

Université de Montréal

***Development of a novel approach for brain delivery:
Dendritic nanocarriers for the enhanced delivery of
methotrexate to the brain tumors***

par

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Université de Montréal
Faculté des études supérieures

Cette thèse intitulée :

Development of a novel approach for brain delivery: Dendritic nanocarriers for the
enhanced delivery of methotrexate to the brain tumors

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Résumé

Dans ce projet, un nouveau dendrimère polyester-co-polyéther (PEPE) a été conçu et évalué comme système de livraison de médicament ayant la capacité de traverser la barrière hémato-encéphalique (BHE). Une série de dendrimères PEPE dont le noyau est l'acide butane tétracarboxylique a été synthétisée. Des variations de l'architecture ont été faites en changeant le nombre de branches, d'unités de branchement, de générations, les groupes terminaux, et la longueur des chaînes de polyoxyéthylène (PEO). La biocompatibilité du dendrimère a été vérifiée *in vitro* en mesurant la cytotoxicité sur des cellules endothéliales (bEnd.3) et leur potentiel hémolytique sur des hématies de rat (RBCs). L'adsorption des protéines plasmatiques à la surface des dendrimères a aussi été évaluée. Le mécanisme d'internalisation a été élucidé en étudiant le devenir dans des cellules bEnd.3, de dendrimères marqués à la rhodamine. Le méthotrexate (MTX) a été choisi comme modèle thérapeutique en raison de sa faible perméabilité à travers la BHE mais aussi pour sa bonne activité contre les tumeurs cérébrales. La capacité de ces dendrimères de traverser la BHE a été vérifiée en utilisant un modèle *in vitro* de culture conjointe de bEnd.3 et de cellules U373 MG. Finalement, l'efficacité des dendrimères chargés avec du MTX a été établie sur une couche monocellulaire de gliome humain (U87 MG et U343 MG-A) et des sphéroïdes avasculaires de gliome humain. Les dendrimères n'ont montré aucune toxicité sur bEnd.3 à des concentrations aussi hautes que 10 mg/ml. Ils ont provoqué moins de 10% d'hémolyse à 5 mg/ml. Les protéines d'opsonisation tels que les immunoglobulines et les facteurs du complément ne s'adsorbent pas à la surface des dendrimères et le taux de liaisons aux protéines plasmatiques est très bas. Ces dendrimères sont capables de traverser la BHE en grande quantité et d'augmenter significativement la quantité de MTX disponible comparée à du MTX libre. La glycosylation des dendrimères PEPE augmente également le passage trans-BHE comparé à celui des dendrimères non glycosylés. L'IC₅₀ du MTX après encapsulation dans les dendrimères est plus faible que celle du MTX libre, ce qui suggère un meilleur potentiel. De même, les dendrimères

glycosylés ont montré une meilleure activité quand on mesure la réduction de volume des sphéroïdes tumoraux. Ces dendrimères sont même capables de tuer des cellules résistantes au MTX, mettant ainsi en lumière une possibilité de contrer cette résistance. Aussi intéressante est la possibilité d'atteindre même les régions centrales et non oxygénées des tumeurs non-vascularisées. Finalement les dendrimères PEPE glycosylés constituent un système de livraison de médicament très prometteur pour le traitement des gliomes comme pour d'autres agents thérapeutiques dans le cerveau.

Mots-clés : dendrimère, architecture, cerveau, gliomes, méthotrexate, BHE modèle

Abstract

In the present project, the suitability of novel polyether-co-polyester (PEPE) dendrimers for drug delivery across the blood brain barrier (BBB) and in the treatment of gliomas was evaluated. A series of PEPE dendrimers consisting of butane tetracarboxylic acid as the core molecule were synthesized. Modifications in the architecture were accomplished by varying the number of branches, branching units, terminal functional groups, generation and the chain length of polyethylene oxide (PEO) linking the branches to the interior cavity. The biocompatibility of the resulting dendrimers was evaluated *in vitro* by assessing their cytotoxicity on brain endothelial cells (bEnd.3) and their hemolytic potential against rat red blood cells (RBCs). Plasma protein adsorption on the surface of dendrimers was also evaluated. The internalization of rhodamine labeled dendrimers into bEnd.3 cells was studied to comprehend the mechanism of uptake. Methotrexate (MTX) was selected as model chemotherapeutic agent because of its poor BBB permeability but at the same time good activity against brain tumors. The ability of the dendrimers to cross BBB was ascertained by using an *in vitro* model consisting of co-culture of bEnd.3 and U373 MG cells. Finally, the efficacy of MTX loaded dendrimers was established against monolayer human glioma cell lines, namely U87 MG and U343 MG-A and their avascular human glioma tumor spheroids. PEPE dendrimers showed no toxicity towards the bEnd.3 cells at concentration as high as 5 mg/mL and also produced less than 10% lysis of rat RBC's even at 5 mg/mL. Opsonic proteins like immunoglobulin and complement factors were not adsorbed on the surface of the dendrimers; additionally, the total amount of protein adsorbed was also low. The dendrimers were able to permeate BBB in large amount and thus, significantly increase the availability of MTX across BBB model as compared to free MTX. Glucosylation of PEPE dendrimers was found to further increase their permeation across BBB as compared to non-glucosylated dendrimers and hence, transport of MTX into the receiver compartment. The IC_{50} of MTX after loading in dendrimers was lower than that of free MTX, suggesting that loading MTX in PEPE dendrimers increased

its potency. Similar higher activity of MTX loaded glucosylated and non-glucosylated dendrimers was found in the reduction of volume of tumor spheroids. The MTX loaded dendrimers were able to kill even MTX resistant cells, highlighting, there ability to overcome MTX resistance. Most interestingly, glucosylation augmented the rate and extent of delivery of dendrimers to the central and hypoxic regions of the avascular tumor spheroids. Thus, glucosylated PEPE dendrimers can serve as a promising delivery system for the treatment of gliomas and also for delivery of other therapeutic agents into the brain.

Keywords: Dendrimers, architecture, brain delivery, gliomas, methotrexate, BBB model

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List of abbreviations

ADEPT	Antibody-directed enzyme prodrug therapy
AFM	Atomic force microscopy
AME	Absorptive-mediated endocytosis
ANOVA	Analysis of variance
AUC	Area under curve
B.C.	Before christ
BBB	Blood brain barrier
BBBD	Blood brain barrier disruption
BCA	Bicinchonic acid
BCB	Blood-cerebrospinal fluid barrier
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BH ₃ -THF	Borane-tetra hydrofuran complex
BHBA	Bis(hydroxyl methyl) butyric acid
BMEC	Brain microvessel endothelial cells
BTB	Brain tumor barrier
CED	Convection enhanced delivery
CDCl ₃	Deuteriated chloroform
CNS	Central nervous system
CO ₂	Carbon dioxide
CrO ₃	Chromium trioxide
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
¹³ CNMR	Carbon nuclear magnetic resonance imaging
Da	Dalton
DAB	Diaminobutane

DAE	Diaminoethane
DHBA	Dihydroxy benzoic acid
DHFR	Dihydrofolate reductase
DIC	Differential interference contrast
DLS	Dynamic light scattering
DMAP	Dimethyl amino pyridine
DMEM	Dulbecco's modified eagle's medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide
EDCU	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide urea
EGFR	Endothelial growth factor receptor
FBS	Fetal bovine serum
FDA	United states food and drug administration
FTIR	Fourier transform infrared spectroscopy
GDEPT	Gene directed enzyme prodrug therapy
GLUT	Glucose transporter
GPC	Gel Permeation chromatography
¹ HNMR	Proton nuclear magnetic resonance imaging
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HOBT	N-Hydroxy benzotriazole
HPLC	High performance liquid chromatography
I.V.	Intravenous

IC ₅₀	Inhibitory concentration-50%
IFN	Interferon
LDH	Lactate dehydrogenase
L-DOPA	L-dopamine
MAB	Monoclonal antibody
MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
MPS	Mononuclear phagocytic system
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
MW	Molecular weight
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NO	Nitric oxide
O ₂	Oxygen
OAT	Organic anion transporters
ODN	Oligonucleotide
P85	Pluronic [®] 85
PBCA	Polybutylcyanoacrylate
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PEG-PHDCA	PEGylated-poly(hexadecyl cyanoacrylate)
PEI	Polyethylenimine
PEO	Polyethylene oxide
PEPE	Polyether-co-polyester

P-gp	P-glycoprotein
PI	Polydispersity
PMAM	Polyamido amine
PS	Polysorbate
RBC	Red blood cells
RH	Relative humidity
RM ANOVA	Repeated measure analysis of variance
RME	Receptor-mediated endocytosis
RNA	Ribonucleic acid
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
Si RNA	Small interfering ribonucleic acid
TEER	Transendothelial electrical resistance
T _g	Glass transition temperature
THF	Tetra hydrofuran
TMS	Tetramethyl silane
TsOH	p-Toluene sulfonic acid monohydrate
UV	Ultra violet
VEGF	Vascular endothelial growth factor
w/w	Weight by weight
ZO	Zonula occludens protein
λ	Wavelength
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength
λ_{max}	Maximum wavelength of the absorbance

List of equations

Acid value = (*Weight of acid*)/(*NaOH normality*)*(*Volume of NaOH*).....Equation 1.....93

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To my loving parents.....

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INTRODUCTION

1.1. Cancer

Cancer has afflicted humans throughout the recorded history (Gallucci, 1985; Diamandopoulos, 1996). Our oldest description of cancer dates back to approximately 1600 B.C. by Egyptian, Edwin Smith Papyrus, who described it as a disease with “no treatment.” Even physicians like Hippocrates (460-370 B.C.) in most part considered it an incurable disease. However, back then disease was viewed in terms of four bodily fluids—blood, phlegm, yellow bile, and black bile. Over the centuries numerous inventions and discoveries have set a new perspective for medicine and radical changes in the understanding of diseases have transpired. In the nineteenth century, many striking developments in science such as bacteriology, cell pathology, discovery of anesthesia, X-rays and radioactivity immensely contributed to the growth of cancer research (Diamandopoulos, 1996). However, it was after German zoologist, Theodor Boveri, related cancer to abnormal chromosomes that understanding of cancer and its treatments gained impetus (Balmain et al., 2003). It was later understood that cancer is a proliferative disease of cells, characterized by uncontrolled growth. Under normal physiological conditions, all cells divide and reproduce in an orderly and controlled manner. In cancer, however, cells multiply without proper control to form a lump (which is called a primary tumor). Sometimes cancer cells detach from the tumor and travel to other parts of the body *via* bloodstream or lymphatic system, called metastasis, where they may settle and start to develop into new tumors. These are known as secondary cancers or metastases. There are around 200 different types of cancer, some are very common while others are extremely rare.

