SYNTHESIS AND CHARACTERIZATION OF DOXORUBICIN CARRYING CETUXIMAB-PAMAM DENDRIMER BIOCONJUGATES

GUNJAN SAXENA
Virginia Commonwealth University

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SYNTHESIS AND CHARACTERIZATION OF DOXORUBICIN CARRYING CETUXIMAB-PAMAM DENDRIMER BIOCONJUGATES

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University

By

Gunjan Saxena
Bachelor of Engineering, Rajiv Gandhi Technological Institute, India, 2008

Director: Dr. Hu Yang, Ph.D., Associate Professor, Biomedical Engineering

Virginia Commonwealth University
Richmond, Virginia
May 2012
Acknowledgement

To my parents - Every bit of me is a little bit of you. This thesis is dedicated to my father, Nawal Saxena, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, Preety Saxena, who taught me to dream big and that even the largest task can be accomplished if it is done one step at a time. I am most grateful to them and my sister, Nikita Saxena, for having faith in me. I would not have been able to reach here without your love and support.

I could not be thankful enough for having had Dr. Hu Yang as my supervisor. Under his guidance, I have learnt to identify and approach research problems, and to present the solutions in a comprehensible manner. He has been a constant source of inspiration and his immense support and guidance helped me deal positively with all crests and troughs throughout this period.

I would like to thank Dr. Gary Bowlin and Dr. Andrew Yeudall for serving on my committee. Their insightful feedback and approval of my research goals and objectives have helped me to complete this thesis. I am also very grateful to both of them for allowing me to work in their respective labs. I am very thankful to Dr. Joseph B. Turner; fluoroscopy wouldn’t have been possible without him.

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Abstract

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By Gunjan Saxena

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Virginia Commonwealth University, 2012

Director: Hu Yang, Ph.D., Associate Professor, Biomedical Engineering

A tumor targeted dendrimer based drug delivery system was designed and synthesized to carry chemotherapy drug doxorubicin. Polyamidoamine (PAMAM) dendrimer G4.5 was chosen as the underlying carrier. Anionic G4.5 is a good option for drug delivery as it consists of 128 surface groups, is less cytotoxic and favorably biodistributed. The delivery system was synthesized using a layer-by-layer arrangement of three functional entities: chemotherapy drug doxorubicin, monoclonal antibody Cetuximab against EGF receptor, and polyethylene glycol (PEG). Doxorubicin was attached via an acid-sensitive hydrazon linkage to the dendrimer. Macromolecules are taken in by cells through endocytosis. pH inside the early endosomes to lysosomes ranges from pH 6 to 4.5. These acidic conditions are favorable for release of drug bound to the dendrimer vehicle through acid-sensitive linkage. 35% of all solid tumors of brain express exceptionally high EGF receptors whereas normal brain tumors express less EGFR. This makes the EGFR a potent targeting moiety for targeted drug delivery. Cetuximab will serve as a targeting ligand to help the delivery system target tumor cells. PEG was incorporated as a linker.
between Cetuximab and dendrimer to avoid reticuloendothelial system (RES) uptake of the system, increase biocompatibility, increase drug half-life and other shortcomings associated with nanomaterials. Nuclear magnetic resonance spectroscopy (NMR), fluorescence anisotropy, and western blotting were used to confirm the conjugation of PEG, doxorubicin and cetuximab to the dendrimer. The synthesized delivery system was characterized using ultraviolet-visible spectroscopy (UV-Vis) to approximate the number of doxorubicin attached. Dynamic light scattering (DLS) and zeta potential were used to analyze the change in size and surface properties of dendrimer during the synthesis. Doxorubicin release studies were conducted at different pHs. Maximum doxorubicin was released at pH 4.5 indicating the successful acid-sensitive linkage between the drug and dendrimer. Cytotoxicity studies indicated that the addition of PEG increased the biocompatibility as compared to free doxorubicin whereas; combination of doxorubicin and cetuximab exerted a significant toxic effect over a period of 72 hours. The cellular uptake of the delivery system was higher than that of free doxorubicin. Free DOX localized mainly in the nucleus whereas, CTX-G4.5-PEG-DOX conjugate localized within both cytoplasm and nucleus after 6 hour incubation. The synthesized delivery system represents a potential targeted drug delivery system.
CHAPTER 1 INTRODUCTION

According to the World Health Organization (WHO), brain diseases such as epilepsy, Alzheimer’s disease, cerebrovascular disease, brain cancers are a major healthcare problem worldwide. Particularly, brain cancer is one of the most complex and deadly diseases known to human beings. According to the Central Brain Tumor Registry of The United States, over 612,000 people are living with primary brain and central nervous system (CNS) tumor being diagnosed. It was estimated that 64,530 new patients would be diagnosed with primary non-malignant and malignant brain tumor in the US alone in 2011 (http://cbtrus.org/factsheet/factsheet.html, cited December 3, 2011).

The major factor limiting therapeutic treatment of CNS diseases is the brain’s effective protective mechanism known as the blood-brain barrier (BBB) (Pardridge et al., 2005). With the presence of BBB, only 2% of small-molecular-weight drugs pass through, whereas nearly all large-molecular-weight neurotherapeutics are excluded from the brain (Pardridge et al., 2005). Thus the treatment efficacy is largely dependent on drug delivery efficiency. Brain drug delivery routes such as trans-cranial drug delivery, trans-nasal brain drug delivery, and BBB disruption are invasive, potentially leading to irreversible side effects or allow a very limited distribution of drug in the brain. Some notable examples are implanting drug eluting wafers after surgical tumor removal, insertion of catheters for pumping drugs into the brain (Abbott et al., 2004; Pardridge et al., 2007). Some relatively less invasive delivery systems have been developed to utilize receptor- targeted delivery mechanism for enhanced brain drug uptake (Yan et al., 2011). In this approach, drug carriers, particularly nanocarriers, play an important role. Nanocarriers are unique as they are small in size. They help increase the permeability, half-life, solubility and stability of the drug in the body. Being the most versatile nanoscale building blocks, dendrimers
have received considerable attention. Among them polyamidoamine (PAMAM) dendrimers are most investigated (Yan et al., 2011; Yang et al., 2010; Lee et al., 2005). As opposed to linear polymers, dendrimers have highly branched, well-defined architecture with very low polydispersity (PDI) (Svenson et al., 2009). Furthermore, availability of a number of functional groups on their periphery can be used for attachment of drugs (Kolhe et al., 2006; Kurtoglu et al., 2009; Kono et al., 2008), solubilizing groups (Gillies et al., 2005), targeting ligands (Chandrasekar et al., 2007), or imaging agents (Patri et al., 2004) for efficient drug delivery or imaging.

In this thesis research, we designed and developed a brain-targeted PEGylated dendrimer drug delivery system for anticancer drug delivery. Biocompatible polyethylene glycol (PEG) was incorporated into the system aiming at reducing immunogenicity, minimizing particle aggregation, improving the carrier’s water solubility, decreasing reticuloendothelial system (RES) opsonization, and strengthening the enhanced permeability and retention (EPR) effect (Boswell et al., 2008). Doxorubicin (DOX), an anti-cancer drug, was used as a model drug. Chimeric monoclonal antibody Cetuximab (CTX) was used as a targeting ligand. It is a 150kDa IgG1K, produced by Sp2/0 mammalian cell line, against the epidermal growth factor receptor (EGFR) and its mutant form EGFRvIII over-expressed in 35% of all solid tumors (Lahlou et al., 2009). The synthesis involved step-by-step assembly of functional groups in sequence including PEG, DOX, CTX on the surface of PAMAM dendrimer G4.5. Particularly DOX was attached to the dendrimer via an acid-labile hydrazone linkage. CTX-PEG-G4.5-DOX bioconjugates and intermediates were characterized using $^1$H-NMR, fluorescence anisotropy, western blotting, dynamic light scattering and zeta potential. The cellular uptake and cytotoxicity of the delivery system was investigated. The in vitro release kinetics of doxorubicin was also studied.
CHAPTER 2 BACKGROUND

2.1 Blood brain barrier (BBB)

The brain is a delicate organ, and evolution built very efficient ways to protect it. The physiological mechanism that alters the permeability of brain capillaries so as to maintain homeostasis of the brain is collectively referred to as the “blood-brain barrier.” A journey to understanding of BBB started a century ago. A German bacteriologist, Paul Ehrlich studied staining of brain in 1885. He observed that dyes did not have a staining affinity to the brain because intravenously administered aniline dyes stained every organ but the brain (Wells et al., 2005). In 1913, his student Edwin Goldman experimentally proved that dye could stain brain tissue but did not reach the brain. Furthermore, he found that dye injected through subarachnoid space stained cerebrospinal fluid (CSF) but not peripheral tissues. This demonstrated the existence of the ‘barrier.’ It was not until 1967 and introduction of high resolution microscopy that Resse and Karnovsky revealed that endothelial cells in mouse cerebral capillaries formed a structural barrier to horseradish peroxidase (HRP) (Reese and Karnovsky et al., 1967). Thus the statement that the BBB is “an endothelial barrier present in capillaries that course through the brain” was made (Rubin et al., 1999).

Although the BBB is an important mechanism to protect the brain, it also represents a formidable barrier to therapeutics required for treatment of CNS diseases (Abbott et al., 1996). Nearly 100% large-molecular-weight pharmaceutics such as proteins, peptides, RNA interference (RNAi)-based drugs have limited transport across the BBB. Only small lipophilic molecules with molecular weight around or under 400-500 daltons can cross the BBB (Pardridge et al., 2007). But, these small molecules can only be effective in treating certain CNS diseases
such as chronic pain, epilepsy and insomnia (Pardridge et al., 2007). The presence of P-glycoprotein on the luminal side of the BBB expels a variety of substances out of the brain to maintain homeostasis, making drug delivery to the brain even more challenging (Pardridge et al., 1997; Golden et al., 1999; El Hafny et al., 1997). Transporter proteins or receptors present on the BBB help essential nutrients such as glucose, amino acids and transferrin to get into the brain (Pardridge et al., 1997; Abbott et al., 2006). Even with the availability of various transporting systems on the BBB, no such ‘transporter’ has been successfully used in transporting large molecules to the brain.

A well accepted BBB model is a three-cell type model. In this model, the BBB comprises brain capillary endothelial cells, astrocytes and pericytes as shown in Figure 2.1 (Garberg et al., 2005; Flaten et al., 2006; Pardridge et al., 1999).
2.2 Brain tumor

Brain tumor is an intracranial mass produced as a result of an uncontrolled growth of cells either normally found in the brain such as neurons, lymphatic tissue, blood vessels, or from cancers primarily located in other organs. Brain tumors can be classified based on whether it is benign (non-cancerous, do not invade or spread to surrounding tissues) or malignant (invasive), the location of the tumor or the type of tissue involved.

Primary brain tumors are the tumors which originate in the brain. Secondary brain tumors originate from the tumor cells which spread to brain from another location in the body. These
mostly originate from organs like breast, kidney, lungs, renal or from melanomas in the skin (Sawaya et al., 2001). Primary brain tumor may be associated with edema and necrosis. Edema is generally found in the white matter regions around the tumor (Prastawa et al., 2005). By definition, brain edema is an increase in brain volume resulting from increased sodium and water content and results from local disruption of the blood brain barrier (BBB). Necrosis is composed of dead cells in the middle of the brain tumor. It is one of the leading causes of mortality resulting from brain tumors.

