>
In order to investigate the efficacy of MXN loaded gelatin mesosphere compositions in the treatment of malignant gliomas, a series of 12 baseline in vitro RG-2 cells studies excluding respective controls was postulated to study cytotoxicity of localized MXN treatments at varying doses (25.0, 12.5, and 0.5 ppm) of MXN: 1) MXN only, 2) MXN and gelatin, 3) MXN loaded GTA C1 crosslinked gelatin mesospheres, 4) MXN loaded GTA C2 crosslinked gelatin mesospheres, 5) MXN loaded GEN C1 crosslinked gelatin mesospheres, 6) MXN loaded GEN C2 crosslinked gelatin mesospheres, 7) MXN-GCMS, 8) Free MXN plus MXN loaded GTA C1 crosslinked gelatin mesospheres, 9) Free MXN plus MXN loaded GTA C2 crosslinked gelatin mesospheres, 10) Free MXN plus MXN loaded GEN C1 crosslinked gelatin mesospheres.

Figure 4.10  MXN-GCMS in vitro release profiles
mesospheres, 11) Free MXN plus MXN loaded GEN C2 crosslinked gelatin mesospheres, 12) Controls (non-treatment and non-MXN modalities).

After treatment, RG-2 cells metabolic activity was monitored using a colorimetric assay, MTT assay. The MTT assay was used to quantitate cellular proliferation and cytotoxicity. This assay used a tetrazolium salt, MTT, which was reduced by proliferating cells to a colored water-insoluble formazan salt. After the formazan salt was solubilized, the metabolic activity of the treated RG-2 cells was quantitated by monitoring the absorbance at 550 nm. Day 4 experimental absorbance values were normalized to their respective controls. Cellular proliferation was based on absorbance values between 0.00 to 1.00 a.u.

The first study investigated localized treatments of free MXN. Three doses (25.0, 12.5, and 0.5 ppm) of MXN were administered to RG-2 cells to establish a dose response. Figure 4.11 is a graphical representation of the RG-2 cell proliferation with time. A two-way ANOVA followed by a Tukey Multiple comparison test was used to compare the effects of treatment and dose on cell proliferation at day four. The analysis suggested the dose had a statistically significant effect on cell proliferation (p<0.001). The pair-wise multiple comparison tests showed that increasing the dose significantly reduces RG-2 cellular proliferation from 90 to 12 %, as seen in Table 4.6 (p<0.05). Figure 4.12 is bright field images of the morphology of the RG-2 cells on day 2. The controls and the low dose were confluent, where as, the high and middle dose displayed ameboid morphology with retracted processes. The high dose exhibited evidence of cellular debris and drug uptake as shown by the blue staining of the cellular matrix.
Table 4.6  RG-2 cellular proliferation with varying doses of MXN

<table>
<thead>
<tr>
<th>Treatment: Free MXN</th>
<th>Dose of MXN</th>
<th>Absorbance</th>
<th>Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppm</td>
<td>Mean</td>
<td>Stdev</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>1.00</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>0.90</td>
<td>0.04</td>
<td>90</td>
</tr>
<tr>
<td>12.5</td>
<td>0.29</td>
<td>0.03</td>
<td>26</td>
</tr>
<tr>
<td>25.0</td>
<td>0.12</td>
<td>0.01</td>
<td>12</td>
</tr>
</tbody>
</table>

The second baseline study evaluated the effects of free MXN and gelatin on RG-2 cellular proliferation. Similar to the first study, the same doses (25.0, 12.5, and 0.5 ppm) of MXN were administered locally to RG-2 cells to establish a dose response. The importance of this study was to determine the effects of gelatin and gelatin/MXN complexes on cellular proliferation. A comparable amount of gelatin that was added in the gelatin mesosphere study was used. Figure 4.13 is a graphical representation of the cell proliferation with time. The statistical analysis suggested the dose had a statistically significant effect on cell proliferation (p<0.001). The pair-wise multiple comparison tests showed that increasing the dose significantly reduces RG-2 cellular proliferation from 43 to 0 %, as listed in Table 4.7 (p<0.05). Figure 4.14 is bright field images of the morphology of the RG-2 cells on day 2. These images display ameboid RG-2 morphology with retracted processes for gelatin/MXN treatment groups. The high and medium doses also exhibited evidence of cellular debris and drug uptake.
Table 4.7  RG-2 cellular proliferation with varying doses of free MXN and gelatin