In terms of incidence, mortality, and prevalence in human population cancer is a dreadful disease. The World Health Organization Globocan database estimates that there were over 58 million deaths worldwide in 2005, cancer accounted for 7.6 million (or 13%) of all deaths. Deaths from cancer in the world are projected to continue rising, with an

estimated 9 million people dying from it in 2015 and 11.4 million dying in 2030. (<http://www.who.int/whosis>). Advances in the cancer diagnosis and treatment together with the high mortality rate of cancer patients have resulted in vigorous research and significant progress have been made in the treatment of some neoplasms such as testicular germ cell tumors, choriocarcinoma, Burkitt's lymphoma, Hodgkins disease and several childhood cancers. However, progress in treating solid malignancies of lung, colon, breast and brain cancer is less impressive (Giaccone, 2002). In fact, in no cancer has the inconsistency been more pronounced or more tragic than in the treatment of brain cancer. As of today, brain cancers stand as one of the most challenging cancer to treat.

1.2. Brain cancer

A brain tumor is a noncancerous (benign) or cancerous (malignant) growth in the brain; it can originate in the brain or spread (metastasized) to the brain from another part of the body. Although primary tumors of central nervous system (CNS) are uncommon, yet they are one of the most lethal forms of cancer. The patients with brain tumor, including those with certain "benign" brain tumors, have poorer survival rates than breast cancer patients. Each year approximately 190,000 people in the United States and 10,000 people in Canada are diagnosed with a primary or metastatic brain tumor (Parkin et al., 2005). A minority of patients (approximately 5%) are likely to survive after 5 years of diagnosis. Brain tumors are the leading cause of solid tumor death in children under age of 20 years and are the third leading cause of cancer death in young adults ages 20-39 years (Landis et al., 1999). Glial neoplasms represent one of the most common types of the brain tumors (Figure 1.1). They consist of astrocytomas, oligodendrogliomas, anaplastic oligoastrocytomas, ependymomas and glioblastoma multiforme and account for more than 60% of the primary brain tumors.

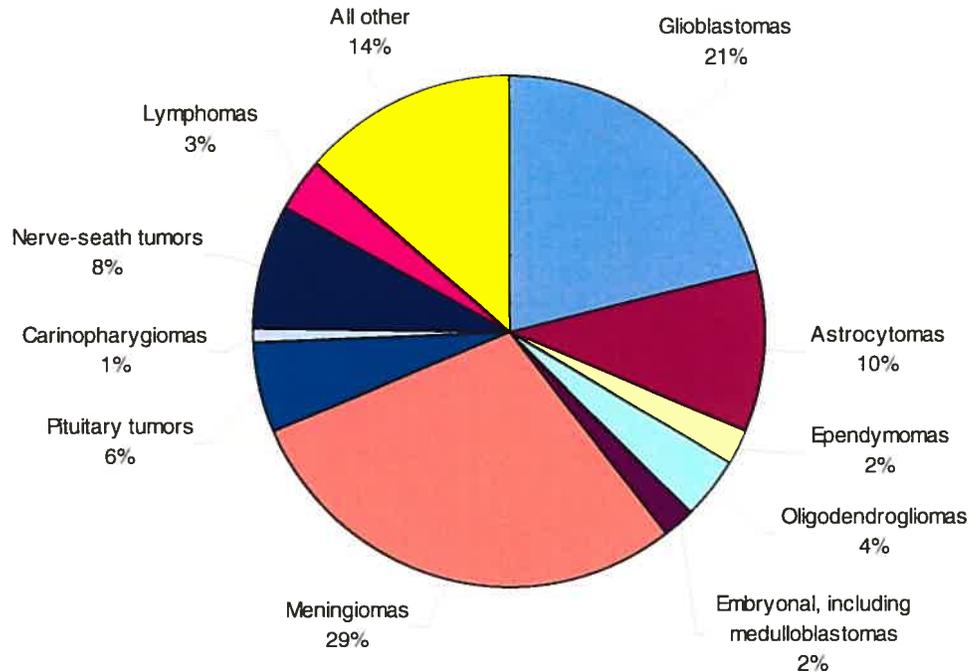


Figure 1.1. Distribution of all primary brain and CNS tumors by histology. Modified from (Landis et al., 1999) and (Parkin et al., 2005).

Due to the strategic location of these tumors they produce profound and progressive disability and lead to death in most cases (Morantz and Walsh, 1994). In addition, to the effect on performance and ability of the patient, brain tumors also exhibit unique challenges in treatment due to their location, aggressive biological behavior and diffuse infiltrative growth. So far, the partial control of tumor growth for a brief period of time has been possible. Most of the chemotherapeutic drugs reach these tumors in limited amounts. This restricted drug delivery is largely due to the biological characteristics of brain and its protective barriers namely, blood-brain barrier (BBB), blood-cerebrospinal-fluid barrier (Scherrmann, 2002).

1.3. Physiological barriers in delivery of therapeutics to the brain tumors

1.3.1. Blood-brain barrier

The blood-brain barrier (BBB) is a membranous structure that acts primarily to protect the brain from chemicals in the blood, while still allowing essential metabolic functions. It is composed of endothelial cells which are packed very tightly in brain capillaries (Figure 1.2). Outgrowths from astrocytes called astrocytic feet surround the endothelial cells, providing biochemical support to these cells. In addition to tight junctions which prevent transport in between epithelial cells, there are two barriers which prevent passive diffusion through the BBB (Wolburg and Lippoldt, 2002). The first barrier includes, glial cells surrounding capillaries in the brain which pose a secondary hindrance to the hydrophilic molecules. The second barrier is the metabolism of certain molecules by endothelial cells to prevent their entry into the central nervous system (Demeulea et al., 2002). For example, L-DOPA, the precursor to dopamine can cross the BBB, whereas dopamine itself cannot, as a result, L-DOPA is administered for dopamine deficiencies. The low concentration of interstitial proteins in the brain is also reported to prevent access of hydrophilic molecules into the brain. BBB blocks all molecules except those that cross cell membranes by means of lipid solubility (such as ethanol and steroid hormones) and those that are allowed in by specific transport systems (such as sugars and some amino acids). Substances with a molecular weight higher than 500 Da generally cannot cross the BBB.

1.3.2. Blood-cerebrospinal fluid barrier (BCB)

BCB is the additional obstacle that impedes the delivery to the CNS. It comprises mainly the choroidal and arachnoidal epithelium, giving access to the ventricular and subarachnoidal cerebrospinal fluid (CSF), respectively (Masserman, 1935). The choroid

epithelial cells, which line the ventricles and produce CSF form a tight boundary that regulates the transfer of molecules into the interstitial fluid that surrounds the brain parenchyma (Cserr, 1971). In addition to these tight junctions there are organic-acid transport receptors which efflux the chemotherapeutic molecules from the CSF. However, the (gap)-junctions of the choroid epithelium are more permeable than the tight junctions of the BBB-endothelium (Felgenhauer et al., 1982). Moreover, blood flow in the choroid plexus blood capillaries seems to be 5 to 10 times higher than the mean cerebral blood flow. Thus, it is more easily permeable barrier as compared to BBB.

1.3.3. Blood-tumor barrier (BTB)

It is a pathological barrier formed by the abnormally dilated and tortuous microvasculature of the brain tumors that limits the effectiveness of the brain tumor therapy (Groothuis, 2000). Though the blood capillaries in the tumors are leaky, the blood flow velocity is significantly low which results in increase in the intratumoral interstitial pressure; thus, compromising the drug delivery. Also the hypoxic environment caused by the erratic blood flow induces expression of many biologically reductive enzymes, allows tumors to become more resistant to chemo and radio-therapy.

1.3.4. Efflux transporters

Carrier mediated efflux is involved in extruding drugs from the brain and is a major obstacle for many pharmacological agents, with the ATP binding cassette transporter P-glycoprotein (P-gp) being the principle efflux mechanism of these agents (Cordon-Cardo et al., 1989). There also exists efflux transporters for organic anions via multidrug resistance associated proteins (Kusuhara et al., 1998), and anionic and cationic cyclic peptide (Tamai and Tsuji, 2000).