Primary brain tumors can be further classified by tissue origin. The major primary brain tumor types based on tissue origin are summarized in Table 2.1 (CBTRUS, 2002; Doolittle et al., 2004). On the basis of location tumors can be classified into 3 classes: local tumors, regional tumors and distant tumors. Local tumors are confined to one hemisphere in part of brain. Regional tumors are those which cross midline and invade bone, blood vessel, nerves and spinal cord. Distant tumors can extend up to the nasal cavity, nasopharynx and outside the CNS.
Table 2.1 Primary brain and CNS tumors by histology and percent reported (Redrawn from Doolittle, 2004)

<table>
<thead>
<tr>
<th>Histology</th>
<th>% of Reported Brain Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumors of neuroepithelial tissue</strong></td>
<td>48.1</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>23.0</td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>4.2</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>3.7</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>2.9</td>
</tr>
<tr>
<td>Glioma malignant</td>
<td>2.7</td>
</tr>
<tr>
<td>Others</td>
<td>10.6</td>
</tr>
<tr>
<td><strong>Tumors of the meninges</strong></td>
<td>28.7</td>
</tr>
<tr>
<td>Meningioma</td>
<td>27.4</td>
</tr>
<tr>
<td>Other mesenchymal, benign and malignant</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Lymphomas and hematopoietic neoplasms</strong></td>
<td>2.7</td>
</tr>
<tr>
<td>Germ cell tumors and cysts</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Tumors of the sellar region</strong></td>
<td>7.4</td>
</tr>
<tr>
<td>Pituitary</td>
<td>6.6</td>
</tr>
<tr>
<td>Craniopharyngioma</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Local extensions from regional tumors</strong></td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Unclassified tumors</strong></td>
<td>5.0</td>
</tr>
<tr>
<td>Hemangioma</td>
<td>0.4</td>
</tr>
<tr>
<td>Neoplasm, unspecified</td>
<td>4.5</td>
</tr>
<tr>
<td>Other</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100.0</td>
</tr>
</tbody>
</table>
2.3 Conventional and modern treatments

Brain tumors can be treated by various methods including surgery, radiation therapy, chemotherapy, and drug delivery. In this section, conventional and modern treatment strategies for the treatment of brain tumors are discussed briefly.

2.3.1 Surgery

Surgery is usually the first step in brain tumor treatment. It is preferred to remove as much tumor as possible and to reduce the intracranial pressure. Surgical removal of brain tumors is expected to control tumor-induced seizures and is a necessary step before radiation and chemotherapy. Some tumors are difficult to resect when they are located at an inaccessible site such as brain stem and thalamus. If a large number of tumors are present or if their borders or edges are poorly defined, it will be more challenging for safe removal. Many other factors such as patient’s general health, neurological status, and history of recovery from surgery should be taken into consideration and can hinder surgery as an option for tumor removal (American Brain Tumor Association. Surgery Brochure, 2004).

Surgery poses both general and specific risks. Common risks include infection, bleeding, blood clots, pneumonia and blood pressure instability. In addition, partial or complete loss of sensation, vision, movement, hearing or other functions are the possible consequences of surgery. Surgery on brain tumors located deep inside the brain is more challenging and potentially causes more serious side effects including seizures, weakness, spinal fluid leakage, brain swelling, stroke, coma, and even death (American Brain Tumor Association, Surgery Brochure, 2008). There are many different types of surgery for brain tumors as summarized in Table 2.2.
Table 2.2 Types of surgery with their procedure and purpose (based on American Brain Tumor Association, Surgery Brochure, 2008)

<table>
<thead>
<tr>
<th>Type of Surgery</th>
<th>Procedure and Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy</td>
<td>To obtain tissue sample for pathology</td>
</tr>
<tr>
<td>Needle biopsy</td>
<td>Hole drilled into skull, sample tissue drawn up by hollow needle</td>
</tr>
<tr>
<td>Stereotactic biopsy</td>
<td>Computer-assisted needle biopsy</td>
</tr>
<tr>
<td>Open biopsy</td>
<td>Tissue sample taken during surgery</td>
</tr>
<tr>
<td>Craniotomy</td>
<td>Opening and removing a part of skull for surgery and replacement of removed skull</td>
</tr>
<tr>
<td>Craniectomy</td>
<td>Similar to craniotomy with bone not being replaced before closing incision</td>
</tr>
<tr>
<td>Debulking</td>
<td>Surgically reduce size of tumor</td>
</tr>
<tr>
<td>Partial removal</td>
<td>Partial removal of tumor, requires additional treatment</td>
</tr>
<tr>
<td>Complete removal</td>
<td>Gross total resection, tumor cells might remain</td>
</tr>
<tr>
<td>Shunt</td>
<td>Remove excess fluid, reduces intracranial pressure</td>
</tr>
<tr>
<td>Ommaya Reservior</td>
<td>Implanted container used to deliver chemotherapy, remove CSF and cystic fluid</td>
</tr>
<tr>
<td>Skull base</td>
<td>Special technique to remove tumors in delicate bony area that supports the bottom of the brain</td>
</tr>
<tr>
<td>Transphenoidal</td>
<td>Incision is under the upper lip and over the teeth, or directly through the nostril</td>
</tr>
</tbody>
</table>

2.3.2 Radiation therapy

The purpose of radiation therapy is to control or prevent brain tumor growth. Radiation therapy is often used after surgery to treat inoperable tumors or metastatic tumors. It can also be applied during or after chemotherapy or along with drugs that make tumor cells more sensitive to radiation. In principle, radiation (x-rays, gamma rays, or photons) either kill tumor cells directly or interferes with their ability to grow (American Brain Tumor Association. Radiation therapy Brochure, 2004). Normal brain tissue can tolerate up to 60 Gy (Gray units) of radiation per dose, different tumors require different doses of radiation. For example, glioblastoma (GB), typically
receive 180-200 cGy (centi-Gray units) per dose, 5 days a week for 6 weeks, totaling 5400-6000 cGy over the course of radiation therapy (Castro et al., 2003; American Brain Tumor Association, Radiation therapy Brochure, 2009). Like any other radiation treatment, brain tumor radiation causes side effects including fatigue, hair loss, changes in skin, swelling, nausea, sexual effects, blood clots and post-treatment depression.

2.3.3 Chemotherapy

Chemotherapy is the method to treat benign or malignant brain tumors with the use of drugs. There are different types of chemotherapy drugs available such as drugs that stop cells from starting the cycle, “targeted or biological agents”, and that which act during a particular phase of cell cycle, “cell-cycle specific” drugs. There are also some “non cell-cycle specific drugs” which are not cycle dependent. Chemotherapy drugs can be delivered to the body via systemic or local delivery. Systemic delivery is where the drug is administered orally or injected into the body and circulates via bloodstream before it crosses the BBB and enters the brain. Local delivery places the drug within or near the tumor in the brain. This produces a higher concentration of drug at the tumor site and minimizes toxic effects on normal cells. Chemotherapy drugs are classified into two types - cytotoxic drugs causing cell death and cytostatic agents preventing cell division (American Brain Tumor Association. Chemotherapy Brochure, 2004). The subgroups of each type are summarized in Figure 2.2.
Anti-tumor antibiotics stop the activity of enzymes needed for cell growth. One example is an anthracycline antibiotic, doxorubicin (DOX) (Figure 2.3). It is closely related to the natural product daunomycin. It intercalates with DNA (Beer et al., 2001) and stops the macromolecular biosynthesis (Fornari et al., 1994; Momparler et al., 1976). Topoisomerase II relaxes the DNA supercoils, after which DOX stabilizes the topoisomerase II complex thus inhibiting transcription. The aromatic portion of DOX intercalates with the base pairs of the DNA, whereas daunosamine sugar interacts with base pairs adjacent to intercalation site (Frederick et al., 1990; Pigram et al., 1972).

**Figure 2.2** Classification of chemotherapy drugs (based on American Brain Tumor Association, Chemotherapy Brochure, 2009)
Figure 2.3 Structure of doxorubicin (Redrawn from DrugBank)

The side effects of chemotherapy are closely related to the type of drug used. Common side effects include nausea, vomiting, sores in the mouth and throat, loss of appetite, diarrhea dizziness, hair loss and fatigue. Serious side effects include hives, skin rash, itching, difficulty breathing or swallowing, seizures, heart arrhythmias and neutropenia (decrease in white blood cells) (Castro et al., 2003; American Brain Tumor Society, Chemotherapy Brochure, 2009). Chemotherapy drugs can be delivered to the brain via different routes such as BBB disruption, blood or marrow stem cell transplantation, convention enhanced delivery (CED), high dose chemotherapy, intracavitary/ polymer wafer implants, interstitial therapies and reservoirs and pumps (American Brain tumor Society, Chemotherapy Brochure, 2009). Not all of the methods mentioned above have been adopted as standard methods.

2.3.4 Drug delivery

Nanotechnology and polymers have been applied to develop efficient and novel drug delivery systems. Some delivery strategies are shown in Table 2.3 (Jain et al., 2005).
Particularly, nanoparticulate drug delivery systems have drawn considerable attention. A variety of materials such as linear polymers, hyperbranched polymers, micelles, dendrimers and lipids can be used to make nanoparticulate drug delivery systems (Kwangjae et al., 2008).

2.3.4.1 Polymer-based drug carriers

Drugs can be either physically encapsulated or covalently conjugated to polymers (Rawat et al., 2006). Both natural and synthetic polymers have been used in drug delivery.