<table>
<thead>
<tr>
<th>Treatment: Free MXN/Gelatin</th>
<th>Dose of MXN (ppm)</th>
<th>Absorbance Mean (a.u.)</th>
<th>Absorbance Stdev (a.u.)</th>
<th>Normalized %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
<td>1.00</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.43</td>
<td>0.01</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
</tbody>
</table>

The free gelatin was dosed comparable to above study. Figure 4.15 is a graphical representation of the cell proliferation with time of free gelatin (no MXN). The statistical analysis suggested that free gelatin had no statistical effect of cell proliferation (p=1.00). RG-2 cells reach maximum proliferation (100 %), regardless the dose of gelatin. RG-2 cellular morphology is depicted in Figure 4.16.

After the completion of the baseline studies, MXN loaded gelatin mesospheres were investigated as possible alternative to free MXN localized therapies. It was hypothesized that these compositions may be more efficacious by affording sustained and controlled release of therapeutic doses of MXN. *In situ* MXN loaded gelatin mesospheres with varying crosslinker (GEN and GTA) and crosslinker concentration were synthesized with a mean particle size of 2 um. Particle size data is listed in Table 4.2. The SEM images of MXN-loaded mesospheres used in these studies are shown in Figure 4.3. GEN was studied as a possible alternative crosslinker to GTA. GEN has been reported to have similar characteristics to GTA, but may have enhanced properties, i.e., release rate, loading efficiency, biocompatibility. Also, the therapeutic effects of crosslink density were examined.
This facet investigated the effects of MXN loaded gelatin mesospheres on RG-2 cellular proliferation. Comparable doses (25.0, 12.5, and 0.5 ppm) of MXN were administered via MXN loaded gelatin mesospheres (GEN and GTA) with varying crosslinker concentration (C1 and C2) to the cells to establish a dose response. Figure 4.17 and Figure 4.18 are graphical representations of the cell proliferations of RG-2 cells after being treated with GEN and GTA crosslinked gelatin mesospheres.

The statistical analysis suggested the dose had a statistically significant effect on cell proliferation (p<0.001). The pair-wise multiple comparison tests showed that increasing the dose significantly reduces cell proliferation (p<0.05). The only statistical difference observed with varying crosslinker and crosslinker concentration on cellular proliferation was at the lowest MXN concentration. GEN crosslinked MXN mesospheres decreased the overall cellular proliferation (58 and 67 %, with increasing crosslinker concentration) compared to GTA crosslinked MXN mesospheres (93 and 83 %, with increasing crosslinker concentration) (p<0.05). Decreased concentrations of GEN also further decreased the cell proliferation, where as, the opposite effect was observed for GTA (p<0.05), as seen in Table 4.8. Figure 4.19 and Figure 4.20 displays ameboid morphology with retracted processes retraction of all MXN loaded mesosphere treatment groups. The high and median dose for both GTA and GEN displayed evidence of cellular debris and drug uptake.

Figure 4.21 and Figure 4.22 are graphical representations of the cell proliferation with time of unloaded gelatin mesospheres. The unloaded gelatin mesospheres were dosed comparable to above study. Compared to the control, none of the treatment groups
significantly hindered proliferation (p=1.00). Figure 4.23 and Figure 4.24 shows cellular confluence all treatment groups.

Table 4.8  RG-2 cellular proliferation with varying doses of MXN-GMS

<table>
<thead>
<tr>
<th>Crosslinker</th>
<th>Crosslinker Conc.</th>
<th>Dose of MXN ppm</th>
<th>Absorbance Mean a.u.</th>
<th>Absorbance Stdev a.u.</th>
<th>Normalized %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.0</td>
<td>0.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GEN C1</td>
<td></td>
<td>0.5</td>
<td>0.58</td>
<td>0.04</td>
<td>58</td>
</tr>
<tr>
<td>GEN C1</td>
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<td>0.10</td>
<td>0.02</td>
<td>10</td>
</tr>
<tr>
<td>GEN C1</td>
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<td>25.0</td>
<td>0.04</td>
<td>0.00</td>
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<tr>
<td>GEN C2</td>
<td></td>
<td>0.5</td>
<td>0.67</td>
<td>0.21</td>
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<tr>
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<td>0.10</td>
<td>0.03</td>
<td>10</td>
</tr>
<tr>
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<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>GTA C1</td>
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<td>0.93</td>
<td>0.02</td>
<td>93</td>
</tr>
<tr>
<td>GTA C1</td>
<td></td>
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<td>0.13</td>
<td>0.02</td>
<td>13</td>
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<tr>
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<tr>
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<td>0.14</td>
<td>0.01</td>
<td>14</td>
</tr>
<tr>
<td>GTA C2</td>
<td></td>
<td>25.0</td>
<td>0.06</td>
<td>0.09</td>
<td>6</td>
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</table>