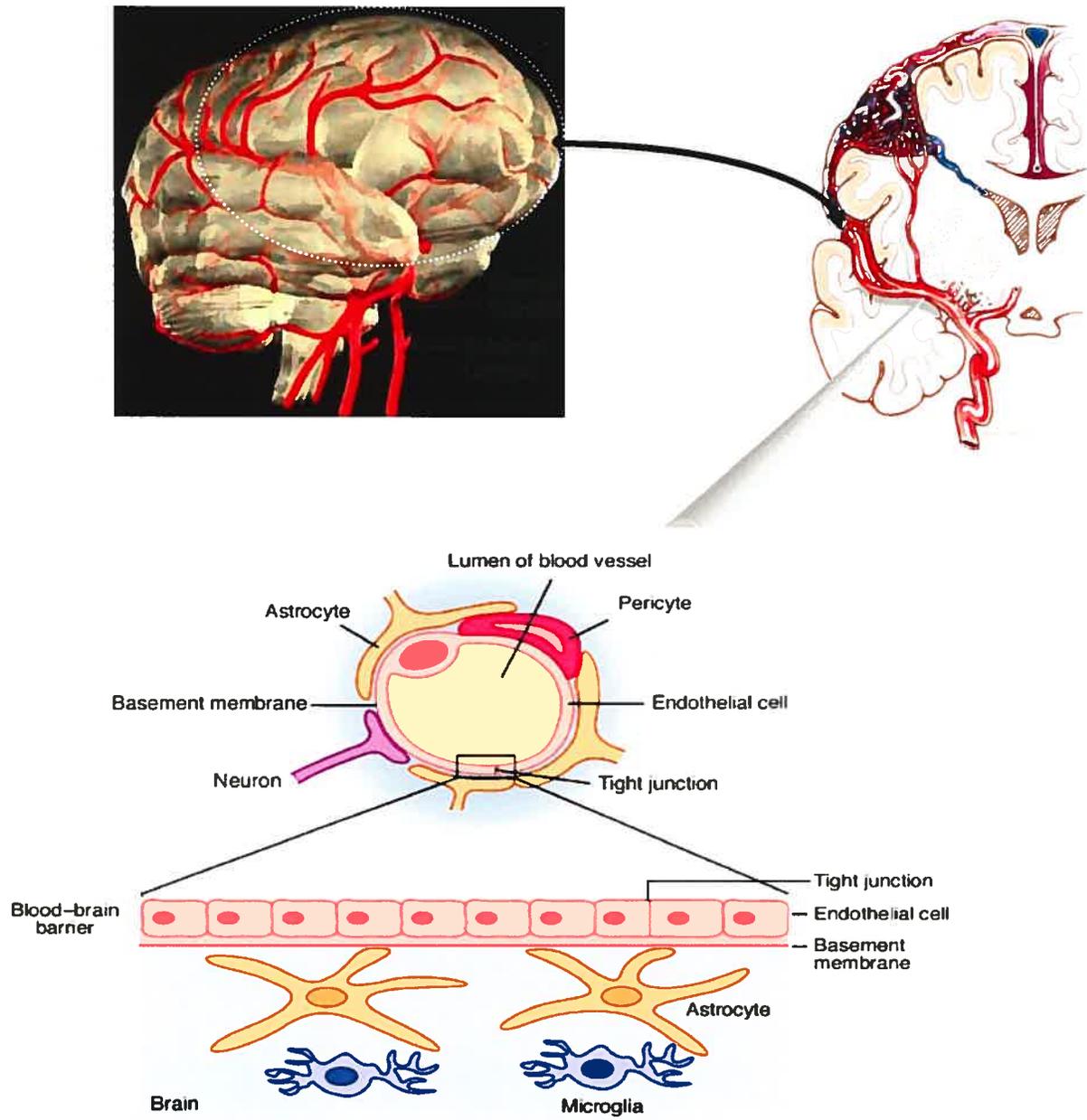


Figure 1.2. BBB and its anatomical characteristics. Modified from <http://www-ermm.cbcu.cam.ac.uk/03006264h.htm>

1.4. Drug transport at the BBB

BBB has a number of highly selective mechanisms for transport of nutrients into the brain, as shown in figure 1.3.

1.4.1. Diffusion

Diffusion of substances into the brain can occur by paracellular (i.e. between cells) and transcellular (i.e. across cells) routes, both of which are non-saturable and non-competitive pathways. However, paracellular diffusion does not occur to a great extent at the BBB due to the presence of tight junctions. On the other hand, transcellular diffusion is restricted to the substances with higher lipophilicity and small size (Pardridge, 1998). The smaller substances penetrate more rapidly and hence, small inorganic molecules (i.e. O₂, CO₂, NO, and H₂O) are highly permeable into the brain. For the transport of large molecules, the hydrogen bonding potential of a compound also plays a crucial role in the BBB permeability, substances with lower hydrogen bonding potential have greater membrane permeability (Chikhale et al., 1994).

1.4.2. Endocytosis

In addition to these diffusive mechanisms, entry of molecules into brain can occur by endocytosis. *Bulk-phase endocytosis* (pinocytosis), a non-saturable, nonspecific uptake of extracellular fluids (Simionescu et al., 1987) which occurs readily and to a large extent in other cells of the body, occurs to a very limited degree in the endothelial cells of brain microvasculature (Pardridge, 1995).

Absorptive-mediated endocytosis (AME) is the mechanism by which positively charged substances are taken up by BBB. AME is reported to be triggered by the electrostatic interaction of positively charged moiety with the negatively charged plasma

membrane surface (i.e. glycocalyx) (Stieber et al., 1984). Due to its non-specific and non-saturable nature, AME has been the focus for the development of many new drug delivery technologies for delivery across BBB (Pardridge, 1999).

Carrier-mediated endocytosis is one of the most prevailing modes of uptake into the brain. It is used for the delivery of nutrients, such as monocarboxylates, hexoses (glucose), amines, amino acids, nucleoside, and purine bases to the brain (Pardridge, 1998). More than eight different nutrient transport systems have been identified at BBB, with each transporting a group of nutrients of the similar structure. Carrier-mediated transcytosis is substrate selective and the transport rate is dependent on the degree of occupation of the carrier.

Receptor-mediated endocytosis (RME) provides a means for selective uptake of macromolecules into the brain. It occurs for substances, such as transferrin (Fishman et al., 1987), insulin (Duffy and Pardridge, 1987), leptin (Banks et al., 1996), and IGF-I & IGF-II (Duffy et al., 1988). RME is a highly specific energy dependent transport mechanism. Substances that enter a cell by means of RME become bound to the receptors that collect in specialized areas of the plasma membrane known as coated pits (Moore et al., 1987). When bound to ligand these pits invaginate into the cytoplasm and then pinch free of the plasma membrane to form coated vesicles. These clathrin vesicle coats are rapidly removed to form smooth-coated endosomes, thereby allowing release of substances/ligand into the cell (Stahl and Schwartz, 1986).

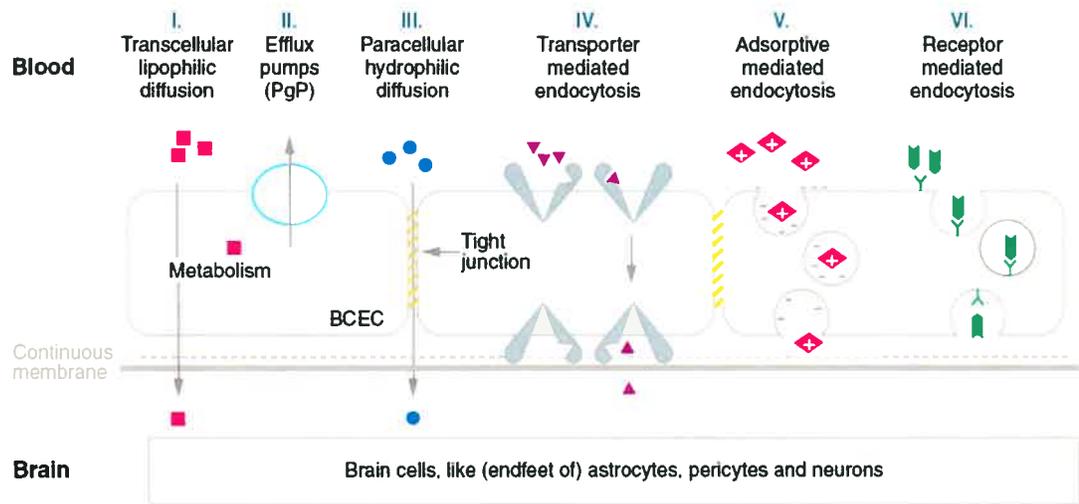


Figure 1.3. The various transport processes that may occur at the BBB. Reproduced from (Pardridge, 1995).

1.5. Standard therapies for brain tumors

The treatment, a cancer patient receives is determined by many related factors such as, the kind of cell from which cancer is derived, its size, the presence or absence of metastatic spread etc. Multiple treatment modalities have been developed in an attempt to eradicate brain tumors. Some of the commonly used therapies are enlisted below:

1.5.1. Surgery

Surgery is one of the first treatment modality used in clinics for the treatment of most of the cancers. It not only serves as a tool for pathological diagnosis, but also for immediate relief of the symptoms. Surgery reduces the number of cancer cells requiring treatment and often serves to remove the hypoxic core of the tumors that are relatively

resistant to radiation and inaccessible to chemotherapy (Siker and Mehta, 2007). However, damage to the neurological tissues during resection remains as a major challenge in the surgical procedures. A surgical resection with tumor free margins would increase the risk of removing normal brain tissue resulting in serious neurological damage (Nesbitt, 2007).

1.5.2. Radiation therapy

Few decades before, primarily in 1970 and 1980's postoperative irradiation of the organs was established as a standard protocol for patients with high-grade gliomas (Brada, 2006). It was believed that increasing the dose of radiation would improve the patient survival (Chang et al., 2007). But now, it is established that tumors reoccur in patients treated solely with radiation therapy. The major limitation to this preferred modality for the treatment of the tumors is the fact that the dose optimal for eradication of brain tumor might damage the surrounding brain tissue. Therefore, the adjuvant treatment with chemotherapeutic agents is often recommended. Because of the limitations of whole brain radiotherapy, newer non-invasive methodologies called "stereotactic radiosurgery" have been developed for delivering radiation locally using techniques like Gliasite brachytherapy in which liquid radiation is placed into a balloon that is implanted in the surgical cavity of a resected tumor (Laperriere et al., 1998; Souhami et al., 2004). Alternative radiation delivery methods for stereotactic radiotherapy including, intensity modulated radiotherapy, implanted seeds and Novalis have been developed to improve the efficacy of the radiotherapy and reduce toxicity. Drugs called radiation sensitizers which sensitize the tumor tissue to radiations so that effect can be obtained at significantly lower dose of radiation have also been developed (Brada, 2006). They are given at the time of radiation and also are used in a clinical research setting as part of a clinical trial (Huncharek, 1998; Huang et al., 2007). These stereotatic methods of radiotherapy allow outpatient procedure, decrease recovery period from radiosurgery and improve patients' quality of life due to reduced side-effects and thus, are been increasingly used in brain tumor treatments.