2.3.4.1.1 Polymeric nanoparticles

Albumin, chitosan and heparin are naturally occurring polymers, and they have been used to deliver drugs eg. Albumin-bound-paclitaxel (Abraxane) for metastatic breast cancer (Gradishar et al., 2005), non-small-cell lung cancer (phase II trial; Green et al., 2006) and nonhematologic malignancies (phase I, pharmacokinetics trials; Nyman et al., 2005). Side effects of chemotherapy drugs can be reduced through conjugation with synthetic polymers such as polymer poly-L-glutamic acid (PGA) and N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) (Li et al., 2002). PGA conjugated with taxol (Xyotax, Sabbatini et al., 2004) and with camptothecin (CT-2106, Bhatt et al., 2003) is in clinical trials. DOX conjugated with HPMA (PK1) is in clinical trial for treating a variety of tumors (Vasey et al., 1999). Recently, DOX conjugated with HPMA (HPMA-DOX, PK1) was further conjugated to galactosamine (HPMA-DOX-galactosamine, PK2) and has cleared Phase I/II trials (Seymour et al., 2002) as mentioned in Table 2.4.
**Table 2.3 Drug delivery strategies in cancer (Redrawn from Jain et al., 2005)**

<table>
<thead>
<tr>
<th><strong>Direct introduction of drug</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Direct injection into tumor</td>
</tr>
<tr>
<td>• Tumor necrosis therapy</td>
</tr>
<tr>
<td>• Local injection for radio-potentiating</td>
</tr>
<tr>
<td>• Electro-chemotherapy</td>
</tr>
<tr>
<td>• Local delivery by implants</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th><strong>Routes of drug delivery</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Intraperitoneal</td>
</tr>
<tr>
<td>• Intrathecal</td>
</tr>
<tr>
<td>• Nasal</td>
</tr>
<tr>
<td>• Oral</td>
</tr>
<tr>
<td>• Subcutaneous injection/ implant</td>
</tr>
<tr>
<td>• Trans-dermal drug delivery</td>
</tr>
<tr>
<td>• Intravenous, intra-arterial vascular route</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Systemic targeted delivery</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Heat-activated</td>
</tr>
<tr>
<td>• Tissue-selective using carrier-mediated transport systems</td>
</tr>
<tr>
<td>• Tumor-activated drug therapy</td>
</tr>
<tr>
<td>• Pressure-induced filtration of drug across vessels to tumors</td>
</tr>
<tr>
<td>• Two-step targeting using bi-specific antibody</td>
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<tr>
<td>• Site-specific delivery</td>
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<tr>
<td>• Light-activation</td>
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<table>
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<tr>
<th><strong>Targeted to blood vessels of tumor</strong></th>
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</thead>
<tbody>
<tr>
<td>• Antiangiogenesis therapy</td>
</tr>
<tr>
<td>• Angiolytic therapy</td>
</tr>
<tr>
<td>• Induce clotting</td>
</tr>
<tr>
<td>• Vascular targeting agents</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Special formulations and carriers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Albumin-based carriers</td>
</tr>
<tr>
<td>• Carbohydrate-enhanced chemotherapy</td>
</tr>
<tr>
<td>• Protein and peptide delivery</td>
</tr>
<tr>
<td>• Fatty acids as targeting vectors</td>
</tr>
<tr>
<td>• Microspheres</td>
</tr>
<tr>
<td>• Monoclonal antibodies</td>
</tr>
<tr>
<td>• Nanoparticles</td>
</tr>
<tr>
<td>• Pegylated liposomes</td>
</tr>
<tr>
<td>• PEG technology</td>
</tr>
<tr>
<td>• Single-chain antigen-binding technology</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Trans-membrane drug delivery to intracellular targets</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Cytoporter</td>
</tr>
<tr>
<td>• Receptor-mediated endocytosis</td>
</tr>
<tr>
<td>• Transduction of proteins and peptides</td>
</tr>
<tr>
<td>• Vitamins as carriers</td>
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<table>
<thead>
<tr>
<th><strong>Biological therapies</strong></th>
</tr>
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<tbody>
<tr>
<td>• Antisense therapy</td>
</tr>
<tr>
<td>• Cell therapy</td>
</tr>
<tr>
<td>• Gene therapy</td>
</tr>
<tr>
<td>• Genetically modified bacteria</td>
</tr>
<tr>
<td>• Oncolytic viruses</td>
</tr>
<tr>
<td>• RNA interference</td>
</tr>
<tr>
<td>System</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
</tbody>
</table>
| Polymeric nanoparticles | Drug conjugated to the polymer via cleavable linker | • Hydrophilic, nontoxic, biodegradable  
• Possibility of surface modification (PEGylation)  
• Selective accumulation and retention in tumor tissue  
• Specific targeting of cancer cells | • Albumin-Taxol, Abraxane (Gradishar, 2005)  
• PGA-Taxol, Xyotax (Sabbatini, 2004)  
• PGA-Camptothecin, CT-2106 (Bhatt, 2003)  
• HPMA-DOX, PK1 (Vasey, 1999)  
• HPMA-DOX-galactosamine, PK2 (Seymour, 2002) |
| Polymeric micelles | Amphiphilic block copolymers assemble to form hydrophobic core and hydrophilic shell micelles | • Carriers for hydrophobic drugs  
• Biodegradable, biocompatible, self-assembling  
• Functional modification  
• Targeting potential | • PEG-pluronic-DOX (Batrakova, 1996)  
• PEG-PAA-DOX, NK911 (Nakanishi, 2001)  
• PEG-PLA-Taxol, Genexol-PM (Kim, 2004) |
| Dendrimers        | Systematic hyperbranched polymer with radially emerging pattern and repeated units | • Tuning of biodistribution and PK  
• Chemical and structural homogeneity  
• Easy functionality and high ligand density  
• Controlled degradation  
• Multifunctional | • PAMAM-MTX (Kukowska, 2005)  
• PAMAM-platinate (Malik, 1999) |
| Liposomes         | Lipid bilayers forming self-assembled closed colloidal structures | • Targeting potential  
• Easy modification  
• Biocompatible, Amphiphilic | • PEGylated liposomal DOX, Doxil (Markman, 2006)  
• Non-PEGylated liposomal DOX, Myocet (Rivera, 2003)  
• Liposomal daunorubicin, DaunoXome (Rosenthal, 2002) |
| Viral nanoparticles | Multivalent self-assembled protein cages | • Modified surface by mutagenesis or bioconjugation  
• Tumor targeting  
• Uniformity and defined structure  
• Inert and biocompatible | • HSP-DOX (Fleniken, 2006, 2005)  
• CPMV-DOX (Manchester, 2006) |
| Carbon nanotubes  | Carbon cylinder consisting benzene ring | • Hydrophilic, biocompatible  
• Multifunctional | • CNT-MTX (Pastorin, 2006)  
• CNT-amphotericin B (Wu, 2005) |
2.3.4.1.2 Polymeric micelles (amphiphilic block copolymers)

Micelles are made of amphiphilic block copolymers. These blocks form nanosized core/shell structures with a hydrophobic core and a hydrophilic shell in an aqueous media. The hydrophobic core helps with encapsulation of hydrophobic drugs (Adams et al., 2003). Polymeric micelle formulations of paclitaxel are in phase I and pharmacokinetic studies for patients with refractory malignancies as mentioned in Table 2.4 (Kim et al., 2004). MRI-ultrasensitive imaging agents carrying multi-functional micelles have also been developed (Nasongkla et al., 2006).

2.3.4.1.3 Dendrimers

Tomalia et. al. developed a class of nanoscale, highly branched, synthetic polymeric macromolecule known as dendrimers (Tomalia et al., 1985). Dendrimers have received significant attention for drug delivery because of its diverse properties: monodisperse size, modifiable surface functionality, multivalency, water solubility, hollow core and low polydispersity (Klajnert et al., 2007; Svenson et al., 2005). Polyamidoamine (PAMAM) dendrimers, most widely used for drug delivery, have either an ethylenediamine (EDA) or an ammonia core with methyl acrylate and ethylene diamine branches (Klajnert et al., 2007; Tomalia et al., 1985). They are commercially available as full generation (cationic) having amine terminal groups and half generations (anionic) having carboxyl terminal groups (Klajnert et al., 2007). PAMAM dendrimer has been conjugated with cisplatin for sarcomas (Malik et al., 1999) and methotrexate (MTX) on animal models for epithelial cancer (Kukowska-Latallo et al., 2005) as mentioned in Table 2.4.
2.3.4.2 Lipid-based drug carriers

Liposomes are closed colloidal structures made of self-assembled lipid bilayers. They have a spherical structure having a hydrophilic core. Many chemotherapeutic drugs can be delivered by liposomes. As summarized in Table 2.4, a few lipid formulations carrying DOX have been approved by FDA, e.g. Doxil (Markman et al., 2006) for treatment of ovarian cancer, Myocet (Rivera et al., 2003) for treatment of metastatic breast cancer and with daunorubicin such as DaunoXome (Rosenthal et al., 2002) for treatment of AIDS-related Kaposi’s sarcoma (Markman et al., 2006; Rivera et al., 2003; Rosenthal et al., 2002).

2.3.4.3 Viral nanoparticles

Researchers have developed virus-based vehicles such as mosaic virus, canine parvovirus for tissue and drug targeting. Specific in vivo tumor targeting has been achieved by conjugating ligands or antibodies such as transferrin, folic acid to viruses (Manchester et al., 2006). Canine parvovirus has a natural affinity for transferrin receptor up-regulated in tumor cells (Singh et al., 2006) as summarized in Table 2.4.

2.3.4.4 Carbon nanotubes

Carbon nanotubes are cylindrical structures made of benzene rings (Bianco et al., 2005). Carbon nanotubes have found applications for detection of DNA (Cai et al., 2003; Wang et al., 2004; Williams et al., 2002), discrimination of protein (Gooding et al., 2003; Wang et al., 2004), ion channel blockers (Park et al., 2003), and drug/ vaccine delivery (Bianco et al., 2005). However, carbon nanotubes have low water solubility and can cause toxicity. Surface modification can improve their water solubility and biocompatibility (Bianco et al., 2005). In
vitro studies involving carbon nanotubes have been promising for drug delivery (Wu et al., 2005; Pastorin et al., 2006). Presence of multiple functionalities on the sidewalls allows them to carry different molecules e.g., drugs and fluorescent agents (Pastorin et al., 2006) at once, thus providing a fundamental advantage for cancer treatment. Methotrexate (Pastorin et al., 2006) and amphotericin B (Wu et al., 2005) that were conjugated to carbon nanotubes showed more efficient cellular internalization compared to free drug.

2.3.4.5 Drug delivery using monoclonal antibodies

Monoclonal antibodies (MAb’s) can function as diagnostic agent and/or therapeutic agent (Jain et al., 2005). Several MAb’s have been approved by FDA for cancer treatment. For example, Cetuximab (Erbitux, CTX) is a chimeric monoclonal antibody of immunoglobulin G\textsubscript{1} class, against the human epidermal growth factor receptor (EGFR) (Baselga et al., 2001; Goldstein et al., 1995; FDA, 2004). CTX has been recently approved by the FDA as an anti-cancer agent for colorectal (FDA, 2004) and head & neck cancers (FDA, 2011). EGFR is over expressed in more than 35% of all solid malignant tumors (Salomon et al., 1995). EGFR is one of the members of the erbB family of receptor tyrosine kinases. It can be divided into an extracellular domain that can bind ligands, a trans-membrane domain and an intracellular tyrosine kinase domain (Yarden et al., 2001). When a ligand binds to EGFR, it causes receptor dimerization leading to tyrosine kinase activation and receptor autophosphorylation which in turn initiates signal-transduction pathways. These pathways are involved in proliferation and survival of cell (Yarden et al., 2001). CTX competitively binds to EGFR and blocks the binding site of ligand. This inhibits dimerization & receptor phosphorylation, thus stopping downstream signaling pathway (Kirkpatrick et al., 2004). The EGFR working mechanism and CTX blocking is mentioned in Figure 2.4.
**Figure 2.4** Schematic diagram representing mode of action of EGFR and CTX (Redrawn from Kirkpatrick et al., 2004)
CHAPTER 3 MATERIALS AND METHODS