Building on the previously described studies, the effects of a free MXN plus MXN loaded mesosphere on RG-2 cells were investigate. Comparable MXN doses (25.0, 12.5, and 0.5 ppm) were administered via the combination of 25 % free MXN with 75 % MXN-GMS to establish a dose response. Figure 4.25 and Figure 4.26 are graphical representations of the cell proliferations of RG-2 cells after being treated with GEN and GTA crosslinked gelatin mesospheres. The statistical analysis suggested the dose had a statistically significant effect on cell proliferation (p<0.001). The pair-wise multiple comparison tests showed that increasing the dose significantly reduces RG-2 cellular
proliferation (30 to 0 %), respectively (p<0.05). No effect of crosslinker and crosslinker concentration on cell proliferation was observed for varying doses, as seen in Table 4.9. Figure 4.27 and Figure 4.28 shows RG-2 cells with ameboid morphology and retracted processes for all MXN and MXN loaded mesosphere treatment groups. The high and median dose for both GTA and GEN displayed evidence of cellular debris and drug uptake.

Table 4.9  RG-2 cellular proliferation with varying doses of free MXN plus MXN-GMS

<table>
<thead>
<tr>
<th>Crosslinker</th>
<th>Crosslinker Conc.</th>
<th>Dose of MXN ppm</th>
<th>Absorbance Mean a.u.</th>
<th>Absorbance Stdev a.u.</th>
<th>Normalized %</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.31</td>
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<td>31</td>
</tr>
<tr>
<td>GEN C1</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>GEN C1</td>
<td>25.0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>GEN C2</td>
<td>0.5</td>
<td>0.26</td>
<td>0.04</td>
<td>0.04</td>
<td>26</td>
</tr>
<tr>
<td>GEN C2</td>
<td>12.5</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
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<td>GEN C2</td>
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<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>GTA C1</td>
<td>0.5</td>
<td>0.24</td>
<td>0.03</td>
<td>0.03</td>
<td>24</td>
</tr>
<tr>
<td>GTA C1</td>
<td>12.5</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>GTA C1</td>
<td>25.0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>GTA C2</td>
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<td>0.33</td>
<td>0.04</td>
<td>0.04</td>
<td>33</td>
</tr>
<tr>
<td>GTA C2</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>GTA C2</td>
<td>25.0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
</tbody>
</table>

After the completion of MXN-GMS in vitro evaluations, the effects of MXN – GCMS with varying concentrations of carboxymethyl cellulose (0.0 and 13.0 w/w %) on RG-2 cellular proliferation were investigated. Comparable doses (25.0, 12.5, and 0.5 ppm) of MXN were administered.
The statistical analysis suggested the dose had a statistically significant effect on cell proliferation (p<0.001). The pair-wise multiple comparison tests showed that increasing the dose significantly reduces cell proliferation (p<0.05). The only statistical difference observed with varying crosslinker and crosslinker concentration on cellular proliferation was at the 0.5 and 12.5 ppm MXN. Increasing the concentration of CMC in MXN mesospheres decreased the overall cellular proliferation (83 and 55 % at 0.5 ppm MXN and 14 to 7 % at 12.5 ppm MXN), as seen in Table 4.10. Figure 4.29 displays ameboid morphology with retracted processes for the MXN-GCMS treatment groups. The high and median dose displays evidence of cellular debris and drug uptake.