1.5.3. Chemotherapy

Chemotherapies have been used to control and ideally irradiate the infiltrative tumor cells. However, the amount of drug reaching the CNS is difficult and incomplete due to the BBB. There are only a few chemotherapeutic agents that readily cross BBB in therapeutic amounts (Figure 1.4), and these include nitrosoureas like 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), procarbazine, topotecan and temozolomide (Muldoon et al., 2007). Nitrosoureas were the first single agent therapy used for the treatment of gliomas because, they readily cross the BBB due their small size, non-ionized state and high lipid solubility. BCNU became the most commonly used single-agent chemotherapeutic agent for recurrent gliomas (Kornblith and Walker, 1988). Later, lipophilic derivatives of BCNU were produced in order to increase its efficacy by enhancing the BBB permeability; however, these agents showed similar results as BCNU. It has been reported that radiotherapy and BCNU produce superior results than radiotherapy alone (Walker et al., 1980). A recent analysis suggests that nitrosourea based adjuvant therapy has modest benefit for patients with anaplastic gliomas and smaller for those with glioblastoma (Levin et al., 1990). Procarbazine is another chemotherapeutic agent which crosses BBB readily. As a single agent, it has been reported to increase the long-term survival in patients with malignant gliomas as compared to BCNU (Newton et al., 1993). Temozolomide, another agent that cross BBB, has shown significantly better survival in patients with glioblastoma multiforme as compared to procarbazine (Macdonald et al., 2005). It is also reported to be effective in patients with newly diagnosed malignant glioma. Unlike other chemotherapeutic agents, temozolomide can be given orally due to its high oral bioavailability. The promising results with temozolomide have established it as new, modestly improved standard of care for patients with newly diagnosed glioblastoma multiforme (Nagasubramanian and Dolan, 2003). Methotrexate (MTX) is another chemotherapeutic agent, which at high dose and in combination with Citrovorum Factor Rescue (Lleucovorin[®]) has clinically treated patient with various brain tumors including glioblastoma multiforme (Abelson et al., 1981a).

paclitaxel, vincristine etc. have not been extensively evaluated for agents for brain tumor therapy due to their poor BBB permeability (Muldoon et al., 2007). Resistance to chemotherapy remains a central reason for the failure to cure patients with cancer. And in the case of brain tumors it is another challenge that needs to be overcome besides poor BBB permeability.

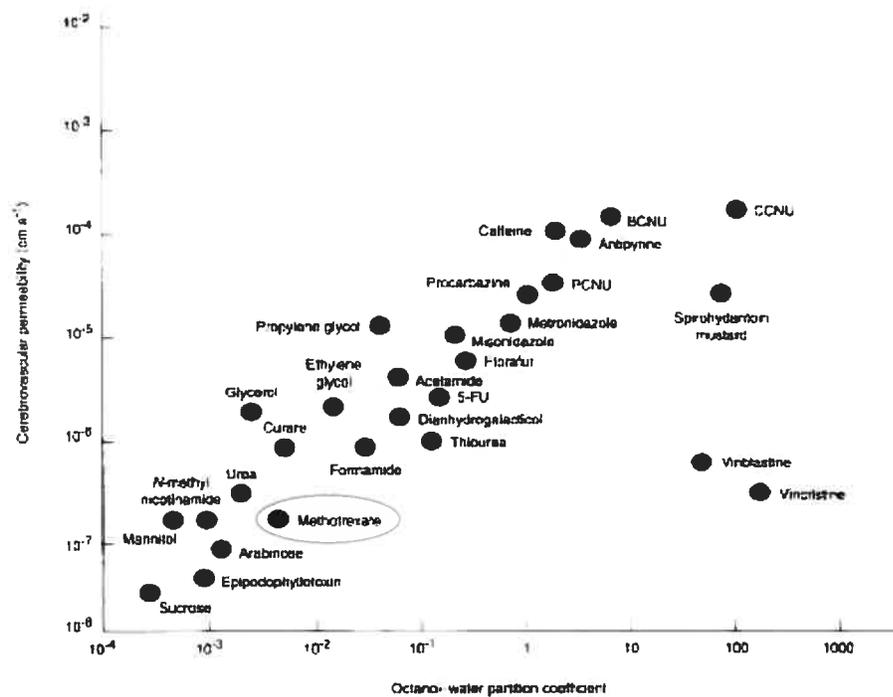


Figure 1.4. Permeability of various chemotherapeutic agents across BBB in relation to partition coefficient. Reproduced from (Muldoon et al., 2007).

1.5.4. Immunotherapy

Immune system is uniquely qualified to be an instrument used in cancer therapy because all the cancer patients including those with brain tumors have some degree of immunosuppression (Wu et al., 2006). In fact, it is speculated that tumors may be able to

grow because they evade the body's immune surveillance. An immune response directed against cells bearing tumor antigens could provide a specific and effective mechanism for killing of residual tumor cells (Gross et al., 2004). One of the strategies in immunotherapy involves delivering antibodies against tumor specific antigens to inactivate and clear highly expressed substances on tumors cells and allow restoration of a more normal immune state. They also help to reduce the tumor-induced immunosuppression that is mediated by soluble factors (Yang et al., 2006a). Monoclonal antibodies (MAb) are also an attractive tool for targeting radio-isotopes, chemotherapy agents, or even activated lymphocytes to the tumor cells (Wu et al., 2006; Sathornsumetee et al., 2007). An antibody specific for tumor antigen conjugated to radio-isotope could deliver high-dose radiation to malignant cells, reducing the morbidity caused by less closely targeted radiotherapy. Problems associated with MAb therapy relate to creating truly tumor-specific antibodies and delivery of sufficient doses of the antibodies to the tumor. Most of the antigens expressed by tumor cells are not tumor specific (Sathornsumetee and Rich, 2006; Wygoda et al., 2006). The epidermal growth factor receptor (EGFR) is one target reported to be specific to the tumor cells, it is found in 17% of glioblastomas. Miyamoto et al., (Miyamoto et al., 1996), conducted a trial using 125I-labeled EGFR-425, a MAb to the human EGFR, as an adjuvant to conventional therapy for patients with anaplastic astrocytoma and glioblastoma multiforme and found it to improve patient survival with minimal toxicity. Injection of antibodies like antitenascin into the resection cavity has also shown improvement in patient survival with moderate side effects (Reardon et al., 2007).

Other strategies in immunotherapy involve activation of immune response to the cancer cells by delivering interferons (IFN), cytokines, interleukins etc. (Edwards et al., 1985; Hill et al., 1992; Boiardi et al., 1994). These agents are given to patients to imitate or influence the natural immune response either by directly altering the cancer cell growth or acting indirectly to help healthy cells to control the cancer. Scientists are also studying vaccines that would boost the body's immune response to cancer cells and thereby prevent cancer from developing. The challenge in immunotherapy has been to identify the putative

target antigens and then direct and stimulate the patients' immune system against their own tumors (Lutz, 1983). The additional immunological disadvantage in the brain is the limited availability of antigen presenting cells and immune stimulatory signals. The success of immunotherapy depends on careful selection of the target antigen, corresponding specific MAb, size and affinity of the antibody and its proper compartmental administration (Ribas et al., 2003). The major limitation of this modality remains the poor permeability of antibodies across BBB after systemic administration which limits its potential for frequent administration.

1.5.5. Gene therapy

Gene therapy is meant to deliver genetic material with a therapeutic goal of encoding proteins (e.g., enzymes) or siRNA to the cells (Kouraklis, 2000). In addition, gene therapy provides the potential for a long-term effect following one single administration. However, genes are hydrophilic, charged, and large molecules that cannot pass cell membranes and tight cellular layers like the BBB. Therefore, their delivery to the desired site of action in brain is challenging. Viruses have evolved over millions of years to obtain optimal mechanisms for gene delivery to host cells, which makes them applicable as a biological vector system to deliver genetic material to brain cells. There is a broad range of viral vectors available, but the most commonly used are adeno-associated viral vectors and lentiviral vectors (Casallo and Alemany, 2004). Current gene therapy for brain tumor patients utilizes scientifically engineered viruses made from strains of the cold sore virus (herpes virus) or common cold virus (adenovirus). When introduced into the human body these engineered viruses are able to recognize cancer cells and kill them. Engineered viruses are also able to self-multiply and thus have a long "life" to kill and keep on killing cancer cells during the time-frame of the virus' life span. However, the immunogenic and pro-inflammatory nature of viral vectors limits their applicability (Tangney et al., 2006). Important issues in viral gene delivery are stable transgene expression, limited immunogenicity, induction of an inflammatory response, the lack of cell specific targeting

efficiency, safety, and toxicity. Due to these limitations of viral gene therapy, non-viral alternatives like liposomes and nanoparticles, for their delivery into brain have been explored (Dachs et al., 1997).

1.5.6. Antiangiogenic therapy

Angiogenesis is a normal process, the growth of new blood vessels, necessary for growth and for wound healing. It has recently been determined that in order for a tumor to grow, it must develop its own blood supply (Folkman, 2006). In contrast to the traditional cancer treatments that attack tumor cells directly, angiogenesis inhibitors target at the formation of tumor-feeding blood vessels that provide continuous supply of nutrients and oxygen. Additionally, antiangiogenic therapies target the endothelial cells thus, delivery challenges are considerably reduced (Miller et al., 2001; Sezer et al., 2001). The formation of new blood vessels is also a hallmark of malignant gliomas. Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis in most of the tumors (Cébe-Suarez et al., 2006). Thus, anti-VEGF therapies have been evaluated clinically and have shown to improve survival. It has been reported that endothelial cells of various tumors also express specific receptors like integrins, selectins etc. which are involved in tumor cell migration and invasion. These receptors are unregulated during neovascularization and tumor neo-angiogenesis and thus targeting them would reduce or inhibit vessel development and tumor spread. Of all the integrins, $\alpha_v\beta_3$ integrins are expressed in small blood vessels surrounding glioblastoma (Gladson, 1996). Antibodies developed to this integrin have been shown to inhibit matrigel invasion of glioma cell lines and primary cultures (Paulus and Tonn, 1994). Recently, a number of naturally occurring endogenous inhibitors of angiogenesis have been discovered and these include molecules like platelet derived factor 4 (Maione et al., 1990; Tanaka et al., 1998), or thrombospondin-1 (Streit et al., 1999) and anti-angiogenic factors such as endostatin (O'Reilly et al., 1997; Sasaki et al., 1998). These antiangiogenic factors can also serve as potential molecules that can be delivered to the tumors for reducing angiogenesis. Antiangiogenic therapies have shown to reduce the

the studies but the cure of cancer from this therapy has never been reported. Indeed, in the clinic, antiangiogenic drugs used as monotherapies have little discernible therapeutic (survival) benefit in the treatment of advanced-stage cancers. Thus, it has been recognized that antiangiogenic therapies might not be effective alone in the treatment of the cancer, the combination with chemotherapy would be needed to enhance their potential.