3.1 Materials and reagents

**Table 3.1** List of materials and reagents used

<table>
<thead>
<tr>
<th>Material</th>
<th>Abbreviation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyamidoamine dendrimer generation 4.5</td>
<td>G4.5</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>N-hydroxysuccinimide</td>
<td>NHS</td>
<td>Fluka</td>
</tr>
<tr>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
<td>EDC</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Amine Polyethylene glycol-maleimide</td>
<td>NH$_2$-PEG-mal</td>
<td>Jen-Kem</td>
</tr>
<tr>
<td>N-methylmorpholine</td>
<td>NMM</td>
<td>Acros</td>
</tr>
<tr>
<td>N,N-dimethylformamide</td>
<td>DMF</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Isobutyl chloroformate</td>
<td>IBCF</td>
<td>Acros</td>
</tr>
<tr>
<td>Carbazic acid-tert-butyl ester</td>
<td>Cat-BE</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>DOX</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>TFA</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Methanol</td>
<td>MeOH</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>CTX</td>
<td>Gifted from Dr. Michael H. Peters’s Lab, Chemical and Life Science Engineering, VCU</td>
</tr>
<tr>
<td>Traut’s reagent</td>
<td>DI water</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>De-ionized water</td>
<td>Ether</td>
<td>EMD Chemicals</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>EMD Chemicals</td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate solution</td>
<td>NaHCO$_3$</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Phosphate buffer saline</td>
<td>PBS</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>
3.2 Equipment

Table 3.2 List of equipment and machines used

<table>
<thead>
<tr>
<th>Equipment name</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotary evaporator, Heidolph LABOROTA 4000</td>
<td>Distillation of low boiling point chemicals from mixture of compounds</td>
</tr>
<tr>
<td>Flexi-dry MP controlled rate freezer, FTS Systems, Inc.</td>
<td>Freeze drying the samples</td>
</tr>
<tr>
<td>Weighing scale</td>
<td>Measuring quantity of chemicals</td>
</tr>
<tr>
<td>Eppendorf centrifuge model-5415D</td>
<td>Density based separation of compounds in a mixture</td>
</tr>
<tr>
<td>Bruker AVANCEIII 600 MHz, Nuclear Magnetic Resonance (NMR) spectrometer</td>
<td>Measuring chemical shifts of protons, ¹H-NMR</td>
</tr>
<tr>
<td>Malvern’s Zetasizer Nano ZS90</td>
<td>Measurement of hydrodynamic radius and molecular weight of nanoparticle and electrokinetic potential in colloidal system</td>
</tr>
<tr>
<td>Genesys 6 UV-Vis spectrophotometer</td>
<td>Quantitative analysis of drug release study and gel filtration chromatography</td>
</tr>
<tr>
<td>Zeiss Invertoskop 40C Microscope</td>
<td>Examining cell growth and for cell counting</td>
</tr>
<tr>
<td>Nexcelom Bioscience Cellometer ® Auto T4</td>
<td>Automated cell counting</td>
</tr>
<tr>
<td>Zeiss Axiovert 200 M fluorescence microscope</td>
<td>Examining of cellular uptake</td>
</tr>
<tr>
<td>Beckman Coulter DU® 640 Spectrophotometer</td>
<td>Measurement of protein content in cell lysis studies</td>
</tr>
<tr>
<td>Incubator</td>
<td>Temperature and humidity control of cell culture</td>
</tr>
</tbody>
</table>
3.3 Experimental methods

3.3.1 Preparation of maleimide bearing PEGylated G4.5 dendrimer (mal-PEG-G4.5)

Maleimide bearing PEGylated PAMAM dendrimer G4.5 (mal-PEG-G4.5) was synthesized by substituting the carboxyl group present on G4.5 PAMAM dendrimer (MW = 26258 dalton) with NH$_2$-PEG-mal (MW = 3500 dalton) group as described in a previously reported method (Yang et al., 2003). As shown in Figure 3.1, after removal of methanol from G4.5 stock solution by rotary evaporation, 0.5 µmol of G4.5 was dissolved in 2ml of DMF. To this solution, 12.8 µmol of EDC and 12.8 µmol of NHS at the feed molar ratio of 25.6:25.6:1 for EDC: NHS: G4.5; (20% w.r.t. G4.5) was added. After an overnight reaction while stirring at room temperature, the resultant G4.5-NHS was precipitated in excess amount of cold diethyl ether. The precipitate was re-dissolved in DI water and then rotary evaporated to obtain G4.5-NHS. The obtained G4.5-NHS and NH$_2$-PEG-mal (2 µmol) were dissolved in 2ml and 1ml of 0.1 M NaHCO$_3$ solution (pH 8.5) respectively. NH$_2$-PEG-mal containing NaHCO$_3$ solution was dropwise added to the G4.5-NHS solution. The mixture was stirred for 3-4 hours at room temperature and the resulting conjugates were dialyzed in DI water using dialysis cassette of MWCO 10K. The purified solution was dried using rotary evaporator to obtain mal-PEG-G4.5.
3.3.2 Conjugation of doxorubicin to G4.5-PEG-mal

DOX was covalently attached to the remaining carboxyl groups on the dendrimer surface of G4.5-PEG-mal. The polymer-DOX conjugates via acid sensitive hydrazone linkage was synthesized as previously reported (Bae et al., 2003; Lai et al., 2007)

As shown in Figure 3.2, 54.4µmol of Cat-BE at a feed molar ratio of Cat-BE: G4.5 as 1:1 (85% of G4.5) and G4.5-PEG-mal were dissolved in 5ml of DMF respectively. 272 µmol of NMM was added to G4.5-PEG-mal solution (5 times of 85% of G4.5 molar quantity). 272 µmol of IBCF (5 times of 85% of G4.5 molar quantity) was added dropwise at 4°C and the mixture was kept for five minutes was added. After this the Cat-BE solution was added dropwise to this solution. The mixture was allowed to react for thirty minutes at 4°C and two more hours at room temperature.
The white sediments of the by-product formed were removed by centrifuging the solution for 10 minutes at 10,000 rpm. The supernatant was added dropwise to diethyl ether and kept at -20 °C overnight. The precipitate was collected by centrifuging the ether solution for 10 minutes at 10,000 rpm and then re-dissolved in 3ml of PBS (pH 7.4). The product was purified in PBS (pH 7.4) using a dialysis cassette of MWCO 10K. mal-PEG-G4.5-hyd-BOC was obtained after rotary evaporating the solution. The synthesized mal-PEG-G4.5-hyd-BOC was further treated with 1ml trifluoro acetic acid (TFA) to remove the BOC protective groups. An excess amount of DOX (65.28 µmol) was added in 20ml of methanol with TFA as an acid catalyst. The solution was stirred for 24 hours in dark. The solution was further concentrated to 2ml and then dialyzed in PBS (pH 7.4) with a dialysis cassette MWCO 7K. The product was further purified using a PD-10 desalting column. The purified product was obtained after rotary evaporation.
3.3.3 Conjugation of Cetuximab to mal-PEG-G4.5-DOX

CTX was thiolated using a similar procedure reported earlier for anti-rat transferrin receptor OX26 mAb (Huwyler et al., 1996). According to Figure 3.3, 0.06 nmol CTX was dissolved in 0.15 M sodium borate buffer/0.1 mM EDTA (pH 8.5) with 2.4 nmol of 2-iminothiolane, Traut’s reagent (2-iminothiolane : CTX molar ratio 40:1) for 60 minutes in dark at room temperature. Afterwards, 0.03 nmol mal-PEG-G4.5-DOX was added to the thiolated CTX mixture to react
overnight with gentle shaking. The mixture was concentrated using a rotary evaporator and purified using Sephacryl S-200 HR column.

![Chemical Diagram]

**Figure 3.3** Conjugation of CTX to mal-PEG-G4.5-DOX

3.4 Characterization

3.4.1 Proton nuclear magnetic resonance spectroscopy (\(^1\)H-NMR)

\(^1\)H-NMR spectroscopy was performed on Brucker AVANCE III 600 MHz NMR spectrometer with D\(_2\)O as the solvent. The data obtained was processed using MestReNova NMR software (licensed software) from Mestrelab research. The chemical shift of D\(_2\)O residue was observed at 4.8 ppm.
3.4.2 Fluorescence anisotropy

Anisotropy was performed to confirm the conjugation of doxorubicin to mal-PEG-G4.5. Doxorubicin is a fluorescent molecule. Fluorescent molecules when struck with polarized light result in polarized fluorescence (Ingersoll et al., 2007). The rotational diffusion of the fluorophore during the excited lifetime is the main cause of this depolarized fluorescence. Thus by measuring these fluorescence polarizations, the rotational mobility of the fluorophore can be easily determined. Anisotropy is the technique to experimentally find out fluorescence depolarization. It is directly related to the polarized-light component and inversely to total light intensity. A schematic diagram representing basic “L-format” fluorescence polarization is shown in Figure 3.4. The sample is first excited using a vertically polarized light and thus the intensity component with both excitation and emission polarizer mounted vertically (I_{VV}) and intensity component with excitation polarizer vertical and emission polarizer horizontal (I_{VH}) are measured.

Anisotropy (r) is given as (Lackowicz et al., 2006)

\[
r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}}
\]

where G, “G-factor” is the ratio of the intensity component with excitation polarizer horizontal and emission polarizer vertical (I_{HV}) to intensity component with both excitation and emission polarizers mounted horizontally (I_{HH}).

\[
G = \frac{I_{HV}}{I_{HH}}
\]
It is dependent on the slit width as well as the monochromator wavelength.

Polarization, P can be found out using

\[ P = \frac{3r}{2 + r} \quad \text{Eq. 3} \]

Samples of both free doxorubicin, 0.01 mg, and mal-PEG-G4.5 bound doxorubicin, 0.01 mg equivalent doxorubicin, were prepared in 1 ml of methanol. The measurements were made using a Cary Eclipse Fluorescence spectrophotometer equipped with an 80 Hz Xenon arc lamp, R928 detector and was operated at 600V. The maximum excitation was found at a wavelength of 480 nm with maximum emission at a wavelength of 585 nm at room temperature.
3.4.3 Ultraviolet-visible spectroscopy (UV-Vis)

UV-Vis spectroscopy was performed to calculate the amount of DOX attached to mal-PEG-G4.5. DOX stock solution was prepared by dissolving 1 mg of DOX in 1 ml of 1X PBS (pH 7.4). Five serial dilutions; 1, 0.5, 0.25, 0.125 and 0.062 mg/ml, were prepared to generate a standard curve, given in Figure 3.5, using a Genesys 6 UV-Vis spectrophotometer. 1.7 mg of mal-PEG-
G4.5-DOX was dissolved in 1 ml of 1X PBS. The absorbance of this mixture was measured at 480 nm. The absorbance value of mal-PEG-G4.5-DOX was compared to the standard curve of DOX. The amount of DOX conjugated was calculated as follows. Using the absorbance (A) determined by UV-Vis spectrophotometer and the equation of line obtained from standard curve, the concentration of DOX was calculated, which gave the amount of DOX in the mal-PEG-G4.5-DOX sample by

\[
\text{Concentration of DOX (mg/ml)} = \frac{\text{Amount of DOX (mg)}}{\text{Amount of solution (ml)}} \tag{4}
\]