Table 4.10 RG-2 cellular proliferation with varying doses of MXN-GCMS

<table>
<thead>
<tr>
<th>CMC Conc. w/w %</th>
<th>Dose of MXN ppm</th>
<th>Absorbance Mean a.u.</th>
<th>Absorbance Stdev a.u.</th>
<th>Normalized %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>Control</td>
<td>1.0</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td>0.0</td>
<td>0.5</td>
<td>0.83</td>
<td>0.01</td>
<td>83</td>
</tr>
<tr>
<td>0.0</td>
<td>12.5</td>
<td>0.14</td>
<td>0.01</td>
<td>14</td>
</tr>
<tr>
<td>0.0</td>
<td>25.0</td>
<td>0.06</td>
<td>0.09</td>
<td>6</td>
</tr>
<tr>
<td>13.0</td>
<td>Control</td>
<td>0.82</td>
<td>0.033</td>
<td>100</td>
</tr>
<tr>
<td>13.0</td>
<td>0.5</td>
<td>0.45</td>
<td>0.31</td>
<td>55</td>
</tr>
<tr>
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<td>12.5</td>
<td>0.06</td>
<td>0.04</td>
<td>7</td>
</tr>
<tr>
<td>13.0</td>
<td>25.0</td>
<td>0.00</td>
<td>0.00</td>
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</tbody>
</table>

In summarizing the efficacy of the various MXN treatment modalities, the statistical analysis suggested the treatment had a statistically significant effect on cell proliferation (p<0.001). The pair-wise multiple comparison tests showed that free MXN plus free gelatin and free MXN plus free MXN loaded mesospheres > MXN loaded
mesospheres > free MXN at reducing RG-2 cellular proliferation (p<0.05). Also, MXN-GCMS were shown to have increased efficacy over MXN-GMS at comparable crosslinker and crosslinker concentration at reducing RG-2 cellular proliferation (p<0.05).

**Murine Mammary Adenocarcinoma *In Vivo* Evaluations**

Preliminary parallel *in vivo* studies evaluated the validity of localized IT therapies using MXN loaded gelatin mesospheres compositions in the treatment of 16/C murine mammary adenocarcinoma (MMAC). This work was performed in conjunction with Shema Freeman and Amanda York using previously established methodologies.\(^8,9\) C3H/HeJ mice were injected subcutaneously into the flank of the mouse. Once the tumor reached a size of 10 mm in its largest dimension approximately 10 to 14 days post-inoculation, the mice was randomly assigned a treatment group. Treatment groups consisted of varying dose regiments of 14 mg/kg or 28 mg/kg of MXN-GMS crosslinked with varying molar concentrations of GEN and GTA (20 and 200 % MEQ) with 4 mg/kg of free MXN were administered IT via a 100 ul injections through a 25 Ga needle. To insure complete perfusion of the tumor 5 injections (4 injections around the perimeter and 1 injection in the center of the tumor) of 20 ul injections for a total of 100 ul were administered. Tumor size and body was monitored daily.

Generally, all MXN treatment groups exhibited tumor regression, with enhanced tumor regression at the high dose MXN groups. The most promising formulations were GTA crosslinked MXN-GMS at the high MXN dose and the with GEN C2 crosslinked MXN-GMS at the high and low MXN dose with approximately 25 % survival, 25 days post treatment.
Summary of Research

MXN-loaded mesosphere particle characterizations

MXN-GMS and MXN-GCMS were synthesized using steric stabilization process, which was adapted from conditions reported in the synthesis of albumin microspheres and casein microspheres. Processing conditions were established to produce smooth, spherical particles, with a mean dry particle size of 2 μm, with a particle size distribution of 0.04 – 20 μm. Investigative studies on processing conditions showed that varying the crosslinker and the crosslinker concentration had little or no effect on particle morphology and no effect on dry particle size or particle size distribution in the synthesis of MXN-GMS.

Swelling studies on MXN-GMS showed that increasing the crosslinker concentration reduced the percent swellability from 750 to 500 %, regardless of which crosslinker was used. The percent swellability of the MXN-GMS was comparable to the percent swellability of GEN crosslinked gelatin films at comparable crosslinker concentrations.