1.5.7. Combination therapy

Active research has failed to identify a single therapeutic approach which can cure malignant gliomas. Due to the failure of single-agent delivery or radiotherapy alone to improve the survival in patients with brain tumors, efforts have been directed to use more than one chemotherapeutic agents alone or in combination with immunotherapy or antiangiogenic therapy (Krauseneck and Mertens, 1987). It has been reported that the delivery of chemotherapeutic agent along with radiotherapy or combination of radiotherapy and immunotherapy are promising approaches. Radiation therapy treats cancer that is confined locally, while chemotherapy also kills cancer cells that may have spread. Sometimes radiation or chemotherapy is given before surgery to shrink a tumor, thereby making the complete removal of the tumor using surgery, or after surgery to destroy any remaining cancer cells (Wheeler and Kaufman, 1981; Sceda et al., 2007). The combination of radiotherapy with temozolomide therapy has shown a promise for patients with newly diagnosed glioblastoma (Addeo et al., 2007). This study has confirmed that combination therapy can improve the outcome for patients with these devastating tumors. The combination of cytotoxic and cytostatic chemotherapeutic agents has also been attempted for improving the efficacy of the treatment. The rationale for combining different chemotherapeutic agents is to use drugs that work on different parts of the cancer cell's life cycle, thereby increasing the likelihood that more cancer cells will be killed (Sceda et al., 2007). When drugs with different toxicities are combined, each drug can be used at its optimal dose, helping avoid intolerable side effects. For instance, combination of

temozolomide, BCNU and thalidomide has shown to improve patient survival than single agents therapy in clinical settings (Parney and Chang, 2003).

A number of studies have shown that targeted antiangiogenic agents and chemotherapy often have additive or synergistic effects when used in combination. Combination of chemotherapy agent with antiangiogenic therapies have been shown to be effective in primary glioblastoma multiforme tumors, producing a patient survival time of 16 months (Wygoda et al., 2006). It has been shown that PTK-787 (a VEGF receptor inhibitor) combined with temozolomide produced a median time to progression of 15.1 weeks. The combination of antiangiogenic therapy has also shown to potentiate the response to radiation therapy. Now it is increasingly realized that combination therapy is one of the most effective way for cure of malignant and aggressive tumors. However, due to complications in dosing schedule, frequency of administration etc., combination therapy is not widely used. Infact, Hildebrand, 1981 has reviewed a number of randomized combination therapy protocols and has concluded that based on the stage and the type of cancer, a specific regimen has to be developed for the success of combination therapy.

1.6. Challenges in delivery to brain tumors

Of all the brain tumors, malignant gliomas are most difficult to treat, this is largely because like most solid tumors they are variably resistant to treatment because of inadequate drug delivery, systemic toxicity, development of resistance to drugs (Krauseneck and Mertens, 1987). The specific considerations for gliomas are;

- BBB restricts the delivery of chemotherapy to brain
- High cellular heterogeneity in the tumor cells
- Defects in immune surveillance ad response
- Low toxicity to non-tumor cells

Clinical failure of many potentially effective therapeutics for the treatment of brain tumors is usually not due to the lack of drug potency, but rather due to the inability of drugs to cross BBB, i.e., can be attributed to shortcomings in the methods by which a drug is delivered to the brain and into the brain tumors (Penas-Prado and Gilbert, 2007). The unique physiological and pathological barriers found in the CNS impede the delivery for the treatment of brain tumors. Orally and intravenously (i.v.) administered chemotherapeutics have difficulty in reaching therapeutic concentrations at the tumors site because of the BBB, BCB, and the BTB. It is recognized that BTB is more permeable than normal brain tissue (Groothuis, 2000). However, the breakdown of the BBB in the area of the tumor is variable and large areas exist where BBB remains intact. In these areas the amount of drug reaching the tumor remains severely restricted. In addition, the brain adjacent to the tumor is the area around the tumor that contains infiltrating tumor cells. These sites may be associated with an intact BBB and drug delivery to these areas is difficult. Finally, although most brain tumors are highly vascular the aberrant tumor induced angiogenic process tends to create abnormal vessels such as blind loops and arteriole-venule shunts, making parenchymal drug delivery even more inefficient (Folkman, 2006).

Though immunotherapy had shown promise in treating these tumors it had failed to deliver the therapeutic benefit mainly due to the inability to produce significant immune response following the administration. Even genetherapy has not shown significant promise in clinical trials; further the need to deliver it invasively has limited its applicability (Kouraklis, 2000). Antiangiogenic therapy on the other hand has also failed to improve survival of patients with gliomas when administered alone. Thus, so far chemotherapy remains as the mainstay of treatment for patients with gliomas because, it has played important role in the treatment of primary brain tumors. However, enhancing permeability of chemotherapeutics across BBB is the major challenge which has been addressed

1.6. Strategies to enhance drug delivery across BBB

Several approaches have been implemented to address the limitations posed by both natural and pathological barriers in the CNS (Table 1.1, Figure 1.5). The strategies used to bypass these boundaries can be divided into chemical and physical based techniques.

1.6.1. Chemical strategies

They focus on improving drug delivery by altering the structure of the drug or by disrupting the BBB.

1.6.1.1. Modification in the drug molecules

BBB is permeable to small, electrically neutral and lipid-soluble molecules. Thus, increasing the lipophilicity of the drug molecule to increase its permeability is one of the preferred enhancement strategies. Such approach was used to enhance the delivery of BCNU by developing its lipophilic analogs like lomustine (CCNU) and semustine (methyl-CCNU). However, clinical trial evaluating the systemic administration of these agents showed no statistical improvement in the efficacy over BCNU in treating glial tumors (Chin et al., 1981; Ushio et al., 1984). Another strategy is linking the drug to

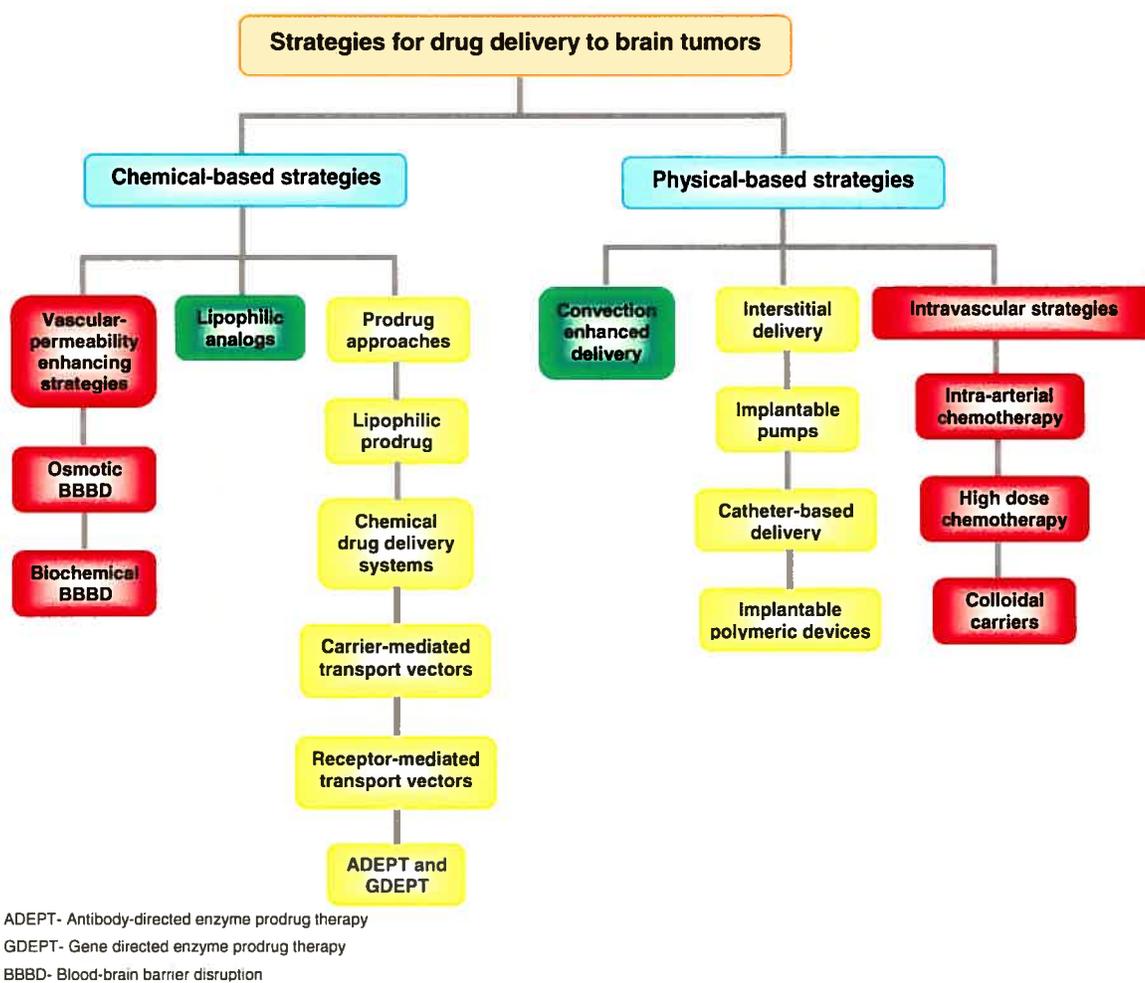


Figure 1.5. Various strategies that have been used to improve delivery of therapeutic molecules to brain.

Table 1.1. Various approaches utilized to enhance the delivery of drugs to the brain

<i>Approaches</i>	<i>Advantages</i>	<i>Limitations</i>
Lipophilic analogs	Readily penetrate CNS	Poor aqueous solubility, molecular weight limit (400-600 Da), enhanced peripheral distribution
Prodrug	High drug residence time	Possibility of reactive metabolite formation, poor selectivity and poor retention
Chemical drug delivery	Specific drug delivery e.g. neuropeptides	Oxidative liability and hydrolytic instability limit the shelf life of these systems
Osmotic BBBD	Alters barrier inducing factors	Breaks down the defense mechanism of the brain, often leads to unfavorable toxic effects
Biochemical BBBD	Selective opening of brain tumor capillaries	Breaks down the defense mechanism of the brain
Olfactory pathway	Direct nose to brain transport and access to CSF	Enzymatically active, low pH, mucosal irritation or variability caused by nasal pathology
Intraventricular/ intrathecal route	Bypasses BBB and provides immediate high CSF concentrations, longer drug half-life, minimized protein binding and decreased enzymatic activity	Increased intrathecal pressure leads to clinical incidences of hemorrhage, CSF leaks, neuro-toxicity and CNS infections
Injections, catheters and pumps	Continuous delivery drug distribution can be maintained	Due to diffusion problems therapeutic agent like to reach only near by sites
Biodegradable polymer wafers, microspheres and nanoparticles	Circumvent BBB, controlled delivery, easily implantable	Useful in very limited number of patients, due to diffusion problems therapeutic agent like to reach only near by sites
Drug delivery from biological tissues	Therapeutic proteins can be released from co-grafted cells	Inefficient transfection of host cells, nonselective expression of transgene and deleterious regulation of transgene by the host
Receptor/vector mediated drug delivery	Allows designing transport linkers for specific functional needs	Saturable process, enzymatic dependent release, attachment to BBB transport drugs renders some drugs inactive
Pegylated liposomes	Capable of receptor mediated transport to BBB	Do not undergo significant transport in absence of vector mediated delivery

Reproduced from (Misra et al., 2003)

lipophilic carrier like dihydropyridine (Kurihara and Pardridge, 1999). Preliminary studies have shown that chemical modification enhances penetration of chemotherapeutic agents into the brain. However, the major problems with lipidization are that it results in increase in the molecular weight of the parent compound and thus, reduces the permeability to certain extent. In addition, lipidization of a molecule increases the uptake into all organs of the body, resulting in decrease in plasma area under curve (AUC). This decrease in plasma AUC offsets the increase in permeability due to lipidization (Ushio et al., 1984).