This amount of DOX in mg was converted to amount in moles by

\[
\text{Amount of DOX (mol)} = \frac{\text{Amount of DOX (mg)}}{\text{Molecular Weight of DOX (mg/mol)}} \tag{5}
\]

where, molecular weight (MW) of DOX = 580 g/mol. Amount of mal-PEG-G4.5 in sample was calculated as,

\[
\text{Amount of mal-PEG-G4.5 (mg)} = \text{Amount of sample (mg)} - \text{Amount of DOX (mg)} \tag{6}
\]

\[
\text{Amount of mal-PEG-G4.5 (mol)} = \frac{\text{Amount of mal – PEG – G4.5 (mg)}}{\text{MW of mal – PEG – G4.5 (mg/mol)}} \tag{7}
\]

The amount of DOX attached to mal-PEG-G4.5 was calculated as a molar ratio,

\[
\frac{\text{Amount of DOX (mol)}}{\text{Amount of mal – PEG – G4.5 (mol)}} \tag{8}
\]

The same standard curve and UV-Vis spectroscopy was later used for analyzing doxorubicin release kinetics from the G4.5-DOX conjugate.
3.4.4 Western blotting

Western blotting is widely used to detect expression of specific protein based on size separation under gel electrophoresis. The protein sample is dissolved in sodium dodecyl sulphate (SDS) buffer. SDS buffer is an anionic detergent, which quantitatively binds to proteins. This binding gives them linearity and a uniform charge, thus they can be separated only based on size (Burnette et al., 1981). Addition of mercaptoethanol in the buffer reduces any disulphide bonds within the protein. Western blotting can also be used to approximate the molecular weight of a protein or a protein conjugate. Samples are loaded into wells in the running gel. One lane generally contains MW markers, a mixture of proteins of defined molecular weights. Analysis of the molecular weight is done after electroblotting the separated protein onto a nitrocellulose or
polyvinylidene difluoride membrane and then photographed. Size approximations are done by referencing the bands of proteins to those of the marker (Burnette et al., 1981).

To confirm the coupling of CTX to mal-PEG-G4.5-DOX, western blotting was performed. Free CTX and CTX-PEG-G4.5-DOX containing CTX equivalent concentration of 10 nmol were dissolved in 20 µl PBS buffer. This mixture was then dissolved in 6X SDS sample loading buffer (375 mM Tris, pH 6.8, 10% SDS, 50% Glycerol, 10% B-mercaptoethanol, 0.03% Bromophenol Blue). Protein samples were run on 8% Tris/ Glycine SDS-polyacrylamide gel (120 minutes, 120 V, room temperature). The proteins were electroblotted using 1X- transfer buffer with 20% methanol (5 mA, overnight, room temperature) on to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA). The membrane was blocked for unspecific binding with 5% non-fat dry milk (1h, room temperature) in Tris-buffered saline. Then, membranes were incubated (1h, room temperature) in blocking buffer with IgE antibodies against cetuximab (1:1000 dilutions). After washing in TTBS, cetuximab were detected using Western Lightning Enhanced Chemiluminescence (ECL; Perkin-Elmer, Waltham, MA).

3.4.5 Size and zeta potential measurements

Molecules in suspension undergo Brownian motion. When such molecules are illuminated with a light source, laser in this case, depending on the size of the particles, the intensity of the scattered light fluctuates (http://www.malvern.com/labeng/technology/dynamic_light_scattering/dynamic_light_scattering.htm, January 19, 2012).
The velocity of Brownian motion is calculated using these intensity fluctuations and hence the hydrodynamic diameter, $d_H$, is calculated using the Stokes-Einstein equation

$$d_H = \frac{kT}{3\pi\eta D} \quad \text{Eq. 9}$$

where, $k$ is the Boltzmann constant, $T$ is the temperature, $\eta$ is the absolute zero-shear viscosity of the medium and $D$ is the diffusion coefficient (Berne et al., 2000).

Particles in aqueous systems generally acquire a surface charge which can be either by surface group ionization or charged species adsorption (http://www.malvern.com/labeng/technology/zeta_potential/zeta_potential_LDE.htm, January 19, 2012). The liquid layer exists in two parts around the diffused particle; an inner region of strongly bound ions (Stern layer) and an outer of loosely bound ions (Diffuse layer). The ions and particles move within the boundary of the diffuse layer. When the particle moves due to Brownian motion, the ions move with it. The point in this layer where the potential moves past the bulk solution is called the zeta potential. A schematic representation of zeta potential is shown in Figure 3.6.

An electric field is applied across the dispersion medium in order to measure zeta potential. The particles in the dispersion will move towards the oppositely charged electrode with a velocity proportional to the magnitude of the zeta potential. The velocity is measured by laser Doppler anemometry. As these particles are moving they cause a phase shift of the incident laser, which is measured as particle mobility. With the use of dispersant viscosity and use of Smoluchowski or Huckel theory, this mobility is converted to zeta potential (Smoluchowski et al., 1903).
Dynamic light scattering (DLS) and zeta potential studies were conducted using a Zetasizer Nano ZS90 equipped with a He-Ne laser from Malvern Instruments (Saovapakhiran et al., 2009). The hydrodynamic radius and zeta potential of G4.5 PAMAM dendrimer and the synthesized products including mal-PEG-G4.5 and mal-PEG-G4.5-DOX were determined. 1X PBS was used as the solvent and filtered through Whatman- Anotop 25 plus, 0.02 μm syringe filter. Samples were prepared by dissolving 1 mg of G4.5, 1 mg of mal-PEG-G4.5 and 1 mg of mal-PEG-G4.5-DOX each in 1 ml of PBS and vortexed for proper mixing. All measurements were taken at 37°C.

**Figure 3.6** Schematic representation of zeta potential (redrawn from [http://www.malvern.com/labeng/technology/zeta_potential/zeta_potential_LDE.htm](http://www.malvern.com/labeng/technology/zeta_potential/zeta_potential_LDE.htm), January 19, 2012)
3.4.6 DOX release studies

The release of DOX from G4.5-DOX was studied at different pHs. 10 mg of CTX-PEG-G4.5-DOX in 10 ml of PBS (pH 7.4, 37°C) was sealed in a dialysis bag (MWCO 1000). The dialysis bag was then submerged in 30 ml of PBS (pH 7.4) in a capped glass bottle and was incubated at 37°C for 192 hours. The released DOX in the incubation buffer was analyzed at predetermined time intervals up to 192 hours. At every time point, 1 ml aliquot of incubation buffer was transferred to a micro-centrifuge tube for doxorubicin content analysis. 1 ml of fresh PBS (pH 7.4) was added to the glass bottle to maintain the volume of incubation buffer. The same procedure was repeated for drug release studies at pH 4.5 and pH 5.5. Doxorubicin was quantified using a UV-Vis spectrophotometer at 480 nm. The standard curve of doxorubicin (Figure 3.5) was used as a reference to calculate the concentration of doxorubicin in each aliquot. Cumulative release of doxorubicin released was calculated as follows:

\[
\% \text{ Cumulative release (t)} = \frac{C_t \times 30ml + \sum_{i=1}^{n-1} C_t \times 1ml}{W_0} \times 100
\]

Eq. 10

where, \(C_t\) is drug concentration in release medium at time \(t\) and \(W_0\) is initial amount of DOX in the sample (mg).

3.4.7 Cell culture

HN12 cells derived from metastatic squamous cell carcinoma were obtained from Dr. Andrew Yeudall’s lab, School of Dentistry, VCU. HN12 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and penicillin-streptomycin (100 units/ml). Cells were grown at 37°C in a humidified atmosphere containing 10% CO₂ (Yeudall et al., 1994).
3.4.8 Cytotoxicity assay

Cytotoxicity of CTX-PEG-G4.5-DOX conjugates was evaluated. HN12 cells were seeded in a twenty-four-well cell culture plate at a density of 5\times10^3 cells/well. After 24 hours of culture, CTX-PEG-G4.5-DOX conjugates and free DOX at DOX equivalent concentrations of (100, 10 and 1 nmol) were added. The cell viability was assessed in triplicates at 24, 48 and 72h respectively with the Trypan blue exclusion assay (Strober et al., 2001). The cells were counted using a Nexcelom Biosciences, Cellometer Auto T4 Cell Counter.

3.4.9 DOX uptake studies

HN12 cells were seeded at a density of 5\times10^3 cells/well in a twenty-four-well cell culture plate or on a 18 mm-diameter glass cover slip positioned in a twelve-well cell culture plate for fluorescence imaging. After 24 hour culture at 37°C, free DOX and CTX-PEG-G4.5-DOX conjugates at DOX equivalent concentration of 10 nmol were added to the wells. Cells were incubated for 6 hours, after which they were washed twice with ice cold 1X DPBS buffer. The cells treated with free DOX or CTX-PEG-G4.5-DOX were then fixed to the cover slip with 100% methanol. After adding DAPI (50µg/ml, diluted to 1:4000) the cover slip was mounted on a glass slide using Vectashield H-1000 mounting medium and fixed with transparent nail polish. The cover slip was examined with a Zeiss Axiovert 200M inverted microscope at the VCU Philips Institute of Oral and Craniofacial Molecular Biology at VCU School of Dentistry. Image analysis was performed using Carl Zeiss Imaging Systems software.
For cell lysis, cells cultured on the plate and treated with free DOX and CTX-PEG-G4.5-DOX conjugates were washed twice with ice cold 1X DPBS buffer and then lysed on ice for 10 minutes using 110 µl of cell lysis buffer (20 mM HEPES, pH 7.5, 10 mM EGTA, pH 8.0, 40 mM β-glycerophosphate, 1% NP-40, 2.5 mM MgCl₂, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 1 mM PMSF). Cells were immediately scraped and transferred to sterile 1.5 ml microcentrifuge tubes. The microcentrifuge tubes were centrifuged for 10 minutes at 12,000 rpm at 4°C. 5 µl of the supernatant was transferred to fresh microcentrifuge tube containing 795 µl of DI water and 200 µl of Bio-Rad blue dye. This mixture was transferred to disposable cuvette the absorbance of protein was measured on a Beckman Coulter DU® 640 Spectrophotometer at 600 nm. A mixture of 800 µl of DI water and 200 µl of Bio-Rad blue dye was used as blank. The protein content was quantified using a modified Bradford assay (BCA; Biorad, Hercules, CA). The amount of DOX taken up by the cells was quantified using a Cary Eclipse Fluorescence spectrophotometer with an excitation wavelength of 480 nm and an emission wavelength of 585 nm.

3.4.10 Statistical analysis

Statistical analysis was performed on anisotropy, hydrodynamic radius, zeta potential, and cytotoxicity studies. All the statistical analysis was based on one way analysis of variance (ANOVA: Single Factor) and Tukey’s test on SigmaPlot software. A p-value less than 0.05 was considered statistically significant for rejecting the null hypothesis. Recording and analysis of data and its graphical representation were done in Microsoft Excel 2007, where the error bars represent standard deviations.
CHAPTER 4 RESULTS AND DISCUSSION

4.1 Preparation and characterization of mal-PEG-G4.5

Bi-functional mal-PEG-NH₂ was conjugated to G4.5 based on EDC/NHS chemistry. 20% of carboxyl surface groups of G4.5 were activated using EDC with NHS. The amine group is highly reactive towards activated carboxyl. Activated carboxyl and amine groups form an amide linkage between mal-PEG and G4.5.