In vitro and in vivo evaluations

MXN loaded gelatin mesosphere compositions were successfully synthesized for use in localized IT therapies. MXN-GMS crosslinked with GEN achieved loadings efficiencies in excess of 85 %, which constitutes 13 w/w % MXN, where as, MXN-GMS crosslinked with GTA only achieved loading efficiencies in excess of 77 %, which constitutes 12 w/w % MXN. Also, MXN-GMS exhibited a decrease in percent release (78 – 65 % MXN) with increasing GEN concentration and (65 – 50 % MXN) with increasing GTA concentration. MXN-GMS’s MXN release was limited to the first 4 hrs
where as, MXN-GCMS exhibited sustained release exceeding 240 hrs with a percent loading of 8 w/w % MXN and percent release of 78 % MXN.

Localized *in vitro* MXN treatments of malignant gliomas were shown to prevent and inhibit cellular proliferation. The most promising responses were seen at high MXN doses and utilizing combinatory treatments. Combinations of free MXN with free gelatin or MXN loaded mesospheres surpassed the single therapies and significantly hindered RG-2 cellular proliferation and prevented cellular regeneration.

Increased efficacy was shown in the treatment of RG-2 cells with the addition of CMC and Genipin to MXN loaded gelatin mesospheres.

The validity of using MXN-GMS compositions for IT therapies in the treatment of high mortality cancers was further supported with the *in vivo* MMAC evaluations. Increased survival was observed with all combinatory therapies, with enhanced efficacy at elevated MXN doses and the incorporation of GEN as a chemical crosslinker.
Figure 4.11 Graphical representation of RG-2 cell proliferation with time for varying doses of free MXN
Figure 4.12  Day two images of RG-2 cells treated with free MXN treatments: A) Control; B) 0.5 ppm MXN; C) 12.5 ppm MXN; and D) 25 ppm MXN
Figure 4.13  Graphical representation of RG-2 cell proliferation with time for varying doses of free MXN and free gelatin
Figure 4.14  Day two images of RG-2 cells treated with free MXN and free gelatin treatments: A) Control; B) 0.5 ppm MXN; C) 12.5 ppm MXN; and D) 25 ppm MXN
Figure 4.15 Graphical representation of RG-2 cell proliferation with time for varying doses of free gelatin
Figure 4.16  Day two images of RG-2 cells treated with free gelatin only: A) Control; B) 0.5 ppm MXN; C) 12.5 ppm MXN; and D) 25 ppm MXN
Figure 4.17  Graphical representations of RG-2 cell proliferation treated with MXN loaded gelatin mesospheres crosslinked with GEN at varying GEN concentrations
Figure 4.18 Graphical representations of RG-2 cell proliferation treated with MXN loaded gelatin mesospheres crosslinked with GTA at varying GTA concentrations
Figure 4.19  Day two images of RG-2 cells treated with MXN loaded gelatin mesospheres crosslinked with GEN: A) C1 25 ppm MXN; B) 12.5 ppm MXN; C) C1 0.5 ppm MXN; D) C2 25 ppm MXN; E) C2 12.5 ppm MXN; and F) C2 0.5 ppm MXN
Figure 4.20  Day two images of RG-2 cells treated with MXN loaded gelatin mesospheres crosslinked with GTA: A) C1 25 ppm MXN; B) 12.5 ppm MXN; C) C1 0.5 ppm MXN; D) C2 25 ppm MXN; E) C2 12.5 ppm MXN; and F) C2 0.5 ppm MXN
Figure 4.21  Graphical representations of RG-2 cell proliferation treated with MXN loaded gelatin mesospheres crosslinked with GEN at varying GEN concentrations
Figure 4.22 Graphical representations of RG-2 cell proliferation treated with MXN loaded gelatin mesospheres crosslinked with GTA at varying GTA concentrations
Figure 4.23  Day two images of RG-2 cells treated with unloaded gelatin mesospheres crosslinked with GEN at comparable concentrations of mesospheres to MXN loaded mesosphere study: A) C1 25 ppm MXN; B) 12.5 ppm MXN; C) C1 0.5 ppm MXN; D) C2 25 ppm MXN; E) C2 12.5 ppm MXN; and F) C2 0.5 ppm MXN
Figure 4.24  Day two images of RG-2 cells treated with unloaded gelatin mesospheres crosslinked with GTA at comparable concentrations to MXN loaded mesosphere study: A) C1 25 ppm MXN; B) 12.5 ppm MXN; C) C1 0.5 ppm MXN; D) C2 25 ppm MXN; E) C2 12.5 ppm MXN; and F) C2 0.5 ppm MXN
Figure 4.25  Graphical representations of RG-2 cell proliferation treated with free MXN and MXN loaded gelatin mesospheres crosslinked with GEN at varying GEN concentrations
Figure 4.26  Graphical representations of RG-2 cell proliferation treated with free MXN and MXN loaded gelatin mesospheres crosslinked with GTA at varying GTA concentrations
Figure 4.27  Day two images of RG-2 cells treated with free MXN and MXN loaded gelatin mesospheres crosslinked with GEN: A) C1 25 ppm MXN; B) 12.5 ppm MXN; C) C1 0.5 ppm MXN; D) C2 25 ppm MXN; E) C2 12.5 ppm MXN; and F) C2 0.5 ppm MXN
Figure 4.28  Day two images of RG-2 cells treated with free MXN and MXN loaded gelatin mesospheres crosslinked with GTA: A) C1 25 ppm MXN; B) 12.5 ppm MXN; C) C1 0.5 ppm MXN; D) C2 25 ppm MXN; E) C2 12.5 ppm MXN; and F) C2 0.5 ppm MXN
Figure 4.29  Day two images of RG-2 cells treated with MXN-GCMS containing 13.0 w/w % CMC: A) Control B) 0.5 ppm MXN; C) 12.5 ppm MXN; D) 25.0 ppm MXN
CHAPTER 5
CONCLUSION