1.6.1.2. Prodrug approaches

1.6.1.2.1. Lipophilic prodrugs

An alternative strategy for lipophilic drug analogs is the design of lipophilic prodrugs. Unlike drug analogs which are active themselves, prodrugs are pharmacologically inactive and require a chemical or biochemical transformation to convert to an active form. Prodrugs are designed to overcome pharmaceutical or pharmacokinetic limitations of the parent molecule such as poor BBB penetration. To enhance drugs' penetration into the brain by passive diffusion the simplest approach is to design lipophilic prodrugs (Bodor and Kaminski, 1987). A well known example of this method is heroin, diacetyl derivative of morphine, which crosses the BBB 100 times more easily than the parent drug morphine. This strategy has also been used for small anticancer drugs (Han and Amidon, 2000). For instance, lipophilic ester prodrugs of chlorambucil have been developed to increase its efficacy in the treatments of brain tumors (Greig et al., 1990). All the prodrugs showed increased accumulation in brain at equimolar doses e.g. chlorambucil-tertiary butyl ester had 35 times higher concentration in brain. However, despite of enhanced brain entry, none of the prodrugs demonstrated superior anticancer activity as compared to chlorambucil at equimolar dose. Thus, improved lipophilicity of the prodrug does not establish improved activity; both the selectivity and rate of bioconversion

of the prodrug in the target tissue should be taken into account when designing bioreversible prodrugs.

1.6.1.2.2. Antibody-directed enzyme prodrug therapy (ADEPT) and Gene directed enzyme prodrug therapy (GDEPT)

Recent prodrug approaches have been actively pursued to achieve very precise and direct effects at the site of action, with minimal side-effects on the rest of the body. This needs the selective cleavage of the drug at the site of action by activating specific enzymes. This could be achieved by designing the prodrug of compounds that are cleaved by specific enzymes, for example in case of tumors this could be done using enzymes endogenous to specific tumors such as glucuronidase, aryl sulphatase and NAD(P)H dehydrogenase isozyme that sensitizes tumors to aniline mustard and 5-aziridinyl-2,4-dinitrobenzamide, respectively. Prodrug-activating enzymes can be targeted to tumor cells by antibodies (ADEPT) (Niculescu-Duvaz and Springer, 1997) or genes (GDEPT) (Denny, 2003). In ADEPT, enzymes that activate prodrug are directed to tumors by conjugating them to the tumor selective MAb (Niculescu-Duvaz and Springer, 1997). An antitumor antibody is conjugated to an enzyme that is not normally present in the extracellular fluid or on cell membranes, and these conjugates are then localized in the tumors by i.v. infusion. After allowing the conjugate to clear from the blood, prodrug is administered. This prodrug is consequently activated in the tumors by the enzyme administered previously. ADEPT has progressed to phase I clinical trials, however, paucity of tumor specific antigens limits its applicability (Sharma et al., 1992).

In GDEPT, an inactive prodrug can be activated to release a cytotoxic drug by an enzyme that has been delivered to tumor for expression. The enzymes of both non-mamalian origin like tyrosine kinase, carboxypeptidase, nitroreductase or of human origin that are expressed in very low concentration or absent like deoxycytidine kinase etc. are used (Denny, 2003). A number of GDEPT approaches have been developed, however, the

most extensively studied approach is centered upon insertion of thymidine kinase gene in herpes simplex virus followed by treatment with cytosine deaminase-5-fluorocytosine (Moolten, 1986; Springer and Niculescu-Duvaz, 2000). In clinical trials with malignant glioma patients, this therapy resulted in moderate response and produced no cure. This poor response was ascribed to difficulty in achieving selective gene delivery to a sufficient number of tumor cells.

1.6.1.3. Chemical delivery systems

Chemical delivery systems are the prodrugs which release the active compound in multiple steps. This concept is designed not only to increase the permeability of the compounds in brain by increasing the lipophilicity but also by incorporating the specific properties in the structure which would lock the drug in the brain and prevent them from crossing the BBB (Bodor and Buchwald, 1997). For instance, linking the drug to a bioremovable lipophilic moiety like dihydrotrigonelline creates a complex that readily distributes throughout the body and brain after administration due to its lipophilic character (Palomino et al., 1989; Misra et al., 2003). Once in the brain parenchyma, the lipophilic dihydrotrigonelline moiety is oxidized to the ionic quaternary salt via ubiquitous $\text{NAD}^+ \leftrightarrow \text{NADH}$ coenzyme system. The acquisition of charge results in accelerating the systemic elimination as well as capturing the drug-salt inside the brain. Drug is slowly released from this salt in sustained manner specifically in brain. This strategy has been applied to lormustine by conjugating it to 1,4- dihydrotrigonelline.

1.6.1.4. Disruption of BBB

Various agents have been used to compromise BBB in order to improve delivery to brain. I.v./intra-arterial administration of hyperosmolar agents like mannitol is used to cause concentration gradient across endothelial cells; the efflux of water from these cells causes them to shrink, thereby loosening tight junctions and creating fenestrations within

the barrier (Rapoport, 2000). Williams et al. have reported that co-administration of mannitol with carboplatin and etoposide resulted in clinical improvements in all the patients with primitive neuroectodermal tumors and half of the patients with lymphoma (Williams et al., 1995). Bradykinin agonists like RMP-7, have also been used to improve concentration of chemotherapeutic agents across BBB (Inamura et al., 1994). The problem with BBB is that it results in the leakage of plasma proteins into the brain, contact of brain with blood results in astrogliosis and presence of albumin leads to toxicity on astrocytes. BBB also leads to vascular pathology and chronic neuropathological changes in the brain (Rapoport, 2000).

1.6.1.5. Conjugating vectors of carrier-mediated endocytosis

Carrier-mediated drug delivery approach takes advantage of facilitative endogenous transport systems that are present in brain endothelial cells. It involves conjugating the vectors of carrier mediated transport at BBB to the drug molecules. A number of carrier transport systems present at BBB are involved in the uptake of various nutrients from systemic circulation (Pardridge, 1995; Bergley, 1996). These transporters include, glucose, aminoacids, choline, vitamins, low density lipoprotein and nucleosides. Of these transport systems, glucose and large nutrient aminoacid transporters have high transport capacity and thus hold promise for significant drug delivery to the brain (Takada et al., 1992; Vannucci et al., 1998). Glucose transporters are very restrictive and transport only the molecules resembling glucose e.g., mannose and galactose. Large neutral amino acid transporters are less specific for their substrates and are capable of transporting numerous endogenous and exogenous amino acids across BBB. D,L-2-amino-7-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid, the derivative of anticancer drug melphalan with amino acid has shown 100-fold greater affinity for aminoacid transporters than the parent drug (Takada et al., 1992). The brain levels of this derivative reached levels 70% of those in plasma following i.v. administration, exceeding 20-40 folds as compared to melphalan. The glucose transporter was employed for facilitating the penetration of glucose

prodrug of chlorambucil into the brain. It was found that these glucose prodrugs inhibited the transport of ^{14}C -glucose by GLUT-1 (glucose transporter) and produced an inhibition profile similar to that of the free glucose (Smith, 2005). It was also found that 6-O-glucose derivate of chlorambucil had activity at 160 fold lower concentration than chlorambucil alone and also it had higher uptake into the brain.

1.6.1.6. Conjugating vectors of receptor-mediated endocytosis

An alternative strategy that takes advantage of endogenous BBB-transport system aims at improving brain uptake by coupling non-transportable therapeutic molecules to drug transport vector. The brain endothelium expresses specific systems for circulating important molecules which cannot diffuse through cerebral microvasculature. These include receptors for transport of insulin, insulin-like growth factors, transferrin and leptin (Pardridge, 1999). Thus, the vectors for these receptors could be insulin, transferrin, a modified protein or anti-receptor specific antibody. Conjugation of drug to these vectors results in transcytosis through BBB by endogenous receptor system in brain capillary endothelium. One of the most commonly used vectors for receptor mediated transcytosis has been transferrin-receptor antibody OX26, which recognizes transferrin receptors (Pardridge et al., 1991). This strategy has been used for transporting peptides, drug-loaded liposomes etc. across BBB. It has been reported that immuno-liposomes targeted by OX26 antibody had higher volume of distribution in brain as compared to non-targeted system. However, saturation of target receptors and competitive inhibition from endogenous substrates may limit the receptor-mediated delivery.

1.6.2. Physical based strategies

These include physical manipulation of the BBB by using catheter, convection-enhanced delivery (CED), polymer or microchip system which directly releases therapeutics in the vicinity of the tumor or intravascular enhancement strategies.

1.6.2.1. Catheter delivery systems

Catheters are placed in the site near the tumor. They deliver intermittent bolus injections of chemotherapeutic agents such as BCNU, MTX, adriamycin and biological agents like interleukin-2 and IFN-gamma (Walter et al., 1995; Boiardi et al., 1999). There have been few case reports indicating their success, however, no large scale clinical trial has proved their efficacy. The major limitation of this delivery system is that it causes considerable discomfort and inconvenience to the patients because patients head is anchored by a catheter. Further, the duration of drug infusion is restricted because of the potential of infection.