The conjugation of mal-PEG and G4.5 was confirmed by ¹H-NMR. In the ¹H-NMR of mal-PEG-G4.5 conjugate (Figure 4.1), the methylene proton peak of PEG (δ 3.69 ppm), multiple proton peaks of G4.5 (δ 2.19-3.47 ppm) and maleimide proton peak of PEG (δ 6.69 ppm, very dim signal) indicated the success of the synthesis of PEGylated dendrimer conjugates. Corresponding peak area was integrated to determine the number of PEG molecules per G4.5 and hence approximate molecular weight of mal-PEG-G4.5 conjugate was calculated (Yang et al., 2006). It was determined that an average of 1.6 PEG chains was coupled to every G4.5 molecule. The approximate molecular weight of mal-PEG-G4.5 conjugate was calculated to be 31,858 g/mol.
4.2 Preparation and characterization of mal-PEG-G4.5-DOX

Hydrazon groups were attached to the G4.5 dendrimer via an acid anhydride reaction as mentioned in Figure 3.2 (Bae et al., 2003; Lai et al., 2007). Synthesized mal-PEG-G4.5-Hyd-BOC was treated with trifluoro acetic acid (TFA) to remove the BOC protective groups. DOX was then attached to the hydrazon residues of the mal-PEG-G4.5-Hyd through an imine, the Schiff base bond as mentioned in Figure 3.2 (Bae et al., 2003; Lai et al., 2007). The formed mal-PEG-G4.5-DOX was dialyzed with PBS (pH 7.4) and applied to a PD-10 column for further purification.

The conjugation of DOX to mal-PEG-G4.5-Hyd was confirmed using fluorescence anisotropy. Fluorescence anisotropy is measure of the fluorescence depolarization of fluorescent
molecules (Ingersoll et al., 2007; Lakowicz et al., 2006). Since fluorescence depolarization is caused by rotational diffusion of the fluorophore during its excited lifetime, the rotational mobility of the fluorophore can be determined with the help of anisotropy, r. If a molecule is freely suspended, it will have a faster rotational diffusion, thus a lower anisotropy value. Thus if it is bound to a large molecule, the rotational diffusion of the fluorophore will decrease and thus anisotropy should increase (Ingersoll et al., 2007). DOX is a fluorophore with an excitation wavelength at 480 nm and an emission wavelength at 585 nm in methanol. The anisotropy of free DOX was found to be 0.032 ± 0.001. The anisotropy value increased by 60% to 0.054 ± 0.002 for DOX-G4.5-PEG-mal (n=6, with each run having 800 internal averages). The increased anisotropy was attributed to the conjugation of DOX to a large molecule mal-PEG-G4.5 (MW = 31,858) and hence confirmed the conjugation of PEGylated dendrimer to doxorubicin. Significant statistical difference was found between the two anisotropy values with p<0.05.

The amount of DOX attached to PEGylated dendrimer was calculated using UV-Vis spectroscopy. 1.7 mg/ml of DOX-G4.5-PEG-mal in 1X-PBS gave an absorbance of 0.666 (n=3) and according to the standard curve of doxorubicin (Figure 3.5) the concentration of DOX in this sample was calculated using Eq. 4 and Eq. 5 to be 0.277 mg/ml or 0.479 µmol/ml. Using the molecular weight of mal-PEG-G4.5 as 31,858 g/mol, calculated earlier, their number of mole in the 1.7 mg/ml sample were calculated using Eq. 6 and Eq. 7 and were found to be 0.045 µmol/ml. Using Eq. 8, it was calculated that approximately 10.7 molecules of DOX were attached per G4.5-PEG-mal molecule.
4.3 Preparation and characterization of CTX-PEG-G4.5-DOX

EGFR is over-expressed in more than 35% of all solid malignant tumors (Salomon et al., 1995) but is very weakly expressed and almost undetectable in the normal brain (Sauter et al., 1996; Schwechheimer et al., 1995). This makes the EGFR an attractive option for targeted delivery of therapeutics to gliomas. Liposomes and PLA micelles have been conjugated with CTX for EGFR selective targeting previously (Pan et al., 2007; Liu et al., 2009). Anti-EGFR monoclonal antibody CTX was assembled on the G4.5 surface via PEG spacer. Thiolated CTX (Pan et al., 2007; Liao et al., 2010) have proved to be efficient in coupling with maleimide-bearing polymer. As thiolation happens on the carbonate part of the Fc portion, the EGF receptor recognizing ability of CTX is preserved (Pan et al., 2007). CTX was first thiolated with Traut’s reagent (2-iminothiolane, Marsh et al., 1988) (Pan et al., 2007; Huwyler et al., 1996). An optimum 2-iminothiolane/CTX molar ratio of 40:1 was used which on an average yields thiolation of one primary amine per CTX (Huwyler et al., 1996). The thiolated CTX to maleimide molar ratio was kept at 2:1 to yield an average of one CTX molecule per mal-PEG-G4.5-DOX molecule. Success of conjugation of CTX to maleimide bearing dendrimer via PEG linker was confirmed using Western blotting (Figure 4.2). Using Western blot, the molecular weight of CTX-PEG-G4.5-DOX was calculated to be around 190 kDa. This molecular weight indicated that on an average, 1 CTX was conjugated to mal-PEG-G4.5-DOX. Using the molecular weights (CTX 150kDa, mal-PEG-NH₂ 3500 Da, G4.5 26258 Da and DOX 580 g) and molar ratios (CTX:PEG: G4.5: DOX as 1:1.6:1:10.7) mathematically the molecular weight of CTX-PEG-G4.5-DOX molecule comes out to be 188 kDa, which is equivalent to that indicated by Western blot.
Dynamic light scattering (DLS) was used to determine the particle size and zeta potential of the conjugates. The reaction chemistry involves multiple reactions and different types of functional moieties, which can lead to undesired cross-reactions. To avoid these unwanted reactions, CTX-PEG-G4.5-DOX conjugate was developed based on a 3-step layer-by-layer design. The first layer composed of mal-PEG conjugated to dendrimer. The second layer consisted of conjugation of DOX on the G4.5 surface. The third functional layer of CTX was assembled on the G4.5 surface via the PEG spacer attached earlier. The hydrodynamic size and zeta potential determined from DLS are summarized in Table 4.1. Coupling of different moieties to the G4.5 surface resulted in a consecutive increase in size and change in zeta potential. The success of the surface modifications is clearly reflected by the changes of size and zeta potential. The size of G4.5 was found to be 3.24 ± 0.46 nm which increased to 5.63 ± 0.23 nm with the addition of mal-PEG on its surface. mal-PEG-G4.5-DOX showed a measurable size of 39.78 ±
0.37 nm, a 12-fold increase after the addition of DOX as compared to G4.5. This significant increase in size can be linked with the addition of mal-PEG and DOX on the dendrimer surface and also can be attributed to flocculation of particles due to mal-PEG chain entanglement. As the surface of G4.5 was modified, the zeta potential changed from -21.02 ± 0.35 mV for G4.5 to -2.53 ± 0.16 mV for mal-PEG-G4.5-DOX. All the conjugate groups showed significant statistical difference with each other with p<0.05.

**Table 4.1** Size and zeta potential of the tested conjugates in pH 7.4 PBS at 37°C

<table>
<thead>
<tr>
<th>Conjugates</th>
<th>Size (nm, n=10) (p&lt;0.05)</th>
<th>Zeta Potential (mV, n=10) (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4.5</td>
<td>3.24 ± 0.46</td>
<td>-21.02 ± 0.35</td>
</tr>
<tr>
<td>mal-PEG-G4.5</td>
<td>5.63 ± 0.23</td>
<td>-18.08 ± 0.95</td>
</tr>
<tr>
<td>mal-PEG-G4.5-DOX</td>
<td>39.78 ± 0.37</td>
<td>-2.53 ± 0.16</td>
</tr>
</tbody>
</table>

4.5 Release kinetics of DOX

Enhanced permeability and retention effect (EPR) plays an important role in tumor targeting in body (Maeda et al., 1989). Macromolecules are engulfed by the cells via endocytosis (Bae et al., 2003). The endocytic pathway undergoes a pH change from 7.4 to 4.5 (Zhu et al., 2010). Release of DOX can be triggered by low pHs. DOX released from DOX-G4.5-PEG-CTX conjugates was measured at different pHs to evaluate the pH sensitivity of the hydrazon linkage between G4.5 and DOX. The pH values of the buffers were chosen as 7.4 (normal blood), 5.5 and 4.5 (endosomal/lysosomal pH) for DOX release. As shown in Figure 4.3, DOX released from DOX-G4.5-PEG-CTX was pH dependent. Less than 20% of DOX was released at pH 7.4 over a period of 192 hours, indicating that DOX-G4.5-PEG-CTX conjugates would be stable in
the blood stream. At pH 5.5 a total of 30% drug was released in 192 hours, whereas almost 90% of the DOX conjugated to G4.5 released in 192 hours at pH 4.5. This proved the success of attaching DOX via an acid liable linkage to G4.5 dendrimer. DOX is sensitive to light, temperature, ph and solvent used. DOX is more stable in acidic medium (pH 7.4 to pH 4.5) with maximum stability at pH 4. Although immense care was taken to conduct the experiment in dark, there was still some exposure to light, and higher pH of 7.4 led to photo-degradation of DOX after 192 hours, which turned it to a deep blue-purple compound. Due to this photo-degradation, the release kinetics studies were stopped at 192 hours. Huge standard deviation observed for pH 4.5 at 192 hours can also be attributed to this photo-degradation. Statistical analysis showed significant difference between pH 4.5 vs. pH 5.5 and pH 4.5 vs. pH 7.4 (p<0.05), where as there was no significant statistical difference between pH 5.5 and pH 7.4.