Initial research focused on the synthesis of mitoxantrone loaded albumin microspheres and the effects of processing conditions on particle size, drug loading and release, and in vivo safety and efficacy. In vivo preclinical evaluations showed that mitoxantrone-loaded albumin microspheres crosslinked with glutaraldehyde were successfully used in the treatment 16/C murine mammary adenocarcinoma. These studies were co-investigated with Drs. Almond and Hadba\textsuperscript{4,5} and demonstrated that high dose chemotherapy can be administered with minimal systemic toxicity to achieve prolonged survival of mice bearing high mortality tumors. The following research was sparked based on the initial successes achieved using albumin microspheres.

The focus of this research was to optimize the processing methods in the preparation of novel casein and gelatin micro/mesospheres and evaluate MXN loaded gelatin mesosphere compositions for use in IT chemotherapy.

The overall aims of this research were as follows:

1. Synthesis and properties of casein and gelatin micro/mesospheres
2. Preparation and characterization of in situ MXN loaded gelatin mesosphere compositions
3. In vitro and in vivo evaluations of MXN loaded gelatin mesosphere compositions
Synthesis and Properties of Casein and Gelatin Micro/Mesospheres

Casein Microspheres

Casein microspheres were synthesized using a steric stabilization process, which was adapted from conditions reported in the synthesis of albumin microspheres and casein microspheres. Initial pilot studies identified casein concentration, d/c ratio, CAB concentration, and stirring rate needed to produce smooth spherical microspheres. After the completion of the initial pilot studies, a 3x3 factorial design study was conducted to evaluate the effects of crosslinker concentrations (1, 2.5, and 4.0 w/w % GTA), [80; 120; and 300 % MEQ] and stirring rates (1000, 1250, and 1500 rpm) on the mean dry particle size, distribution, and morphology of casein microspheres.

The particle size was shown to decrease from 30 to 5 um, and the particle size distribution was determined to shift to binodal distribution, with increased stirring rate and crosslinker concentration. The particle morphology was also shown to vary from smooth, spherical to rough pitted microspheres with decreasing crosslink concentration and stirring rate.

Gelatin Films

GEN and GTA crosslinked films were synthesized and the modulus, tensile strength, and percent swelling were characterized. This study was a 2x3 factorial design, which evaluate the effects of varying crosslinker and crosslinker concentrations.

Under the processing conditions that were established for the synthesis of gelatin films, GEN has been shown not only to be a valid chemical crosslinker but also an alternative to GTA yielding superior physical properties. This may be due in fact to the slow reactivity of GEN, which allows for complete mixing; therefore a higher degree of chemical crosslinking was achieved.
**Gelatin Mesospheres**

Smooth, spherical gelatin mesospheres were developed. A major difficulty in the synthesis of gelatin mesospheres was that gelatin solutions gel at room temperature. Therefore processing temperatures had to be increased. This problem was overcome by using a mixing temperature of 40°C and a heating time 5.0 min to limit evaporation.