1.6.2.2. Implantable Pumps

Recently, a new generation of implantable pumps have been developed that provide a constant infusion of drugs over an extended period of time. These pumps include, Infusaid pump, Mini Med PIMS pump, Medtronic SynchroMed system (Giussani et al., 2003). Continuous infusion of chemotherapeutic agents using these implantable pumps has proven beneficial in brain-tumor models. Unfortunately, pumps have many potential pitfalls, including, clot or tissue debris obstruction, mechanical failure, infection during further operations and undesired alterations in the chemical properties of the drug during its transit from the pump to the target site.

1.6.2.3. Convection enhanced delivery (CED)

This new method allows chemotherapy to be delivered more safely and effectively by pumping the therapeutic agent under pressure directly into the brain. By targeting the tumor cells, CED helps conserve healthy brain cells and prevents adverse side effects commonly found in traditional systematic chemotherapy delivery methods (Bobo et al., 1994). It is a promising technique for drug delivery to the brain, especially in the areas

where surgical intervention is not possible. Animal studies have shown that CED of carboplatin and gemcitabine are effective in the treatment of gliomas. In addition, it is also reported that CED of taxol and IL-13 (immunotoxin) have shown promise in clinical trials for malignant glioma patients (Hall et al., 2003).

1.6.2.4. Polymer based delivery systems /Interstitial chemotherapy

It involves the placement of the delivery systems like biodegradable polymers devices at the resection site (Brem and Langer, 1996). These polymeric devices break down and disintegrate, permitting the gradual release of the anti-cancer drug over weeks (Hanes et al., 1997). By placing the chemotherapeutic drug directly at the site of the tumor instead of injecting it in the veins, higher doses of the drug can be delivered to the cancerous area for a longer period of time with reduced risk of side effects commonly seen with standard chemotherapy delivery methods (Guerin et al., 2004). Biodegradable polymers like polylactic glycolic acid, poly(1,3 bis(carboxyphenoxy)propane-co-sebacic acid) etc. have been used to develop microspheres, rods or films that can release the drug slow at the tumor site and the end polymer breaks down into monomers. Brem's group was the first one to demonstrated the feasibility of polymer-mediated drug delivery by using the standard chemotherapeutic agent BCNU and showed that local treatment of gliomas by this method is effective in animal models of intracranial tumors (Brem and Gabikian, 2001). This has led to clinical trials for glioma patients, and subsequent approval of Gliadel[®] by the FDA (Westphal et al., 2006). Benoit's group has developed a new concept of drug targeting into the CNS by stereotactic implantation of biodegradable microparticles (Menei et al., 1994). Because of their size, these microparticles can easily be implanted by stereotaxy in functional areas of the brain without damaging the surrounding tissue. Compared to large implants, microparticles do not need open surgery. Insterstitial delivery is beneficial in very limited number of patients because the therapeutic agents released from these systems are able to diffuse to only regions in vicinity of the implant. Further due

to the invasive delivery, frequent administration of drug is limited; consequently, recurrence of tumor is the major drawback of this strategy.

1.6.3. Intravascular techniques

1.6.3.1. High-dose intravenous chemotherapy

High-dose chemotherapy has been used for the treatment of gliomas with the objective of increasing the delivery across BBB due to higher plasma levels. Generally, a high dose of chemotherapeutic agent is administered followed by bone marrow transplant or peripheral blood stem cell rescue (Abrey et al., 1999). High-dose chemotherapy has shown encouraging results in pediatric patients and young children but is less promising for adults with malignant gliomas. Therapeutic concentrations of certain chemotherapy drugs such as MTX within the brain can be achieved by giving them into a vein at high doses. Other drugs can only be given at high doses if it is followed by bone marrow transplantation. This approach, though successful for other types of cancer, has not resulted in a better outcome in most primary brain tumors (Petersdorf and Livingston, 1994). The major limitation with high dose chemotherapy has been the increased systemic toxicity and potential for delayed neurotoxicity. The response rates and overall patient survival has not been impressive albeit toxicity has been substantial.

1.6.3.2. Intra-arterial chemotherapy

Intra-arterial chemotherapy can provide several fold advantage over the same drug dose given systemically in terms of peak drug concentrations in the tumor and total area under the curve exposure (Dropcho, 1999). Drugs like nitrosoureas have been delivered using this route however, it was found that patients administered BCNU i.v. had better survival rate than patients treated intra-arterially (Greenberg et al., 1984). The infusion of

chemotherapeutic agents into intracerebral arteries increases the delivery advantage but it increases the risk of other neurotoxicities including seizures, vision loss etc.

1.6.3.3. Colloidal carriers

Colloidal drug carriers are the delivery systems with size below a micron and most often in sub 500 nm range. Commonly used colloidal carriers include micelles, emulsions, liposomes and nanoparticles (nanospheres and nanocapsules). The aim in using colloidal carriers is generally to increase the specificity towards cells or tissues, to improve the bioavailability of drugs by increasing their diffusion through biological membranes and/or to protect them against enzyme inactivation. Moreover, the colloidal systems allow access across the BBB of non-transportable drugs by masking their physico-chemical characteristics through their encapsulation in these systems (Garcia-Garcia et al., 2005). It is noteworthy that only liposomes and nanoparticles have been largely exploited for brain drug delivery.

1.6.3.3.1. Polymeric micelles

Polymeric micelles are formed by the self-assembly of amphiphilic diblock copolymers having a hydrophilic and hydrophobic segments. The hydrophilic portion of the polymer forms the shell while the hydrophobic region forms the core. This organization provides them to encapsulate hydrophobic compounds in their core. Micelles have a size range of several tens of nanometers with a considerably narrow size distribution. Micelles have not been widely used for the delivery of chemotherapeutics to the brain; however, they have shown improvement in the amount of drug available to brain. Studies by Kabanov et al. (Kabanov et al., 1992) have shown that poloxamer (Pluronic™) micelles conjugated with antibodies improve brain distribution and efficacy of haloperidol, a neuroleptic agent. This result indicated that Pluronic™ micelles provide an effective transport of solubilized neuroleptic agents across the BBB. Recent investigations made by

the same group demonstrated that only Pluronic™ unimers allowed cell penetration in bovine brain microvessel endothelial cells (BMEC) monolayers of molecules such as digoxin (Batrakova et al., 2001), doxorubicin (Alakhov et al., 1999) by inhibition of the P-gp mediated drug efflux system.

1.6.3.3.2. Liposomes

Liposomes are small vesicles consisting of unilamellar or multilamellar phospholipid bilayers surrounding aqueous compartments. They are composed of biocompatible and biodegradable lipids similar to biological membranes. Liposomes have been considered for brain targeting in several pathologies through both intracerebral and i.v. administrations. An enhanced transport of liposome-encapsulated drugs into the brain has been observed in several reported studies. Most of the studies have focused on tumor therapies to deliver doxorubicin and other antineoplastic agents with the aid of either cationic or PEGylated liposomes (i.e. liposomes sterically stabilized by a coating of polyethylene glycol (PEG)). In general, these treatments have led to long-term survival and inhibition of tumor growth in patients (Fiorillo et al., 2004).

A). PEGylated liposomes

Liposomes coated with the inert and biocompatible polymer PEG are widely used and are often referred to as “sterically stabilized” or “stealth liposomes”. PEG coating is believed to prevent recognition of liposomes by macrophages due to reduced binding of plasma proteins. These liposomes have proven their ability to deliver the drugs owing to their long blood circulating times and their reduced clearance by the mononuclear phagocytic (MPS) system (Gabizon and Papahadjopoulos, 1988). This allows them to selectively extravasate in pathological sites, like tumors or inflamed regions with a leaky endothelium. The earlier studies in animals demonstrated an enhanced drug exposure and improved therapeutic activity (Gabizon and Papahadjopoulos, 1988). Now, a liposomal

formulation of PEGylated liposomes encapsulating doxorubicin (Caelyx[®]) is used in clinical practice, showing effectiveness in glioblastomas and metastatic tumors.

B). Cationic liposomes

These liposomes are prepared from cationic lipids and are generally used to entrap genetic material. Encapsulation of genetic material into cationic liposomes confers a protection from the extracellular environment and provides a mechanism for genetic material transfer to target cells. The studies on IFN-gene therapy using cationic liposomes have shown interesting results in the treatment of brain tumors (Yagi et al., 1994). To achieve an efficient transfer of the cationic liposomes content into cells, fusogenic liposomes have been prepared using fusogenic lipids, by conjugation of fusogenic molecules to liposome membranes or by incorporation of viral fusion proteins to bilayers (Shangguan et al., 1998). Similarly, Matsuo et al. (Matsuo et al., 2000) have reported the feasibility to introduce oligodeoxynucleotides into mouse brain-capillary endothelial cells by utilizing the hemagglutinin virus of Japan and liposomes with fusogenic activity. Unfortunately, cationic liposomes normally require an invasive way of administration to transfer genes into the brain.

C). Targeted liposomes

Further specific delivery of liposomes to brain after systemic administration has been achieved by active targeting. Active targeting can be achieved by complexing the liposomes with an antibody or a ligand that will be recognized by cell surface receptor in the targeted tissue. This approach may be the most striking advance in BBB targeting and translocation. MAbs have enabled brain targeting of PEGylated liposomes. Immunoliposomes (antibody-directed liposomes) have been recognized as a promising tool for the site-specific delivery of drugs and diagnostic agents (Schnyder and Huwyler, 2005). The MAbs are able to attach a receptor expressed on the BBB and to trigger a receptor-

mediated transcytosis across the BBB. The targeting MAb acts as a molecular Trojan horse to ferry the liposomes across biological barriers in the brain via endogenous transport systems. In this regard, Huwylar et al. (Huwylar et al., 1996) have shown that specific OX26-mediated targeting of daunomycin to the brain may be successfully achieved by the use of PEGylated MAb-liposomes. However, the *in vivo* use of classical immunoliposomes is hampered by the very rapid clearance of immunoliposomes from the circulation by the reticuloendothelial system. Avoidance of this obstacle is possible if gangliosides or PEG-derivatized lipids are inserted within the bilayer of conventional liposomes, as these modifications prolong considerably the liposome half-life in the circulation.

Another approach for targeting liposome's to brain is the use of the transferrin ligand. da Cruz et al. (Cruz et al., 2004) reported that cationic liposomes decorated with transferrin resulted in a significant enhancement of luciferase gene expression activity in C6 glioma cells, primary hippocampal neurons and primary cortical neurons. However, the transfection efficiency of this system was low in comparison with pegylated MAb-liposomes, perhaps due to the fact that transferrin was just electrostatically associated to the cationic liposomes. In conclusion, liposomes have been extensively investigated for the brain delivery of molecules, showing increased drug efficacy and reduced drug toxicity.