**Figure 4.3** Cumulative release profiles of DOX from CTX-PEG-G4.5-DOX conjugates at different pH
4.6 Cytotoxicity studies

HN12 cells were used to perform cytotoxicity tests. Untreated HN12 cells were used as a positive control. The cells were incubated with DOX-G4.5-PEG-CTX or free DOX at DOX equivalent concentration of 100, 10 and 1 nmol (higher concentrations such as 1 and 10 µmol were highly cytotoxic with 0% cells viable after 24 hours). The amount of DOX-G4.5-PEG-CTX equivalent to free DOX also contained CTX at a concentration of 10, 1 and 0.1 nmol, thus cells were also incubated with equivalent free CTX concentration. The cytotoxicity was both dose and time dependent. As seen in Figure 4.4, DOX-G4.5-PEG-CTX conjugate was cytotoxic at all concentrations. A constant reduction in the activity of cells was indicated by Trypan blue test over a period of 72 hours (n=6), which indicated a controlled release of DOX from the nanoparticles which was also seen in the DOX release studies. There was no significant statistical difference within the groups over 72 hours except groups N1, D1, CTX1 and CTX0.1 (p<0.05). When individual concentrations were compared, it was observed that the cytotoxicity of DOX-G4.5-PEG-CTX was much higher than that of the equivalent amount of free CTX. This increased cytotoxicity can be attributed to a combined effect of DOX and CTX as reported earlier (Vega et al., 2003; Liao et al., 2010). However, the cytotoxicity of DOX-G4.5-PEG-CTX was relatively less or equal to free DOX. This can be a result of the presence of biocompatible PEG in the dendrimer conjugates. Significant statistical difference was observed with all groups compared as whole with p<0.05.
4.4 Cytotoxicity studies of DOX-G4.5-PEG-CTX (N), free DOX (D) and free CTX at different equivalent concentration of DOX and CTX. (N100, N10, N1: DOX-G4.5-PEG-CTX at DOX equivalent concentration of 100, 10 and 1 nmol; D100, D10, D1: free DOX at concentration of 100, 10 and 1 nmol; CTX10, CTX1, CTX0.1: free cetuximab at DOX-G4.5-PEG-CTX equivalent concentration of 10, 1 and 0.1 nmol)

4.7 Cellular uptake studies

Fluorescence microscopy was performed to investigate the mechanism behind free DOX and CTX-PEG-G4.5-DOX uptake in the HN12 cells. It was found that free DOX translocated from cytosol to the nucleus after 6 hours incubation as seen in Figure 4.5. It has been reported earlier that DOX penetrates the plasma and then diffuses into the nucleus, where it interacts with the DNA (Alton et al., 1998). On the other hand, after 6 hours of incubation, CTX-PEG-G4.5-DOX was present both in the cytosol and the nucleus. Cells internalize macromolecules via endocytosis. Endosomes have an acidic pH 6 due to the proton pump present on the membrane.
When these endosomes change from early endosomes to late endosomes, their internal pH goes further down to pH 5.5 (Cooper et al., 1977). The hydrazon linkage between the DOX and G4.5-PEG-CTX should start breaking in the early and late endosomes stage inducing death signals in the cells (Rihova et al., 2002). Golgi apparatus releases transport vesicles which carry lysosomal hydrolases, which fuse with late endosomes to form lysosomes. The lysosomes are much more acidic with pH 4.5-pH 5 which would result in more release of DOX bound to the G4.5-PEG-CTX conjugate and resulted in cell death as shown in cell viability. The cellular uptake results are similar to results found in earlier studies done with similar linkage of DOX with other polymers like HPMA (Etrych et al., 2002; Rihova et al., 2001), PEG (Rodrigues et al., 1999), and neuropeptide (Langer et al., 2001). The amount of free DOX and DOX-G4.5-PEG-CTX uptaken by the cells was found out using cell lysis studies. As seen in Figure 4.5 B and E, although equivalent concentrations of DOX were used still the intensity of DOX is much lower in cells treated with free DOX (Figure 4.5–B) to those treated with DOX-G4.5-PEG-CTX conjugates (Figure 4.5-E). It was found that after 6 hour incubation the uptake of DOX-G4.5-PEG-CTX was ten-folds higher at 2.32 nmols/ µg of protein as compared to that of free DOX at 0.25 nmols/ µg of protein, suggesting CTX facilitates particle uptake via receptor mediated endocytosis.

4.8 Conclusion

A tumor-targeted dendrimer-based delivery system carrying CNS drugs was synthesized through a 3-step layer-by-layer design mechanism. Chemotherapy drug doxorubicin was successfully conjugated to the dendrimer via acid-labile hydrazon linker. Monoclonal antibody cetuximab was successfully conjugated to the dendrimer via PEG linker as the targeting ligand. The conjugate was characterized with NMR, fluorescence anisotropy, UV-Vis, DLS, zeta
potential and Western blotting. A controlled release of DOX was observed at pH 4.5. The dendrimer conjugate was very stable at bloodstream pH of 7.4. Cytotoxicity of the dendrimer conjugate was considerably high compared to free cetuximab but was lower than free doxorubicin because of the addition of PEG chains.

Figure 4.5 Cellular uptake of free DOX (A: Nucleus with DAPI, B: free DOX in nucleus, C: overlapped nucleus and free DOX) and DOX-G4.5-PEG-CTX (D: Nucleus with DAPI, E: DOX from DOX-G4.5-PEG-CTX, F: overlapped nucleus and DOX from DOX-G4.5-PEG-CTX)
CHAPTER 5 SUMMARY AND FUTURE WORK

Synthetic polymers coupled with anticancer drugs can increase the efficacy and reduce the side-effects of such drugs (Lai et al., 2007). Polymers can be easily modified as potent drug delivery systems. We designed a brain-targeted drug delivery system for high EGFR expressing tumors. We demonstrated the change in size and surface properties due to addition of functional moieties to dendrimers. Considering endocytic release of drug, we demonstrated pH sensitive drug release of doxorubicin; various other drugs can be incorporated in a similar way for future testing. Different protein assays can be used to characterize monoclonal antibody. All the studies done in this work were using cancer cells, further analysis can be done using in vitro BBB model. Further studies can be done to determine if the drug delivery system can be administered using an alternative drug administration route. BBB administration can be evaluated using only Fab’ fragments of the antibody as evaluated in an earlier work (Mamot et al., 2005).


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APPENDICES

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Interpretation of the statistical analysis shown in Appendix A, B, C, D and E

Analysis of Variance (ANOVA):

- p < 0.05 indicates that the data is statistically significant

Tukey’s Pairwise Comparisons:

- Performed to determine statistical difference between two data values

- Confidence interval (lower to upper) excludes zero: significant statistical difference between data values

- Confidence interval (lower to upper) includes zero: insignificant statistical difference between data values
Appendix A (Statistical data for anisotropy)

One Way Analysis of Variance (ANOVA): Anisotropy

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.598)

Equal Variance Test: Passed (P = 0.758)

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<th>Std Dev</th>
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Source of Variation

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<tr>
<td>Total</td>
<td>11</td>
<td>0.00141</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>r for DOX-G4.5-PEG vs. r for free D</td>
<td>0.0215</td>
<td>25.012</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Appendix B (Statistical data for hydrodynamic size)

**One Way Analysis of Variance (ANOVA) : Particle size**

**Data source:** Data 1 in Notebook2

**Normality Test (Shapiro-Wilk)**  Failed  (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

**Kruskal-Wallis One Way Analysis of Variance on Ranks**

**Data source:** Data 1 in Notebook2

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4.5 (nm)</td>
<td>10</td>
<td>0</td>
<td>3.529</td>
<td>2.780</td>
<td>3.615</td>
</tr>
<tr>
<td>mal-PEG-G4.5 (nm)</td>
<td>10</td>
<td>0</td>
<td>5.606</td>
<td>5.523</td>
<td>5.828</td>
</tr>
<tr>
<td>mal-PEG-G4.5-DOX (nm)</td>
<td>10</td>
<td>0</td>
<td>39.680</td>
<td>39.432</td>
<td>40.140</td>
</tr>
</tbody>
</table>

H = 25.812 with 2 degrees of freedom.  (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Ranks</th>
<th>q</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>mal-PEG-G4.5-DOX vs G4.5 (nm)</td>
<td>200.000</td>
<td>7.184</td>
<td>Yes</td>
</tr>
<tr>
<td>mal-PEG-G4.5-DOX vs mal-PEG-G4.5</td>
<td>100.000</td>
<td>3.592</td>
<td>Yes</td>
</tr>
<tr>
<td>mal-PEG-G4.5 (nm) vs G4.5 (nm)</td>
<td>100.000</td>
<td>3.592</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Note: The multiple comparisons on ranks do not include an adjustment for ties.
Appendix C (Statistical data for zeta potential)

**One Way Analysis of Variance**

**Data source:** Data 1 in Notebook2

**Normality Test (Shapiro-Wilk)** Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

**Kruskal-Wallis One Way Analysis of Variance on Ranks**

**Data source:** Data 1 in Notebook2

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4.5 (mV)</td>
<td>10</td>
<td>0</td>
<td>-21.100</td>
<td>-21.225</td>
<td>-20.875</td>
</tr>
<tr>
<td>mal-PEG-G4.5 (mV)</td>
<td>10</td>
<td>0</td>
<td>-18.650</td>
<td>-18.800</td>
<td>-17.275</td>
</tr>
<tr>
<td>mal-PEG-G4.5-DOX (mV)</td>
<td>10</td>
<td>0</td>
<td>-2.595</td>
<td>-2.612</td>
<td>-2.543</td>
</tr>
</tbody>
</table>

H = 25.864 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

**All Pairwise Multiple Comparison Procedures (Tukey Test):**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Ranks</th>
<th>q</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>mal-PEG-G4.5-DOX vs G4.5 (mV)</td>
<td>200.000</td>
<td>7.184</td>
<td>Yes</td>
</tr>
<tr>
<td>mal-PEG-G4.5-DOX vs mal-PEG-G4.5</td>
<td>100.000</td>
<td>3.592</td>
<td>Yes</td>
</tr>
<tr>
<td>mal-PEG-G4.5 (mV) vs G4.5 (mV)</td>
<td>100.000</td>
<td>3.592</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Note: The multiple comparisons on ranks do not include an adjustment for ties.
Appendix D (Statistical data for cumulative release kinetics of DOX)

One Way Analysis of Variance (ANOVA): Cumulative release kinetics of DOX

Data source: Data 1 in Release kinetics.JNB

Normality Test (Shapiro-Wilk) Failed ($P < 0.050$)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Release kinetics.JNB

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.5</td>
<td>12</td>
<td>0</td>
<td>15.350</td>
<td>13.500</td>
<td>18.300</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>12</td>
<td>0</td>
<td>19.600</td>
<td>14.225</td>
<td>29.400</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>12</td>
<td>0</td>
<td>40.227</td>
<td>33.815</td>
<td>53.149</td>
</tr>
</tbody>
</table>

$H = 22.677$ with 2 degrees of freedom. ($P = <0.001$)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = <0.001$)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Ranks</th>
<th>q</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.5 vs pH 7.5</td>
<td>237.500</td>
<td>6.507</td>
<td>Yes</td>
</tr>
<tr>
<td>pH 4.5 vs pH 5.5</td>
<td>173.500</td>
<td>4.754</td>
<td>Yes</td>
</tr>
<tr>
<td>pH 5.5 vs pH 7.5</td>
<td>64.000</td>
<td>1.754</td>
<td>No</td>
</tr>
</tbody>
</table>

Note: The multiple comparisons on ranks do not include an adjustment for ties.
Appendix E (Statistical data for cytotoxicity assay)