After the processing conditions were optimized, genipin was used as a chemical crosslinker. The effects of varying crosslinker, genipin and glutaraldehyde, and crosslinker concentrations on particle size were evaluated using a 2x4 factorial design experiment.

Smooth, spherical particles, with a mean dry particle size of 2 um, with a particle size distribution of 0.04 to 20 um were synthesized. Processing conditions showed that varying the crosslinker and the crosslinker concentration had little effect on particle morphology and no effect on dry particle size or particle size distribution.

**Carboxymethyl Cellulose Modified Gelatin Mesospheres**

The synthesis of gelatin mesospheres blended with an anionic polysaccharide, CMC, was evaluated as a possible means of enhancing the drug release. The principle for this rationale is contributed to the cationic nature of the drug; therefore increasing the anionic charge of the mesospheres that may create a higher affinity for the drug.

Gelatin/CMC mesospheres were synthesized using a steric stabilization process. The effects of varying crosslinker, GEN and GTA, and CMC concentrations on particle size were evaluated using a 2x4 factorial design.

Processing conditions were established to produce smooth, spherical particles, with a mean dry particle size range of 2 to 4 um. Increasing the amount of CMC increased the total concentration of the aqueous phase for a given concentration of continuous phase,
therefore an increase in particle size was observed, with no change in particle
morphology. Varying the crosslinker had no effect on particle size and particle
morphology.

**Preparation and Characterization of *In situ* MXN loaded Gelatin Mesosphere
Compositions**

MXN-GMS and MXN-GCMS were synthesized using a steric stabilization process,
which was adapted from conditions reported in the synthesis of albumin microspheres\(^4\)
and casein microspheres.\(^{18,160}\) Processing conditions were established to produce
smooth, spherical particles, with a mean dry particle size of 2 \(\mu\)m, with a particle size
distribution of 0.04 to 20 \(\mu\)m. Studies investigating processing conditions showed that
varying the crosslinker and the crosslinker concentration had little or no effect on particle
morphology and no effect on dry particle size or particle size distribution in the synthesis
of MXN-GMS.

Swelling studies on MXN-GMS showed that increasing the crosslinker
concentration reduced the percent swellability from 750 to 500 \%, regardless of which
crosslinker was used. In addition, the percent swellability of the MXN-GMS was
comparable to the percent swellability of GEN crosslinked gelatin films at comparable
crosslinker concentrations.

**In vitro and In vivo Evaluations of Gelatin Mesosphere Compositions**

MXN loaded gelatin mesosphere compositions were successfully synthesized for
use in localized IT therapies. MXN-GMS crosslinked with GEN achieved loadings
efficiencies in excess of 85 \%, which constitutes 13 w/w \% MXN, where as, MXN-GMS
crosslinked with GTA only achieved loading efficiencies in excess of 77 \%, which
constitutes 12 w/w \% MXN. Also, MXN-GMS exhibited a decrease in percent release
(78 – 65 % MXN) with increasing GEN concentration and (65 – 50 % MXN) with increasing GTA concentration. MXN-GMS’s MXN release was limited to the first 4 hrs whereas, MXN-GCMS exhibited sustained release exceeding 240 hrs with a percent loading of 8 w/w % MXN and percent release of 78 % MXN.

*In vitro* MXN treatments of RG-2 cells using mesosphere preparations were shown to be highly cytotoxic and inhibit cellular proliferation. The most promising results were observed at high MXN doses, and utilizing combinatory treatments (Free MXN plus MXN loaded mesospheres). Combinations of free MXN with free gelatin or MXN loaded mesospheres surpassed the single therapies and significantly hindered cellular proliferation and prevent cellular regeneration.

The validity of using MXN-GMS compositions for IT therapies in the treatment of high mortality cancers was further supported with the *in vivo* MMAC evaluations. Increased survival was observed with all combinatory therapies, with enhanced efficacy at elevated MXN doses and the incorporation of GEN as a chemical crosslinker.
CHAPTER 6
FUTURE WORK

The focus of this research was the development of protein micro/mesosphere compositions for localized IT delivery of chemotherapy agents. The following summarizes research interests that may be useful for future studies.

1. Evaluation of longer genipin reaction regimes in the synthesis of mesospheres. All genipin mesosphere synthesis was done in parallel with pre-established glutaraldehyde conditions. Future experiments may be helpful to modify the mesosphere synthesis methods using longer genipin reaction times.