One Way Analysis of Variance

Data source: Data 1 in Notebook3

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook3

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>N100</td>
<td>9</td>
<td>0</td>
<td>131000.00</td>
<td>106000.00</td>
<td>175500.00</td>
</tr>
<tr>
<td>N10</td>
<td>9</td>
<td>0</td>
<td>260000.00</td>
<td>204000.00</td>
<td>325000.00</td>
</tr>
<tr>
<td>N1</td>
<td>9</td>
<td>0</td>
<td>229000.00</td>
<td>204000.00</td>
<td>436500.00</td>
</tr>
<tr>
<td>D100</td>
<td>9</td>
<td>0</td>
<td>173000.00</td>
<td>143000.00</td>
<td>219500.00</td>
</tr>
<tr>
<td>D10</td>
<td>9</td>
<td>0</td>
<td>169000.00</td>
<td>133500.00</td>
<td>270500.00</td>
</tr>
<tr>
<td>D1</td>
<td>9</td>
<td>0</td>
<td>208000.00</td>
<td>129500.00</td>
<td>307500.00</td>
</tr>
<tr>
<td>CTX10</td>
<td>9</td>
<td>0</td>
<td>309000.00</td>
<td>220000.00</td>
<td>429500.00</td>
</tr>
<tr>
<td>CTX1</td>
<td>9</td>
<td>0</td>
<td>347000.00</td>
<td>253500.00</td>
<td>415500.00</td>
</tr>
<tr>
<td>CTX0.1</td>
<td>9</td>
<td>0</td>
<td>329000.00</td>
<td>247500.00</td>
<td>485500.00</td>
</tr>
</tbody>
</table>

H = 30.710 with 8 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Ranks</th>
<th>q</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX0.1 vs N100</td>
<td>406.00</td>
<td>5.752</td>
<td>Yes</td>
</tr>
<tr>
<td>CTX0.1 vs D10</td>
<td>286.50</td>
<td>4.059</td>
<td>No</td>
</tr>
<tr>
<td>CTX0.1 vs D100</td>
<td>286.00</td>
<td>4.052</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX0.1 vs D1</td>
<td>218.50</td>
<td>3.096</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX0.1 vs N10</td>
<td>140.50</td>
<td>1.991</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX0.1 vs N1</td>
<td>109.00</td>
<td>1.544</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX0.1 vs CTX10</td>
<td>52.50</td>
<td>0.744</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX0.1 vs CTX1</td>
<td>26.50</td>
<td>0.375</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX1 vs N100</td>
<td>379.50</td>
<td>5.377</td>
<td>Yes</td>
</tr>
<tr>
<td>CTX1 vs D10</td>
<td>260.00</td>
<td>3.684</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX1 vs D100</td>
<td>259.50</td>
<td>3.677</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX1 vs D1</td>
<td>192.00</td>
<td>2.720</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX1 vs N10</td>
<td>114.00</td>
<td>1.615</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX1 vs N1</td>
<td>82.50</td>
<td>1.169</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX1 vs CTX10</td>
<td>26.00</td>
<td>0.368</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX10 vs N100</td>
<td>353.50</td>
<td>5.009</td>
<td>Yes</td>
</tr>
<tr>
<td>CTX10 vs D10</td>
<td>234.00</td>
<td>3.315</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX10 vs D100</td>
<td>233.50</td>
<td>3.308</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX10 vs D1</td>
<td>166.00</td>
<td>2.352</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Comparison</td>
<td>Rank Sum</td>
<td>p-value</td>
<td>Decision</td>
</tr>
<tr>
<td>---------------</td>
<td>----------</td>
<td>---------</td>
<td>----------------</td>
</tr>
<tr>
<td>CTX10 vs N10</td>
<td>88.000</td>
<td>1.247</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>CTX10 vs N1</td>
<td>56.500</td>
<td>0.801</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>N1 vs N100</td>
<td>297.000</td>
<td>4.208</td>
<td>No</td>
</tr>
<tr>
<td>N1 vs D10</td>
<td>177.500</td>
<td>2.515</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>N1 vs D100</td>
<td>177.000</td>
<td>2.508</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>N1 vs D1</td>
<td>109.500</td>
<td>1.551</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>N1 vs N10</td>
<td>31.500</td>
<td>0.446</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>N10 vs N100</td>
<td>265.500</td>
<td>3.762</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>N10 vs D10</td>
<td>146.000</td>
<td>2.069</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>N10 vs N10</td>
<td>145.500</td>
<td>2.061</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>N10 vs D1</td>
<td>78.000</td>
<td>1.105</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>D1 vs N100</td>
<td>187.500</td>
<td>2.657</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>D1 vs D10</td>
<td>68.000</td>
<td>0.963</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>D1 vs D100</td>
<td>67.500</td>
<td>0.956</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>D100 vs N100</td>
<td>120.000</td>
<td>1.700</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>D100 vs D10</td>
<td>0.500</td>
<td>0.00708</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>D10 vs N100</td>
<td>119.500</td>
<td>1.693</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.
Appendix F (Properties and handling of DOX)

**General Details**

Approved name: Doxorubicin, Doxorubicin Hydrochloride  
Derivation: Strptomycyces peucetius var. caesius  
Proprietary name: Adriamycin  
Diseases: Hematological malignancies, Carcinoma, Soft tissue sarcomas, Metastatic endometrial cancer and advanced ovarian cancer  
Dissolution: Sterile, pyrogen-free, orange-red, freeze dried powder  
Storage and shelf life:  
- Light Sensitive  
- Dry, unopened: Dry place away from light, Three years  
- Solution: 2 to 8 °C, Eighteen months  
- Removed from refrigeration: One Month

**Chemistry**

Type: Cytotoxic antibody containing  
- An aminosugar, daunosamine  
- Glycosidic bond to C7  
- Tetracyclic aglycone, doxorubicinone  
Action  
- Complex with DNA  
- Interferes nucleic acid synthesis  
- Highest activity: S phase

**Molecular Study**

Molecular structure
Molecular Weight: 580.0

Solubility
- Water
- 5% Glucose
- 0.9% Sodium Chloride
- Partially in Alcohol

Insoluble
- Chloroform, ether, other organic solvents

**Stability Profile**

Physical and chemical stability
- 48 hours at room temperature in normal artificial light
- 18 months at 2-8°C at pH 3.0
- One month at room temperature at pH 3.0

Stability depends
- Temperature
- pH
- Solvent

Light sensitive
Absorbs on to glass and certain plastics

**Effect of pH**

More stable in acidic medium (pH 7.4 to pH 4.5)
- Maximum at pH 4.0

Below pH 4.0
- Acidic hydrolysis – red-colored, water insoluble aglycone and water-soluble amino sugar
- Rate proportional to hydrogen ion concentration
- Aglycone is inactive
- Dependent on structural modification in amino sugar

Above pH 4.0
- Color change from red to deep blue-purple
- Rapid degradation occurs
- At pH 8.0, fluorescing compounds

pH ≤ 9.5
- Accelerated by acetate, phosphate and carbonate buffers

pH > 10.0
- No buffer catalysis
Alkaline solution

- Anthracyclines are affected by structural modification of aglycone portion of the molecule

Photodegradation

Substantial photodegradation observed at concentrations below 100 µg/ml.
No special precautions are needed at Concentrations ≥ 500 µg/ml

Effect of Temperature

Stability

- Water, concentration 2 mg/ml, 4°C polypropylene syringe, 6 months
- 25 °C in dark
  - 5% glucose, pH 4.7
  - 3.3% glucose with 0.3% sodium chloride, pH 4.4
  - Polypropylene tubes for 28 days
  - Significant degradation in six days in 0.9% sodium chloride, pH 7.0
- PVC minibags
  - 0.9% sodium chloride, pH 6.7 at 25 °C in dark
  - Loss in potency
  - 5% glucose, pH 4.36 and 0.9% sodium chloride (pH 5.2 and pH 6.47), 43 days at 4°C
- Freezing Doxorubicin
  - 2 mg/ml aqueous solution, one month, -20°C
  - Cannot be frozen with sodium chloride
  - 5% glucose, pH 4.36 and 0.9% sodium chloride (pH 5.2 and pH 6.47), 43 days at -20°C
- Microwave Radiation
  - Concentration reduces after four re-thawing in microwave
  - Stable for 2 weeks thawed by any means
  - Aqueous solutions frozen and thawed seven times
  - Overheating may lead to degradation
  - Thawing in microwave NOT RECOMMENDED

Container Compatibility

Polypropylene, polyethylene, PVC and glass
More stable in plastics than glass
Absorbs on to glass and polyethylene
Diluted solutions absorb more on membranes
Negligible absorbance at concentrations of at least 500 µg/ml
Compatibility with other drugs

Incompatible
- Heparin, dexamethasone sodium phosphate, hydrocortisone sodium succinate and diazepam
- Combination with fluorouracil or aminophylline results in color change

Compatible in
- Vincristine, but with buffer
- Recommended NOT to be mixed with any drug

Destruction

Incineration – 700 °C

Chemical
- Dilute sodium hypochlorite for 24 hours

Contact with skin
- Wash with water, soap or sodium bicarbonate solution

Contact with eyes
- Irrigation with saline
Appendix G (Gravity protocol of PD-10 column)

(Based on GE Healthcare, Instructions 52-1308-00 BB)

PD-10 properties

- Sephadex™ G-25 medium
- Uses: Desalting, buffer exchange and removal of low-molecular weight impurities
- Particle size range: 85-260 µm
- Bed dimension: 1.45 x 5.0 cm, 8.3 ml
- Exclusion limit: 5000
- Chemical stability: All common buffers
- Working pH: 2-13
- Storage temperature: +4 to +30°C
- Storage solution: 0.15% Kathon CG/ICP Biocide

Protocols

- Gravity protocol
- Spin protocol

<table>
<thead>
<tr>
<th>Properties</th>
<th>Gravity protocol</th>
<th>Spin protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Description</strong></td>
<td>Use of Gravity force</td>
<td>Additional force by spinning in centrifuge</td>
</tr>
<tr>
<td></td>
<td>Higher recovery</td>
<td>No dilution of sample</td>
</tr>
<tr>
<td></td>
<td>Applied sample is diluted</td>
<td></td>
</tr>
<tr>
<td><strong>Sample volume</strong></td>
<td>1.0-2.5 ml</td>
<td>1.75-2.5 ml</td>
</tr>
<tr>
<td><strong>Elution buffer</strong></td>
<td>3.5 ml</td>
<td>None</td>
</tr>
<tr>
<td><strong>Dilution factor</strong></td>
<td>1.4 times</td>
<td>None</td>
</tr>
<tr>
<td><strong>Desalting capacity</strong></td>
<td>&gt;98%</td>
<td>&gt;90%</td>
</tr>
</tbody>
</table>
Gravity protocol

1. Remove the top cap and discard the column storage solution
2. Cut the seal at the end of column using scissors
3. Fix the column secure on burette stand
4. Take 25 ml of equilibration buffer equilibrate column up to four times
   a. Fill column with buffer and let the bed be completely soaked.
   b. Discard the flow-through buffer
5. Add 2.5 ml of sample
   a. If sample is lesser than 2.5, after the sample is completely soaked in column, add equilibration buffer to adjust the volume to 2.5 ml
   b. Discard the flow through
6. Use 3.5 ml of buffer to elute samples
7. Place microcentrifuge tubes below the column to collect samples at fixed time intervals
VITA

Gunjan Saxena was born on August 13, 1986 in New Delhi, India. She completed high school from Vidyasagar School, Indore in 2004. She completed her undergraduate degree in Biomedical Engineering from Samrat Ashok Technological Institute, Vidisha, India in 2008. She began her graduate studies in Biomedical Engineering at Virginia Commonwealth University in Fall 2008. During her time at VCU, her work was focused on targeted drug delivery. She is very keen to enhance her knowledge in the field of biomaterials and nanomedicine.