2. Preparation of gelatin and albumin blended mesospheres. Gelatin mesospheres were shown to hydrate rapidly, which may have lead to rapid drug release. The incorporation of albumin may provide modified release profiles and slower rates of degradation. Preliminary studies using gelatin/albumin blends on particle size, distribution, and morphology have been conducted in our laboratory.

3. Synthesis of anionic modified protein mesospheres. Gelatin/CMC mesospheres displayed lower order drug release profiles, but reduced drug loading. Varying the molecular weight of CMC and/or other anionic polymer may improve the drug loading and release of a cationic drug.

4. Preparation of DNA mesospheres. Plant derived DNA has been recently investigated in our laboratory for tissue regenerative applications. Many chemotherapy drugs such as mitoxantrone can be bound to DNA noncovalently.
Therefore, DNA may serve as an excellent drug carrier. Preliminary studies have used herring testes derived DNA for the synthesis of mesospheres.

5. Evaluation of mixed particle size drug loaded mesospheres based on reports release profiles may be tailored by creating mixtures with different particle sizes.

6. Evaluation of free MXN in gelatin solutions for in vivo gelation. This combination may provide a viscous solution to enhance tumor perfusion and controlled release from a gel with minimal systemic toxicity.

7. Evaluation of free genipin itself for localized IT chemotherapy. Genipin has been shown to react with primary amines, which in turn may prevent malignant cells from proliferating when delivered locally. The slow reactivity of genipin would allow for complete perfusion and fixation of the tumor.

8. Combinations of IT immunotherapeutic with MXN mesospheres. Localized IT delivery of MXN mesospheres followed by IT immunotherapy may enhance systemic immune response to help eradicated metastasis.

9. Synthesis of DNA particles for magnetic resonance imaging (MRI) enhancement. MRI enhancement is achieved by the addition of gadolinium. Recent studies have established conditions to form DNA particles using a steric stabilization process followed by ionic stabilization with gadolinium. The delivery of high concentration of gadolinium via DNA particles may provide improved tissue resolution.
GLOSSARY

Adenocarcinoma: carcinoma derived from glandular tissue or in which the tumors cells form recognizable structure.

Aplasia: lack of development of an organ or tissue.

Carcinoma: a malignant new growth made up of epithelial cells tending to infiltrate the surrounding tissues and give rise to metastases.

Glioma: a tumor composed of tissue which represents neuroglia in any one of its stages of development.

Granulocyte: any cell containing granules, insoluble nonmembranous particle found in the cytoplasm, especially a leukocyte containing neutrophil, basophil, or eosinophil granules in its cytoplasm.

Intercalate: to insert between.

Lymphosarcoma: a diffuse lymphoma, a neoplastic disorder of the lymphoid tissue.

Malignant: tending to become progressively worse and to result in death.

Mammary: pertaining to the breast.

Metastasis: the transfer of disease from one organ or part to another not directly connected with it. It may be due either to the transfer of pathogenic microorganisms or to transfers of cells, as in all malignant tumors.

Murine: pertaining to or affecting mice or rats.

Mutant: a gene or organism that has undergone genetic mutation.

Note: These definitions were taken directly from Dorland’s Illustrated Medical Dictionary, 28th edition


BIOGRAPHICAL SKETCH

Brian Joseph Cuevas was born on February 11th, 1977, and raised on a small farm in Southern Mississippi. After graduating Salutatorian from Hancock High School in May 1995, He received his Associate of Arts certificate from Pearl River Community College in May of 1997 and his Bachelor of Science in polymer science from the University of Southern Mississippi (USM) in May of 1999. While at USM, Brian conducted research under the supervision of Dr. Charles McCormick. His senior research thesis was entitled “Reorganization of Unilamellar Phospholipid Vesicles in Aqueous Media by the Amphipathic Fluorescently Modified Apolipoporphin-III.” After graduating magna cum laude from USM, he entered the graduate program under the supervision of Dr. Eugene Goldberg at the University of Florida in the Department of Materials Science and Engineering, in August of 1999. He spent the next four years researching novel protein micro/mesosphere compositions for the localized IT delivery of chemotherapeutic agent in the treatment of high mortality cancers. He received his Doctor of Philosophy degree in August of 2003.