1.6.3.3.3. Nanoparticles

Nanoparticles are submicron drug carrier systems generally of polymeric nature. Nanospheres are nanoparticle system with a matrix character and constituted by a solid core with a dense polymeric network (Yang et al., 2006b). Practically, the nanoparticles have a size around 200 nm and the drugs or other molecules may be dissolved into the nanoparticles, entrapped, encapsulated, adsorbed or attached. These systems are attractive because the methods of preparation are generally simple and easy to scale-up. Nanoparticles can be made from a broad number of materials such as poly(alkylcyanoacrylates), polyacetates, polysaccharides and copolymers (Arayne and

Sultana, 2006). The advantage of using nanoparticles for drug delivery results from their two basic properties. Firstly, due to their small size, nanoparticles penetrate into even small capillaries and are taken up within cells, allowing an efficient drug accumulation at the targeted sites in the body. Secondly, the use of biodegradable materials for nanoparticle preparation, allows sustained drug release at the targeted site over a period of days or even weeks after injection.

A). Coated nanoparticles

Nanoparticles have been long used for delivery of therapeutics to brain, however like liposomes they are taken up rapidly by reticulo-endothelial system resulting in failure of the therapy. The deposition of plasma proteins on nanoparticles can be reduced either by adsorbing at the surface of the colloids some surfactant molecules (such as copolymers of polyoxyethylene and polyoxypropylene, polysorbate (PS)-80 etc.) or by providing a sterical stability by the direct chemical link of PEG at the surface of the particles (Peracchia et al., 1997). The former approach generates coated nanoparticles, such nanoparticles have indeed shown improvement in terms of amount of drug delivered to brain. For instance, doxorubicin loaded nanoparticles after coating with PS-80 showed significantly higher survival times in rats with glioblastoma (Steiniger et al., 2004). Moreover, the acute toxicity of doxorubicin was reduced when it was associated with PS-80-coated nanoparticles. Recently, Olivier et al. (Olivier et al., 1999) have suggested that polybutylcyanoacrylate (PBCA) nanoparticles coated with PS-80 displayed some toxic effect towards the BBB. In addition, it was also suggested that the nanoparticles could open the tight junctions between endothelial cells in the brain microvasculature, thus creating a paracellular pathway for nanoparticles translocation. Nevertheless, desorption of coated polymer from the nanoparticles resulting in unpredictable *in vivo* behaviour is the major limitation of this strategy.

B). PEGylated nanoparticles

In this technology, the PEG is covalently attached to the hydrophobic block of the polymer, rather than adsorbed, to avoid the possibility of PEG desorption. PEGylated-poly(hexadecyl cyanoacrylate) (PEG-PHDCA) nanoparticles have been investigated for the treatment of brain tumors. PEG-PHDCA nanoparticles have been shown to penetrate into the brain to a greater extent than all the other nanoparticles formulations tested, including the above discussed PS-80 nanoparticles (Calvo et al., 2001). Calvo et al. (Calvo et al., 2002) have investigated the accumulation of PEG-PHDCA nanoparticles in the rat brain. After i.v. administration in rats bearing intracerebral well-established gliosarcoma, PEG-PHDCA nanoparticles have accumulated preferentially in the tumoral tissue, rather than in the peritumoral brain tissue or in the healthy controlateral hemisphere. Interestingly, PEG-PHDCA nanoparticles concentrated much more in the gliosarcoma than their non-PEGylated counterparts (PHDCA nanoparticles). These nanoparticles also did not display any toxicity towards the BBB based on sucrose permeability test.

1.6.3.3.4. Nanogel

Vinogradov et al. have developed a new family of carrier systems for the delivery of drugs and biomacromolecules to the brain (Vinogradov et al., 2004). These so called “nanogels” systems, are made from a network of cross-linked ionic polyethylenimine (PEI) and non-ionic PEG chains (PEG-cl-PEI). When a biologically active macromolecule is associated to the nanogel by electrostatic interactions, the PEI chains have a tendency to collapse which results in decreased volume and size of the particles. Because of the steric stabilization of the PEG chains, the collapsed nanogel forms stable dispersions with a mean particle size of 80 nm. To realize active targeting, the surface of the nanogel could be modified with biospecific ligands. For this purpose, various coupling strategies have been used including covalent attachment of the ligand moiety to free amino groups of the PEI fragments in the PEG-cl-PEI nanogel. Another simple way to introduce ligands in the

nanogel particles consists of the partial modification of PEI fragments with biotin moieties allowing attachment of ligand using standard biotin-avidin coupling chemistry (Vinogradov et al., 2002). Nanogels have been tested as a potential carrier for oligonucleotide (ODN) delivery to the brain by using polarized monolayers of bovine BMEC (Vinogradov et al., 2004). The studies performed with that model of BBB have shown an increased transport of ODN across the cell monolayers as a result of their incorporation into the nanogel. Further increase in ODN transport was observed when the nanogel carriers were modified with insulin or transferrin ligands. Permeability assays with mannitol indicated that the increased transport of ODN-nanogels did not result from single paracellular diffusion due to a disruption of the bovine BMEC monolayers. After i.v. injection of ODN-nanogels in mice, no adverse toxic effects were observed and increased brain and decreased liver/spleen accumulations were noted, compared to free ODN (Vinogradov et al., 2004). These preliminary studies suggest that this system could represent a promising carrier for the delivery of ODN to the brain.

1.6.3.3.5. Dendrimers

Dendrimers represent a new class of polymeric carriers which hold a very promising application in drug delivery due to their unique properties. They are monodisperse sphere-like, three dimensional macromolecules built up from simple building blocks that are connected to a small core (Tomalia and Frechet, 2002). The term “dendrimer” meaning “tree-like” was originally coined by Tomalia et al. in 1985 (Tomalia et al., 1985) to describe a family of regularly branched poly(amidoamines) (PMAM) in which all bonds converge to a single point, each repeat unit contains a branch junction and a large number of identical functional groups are present at the chain ends. This highly branched and complex architecture endows dendrimer with globular shape (resembling proteins) and monodisperse size (Klajnert and Bryszewska, 2001). Dendrimers possess properties considerably different from the linear polymers like globular confirmation, higher solubility and presence of internal cavities. By virtue of these properties, numerous

investigators are exploring their application as drug delivery vehicles (Esfand and Tomalia, 2001; Cloninger, 2002; Patri et al., 2002; Aulenta et al., 2003).

A). Molecular structure of the dendrimers

A dendrimer is built up of layers of repeating units around a central core (Figure 1.6). Each layer of monomers builds up one generation. One wedge of a dendrimer is called a dendron. Because of the branching of the repeating unit, the numbers of end-groups

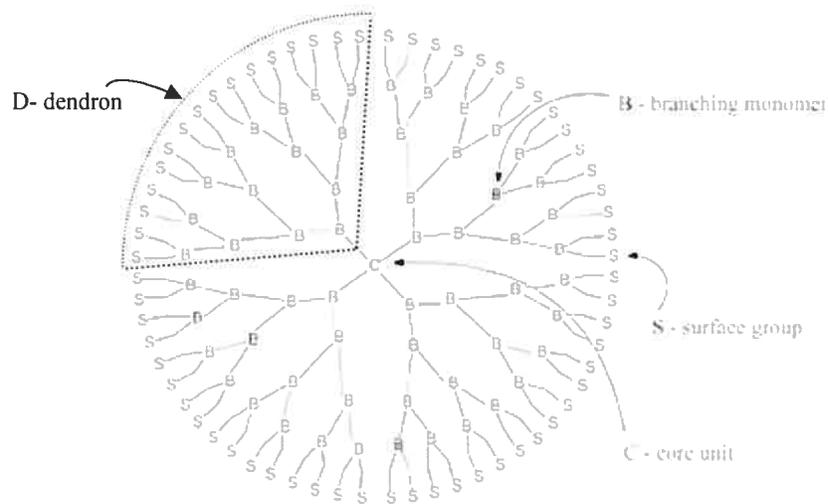


Figure 1.6. Pictorial representation of the molecular architecture of dendrimer. Reproduced from (Boas and Heegaard, 2004).

increases with each generation, resulting in a large number of terminal units at high generations. All the dendrimers have three basic regions consisting (Ballauff and Likos, 2004);

- shell, which is the layer of monomers surrounding core (Figure 1.6)
- surface groups (like $-\text{OH}$, $-\text{NH}_2$, $-\text{COOH}$), which are the reactive sites for further reaction

- core, which consists of region around the starting molecule

Based on the molecular architecture dendrimers can be classified as (Klajnert and Bryszewska, 2001);

Layer-block dendrimer are comprised of concentric rings of different chemistry around the core molecule (Figure 1.7a). They are synthesized by using different monomers in each generation of the dendrimer.

Segment block dendrimers, which have dendritic segments of different composition (Figure 1.7b). They are synthesized by attaching different dendrons to the core molecule.

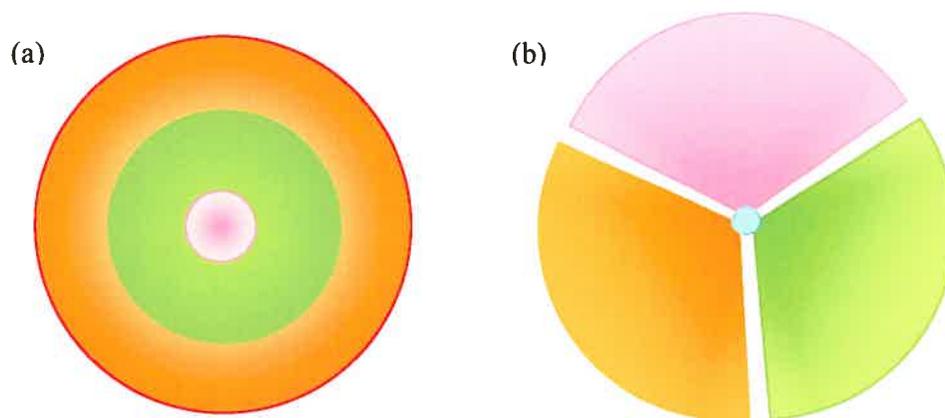


Figure 1.7. (a) Layer-block and (b) Segment-block dendrimers. Adapted from (Klajnert and Bryszewska, 2001).

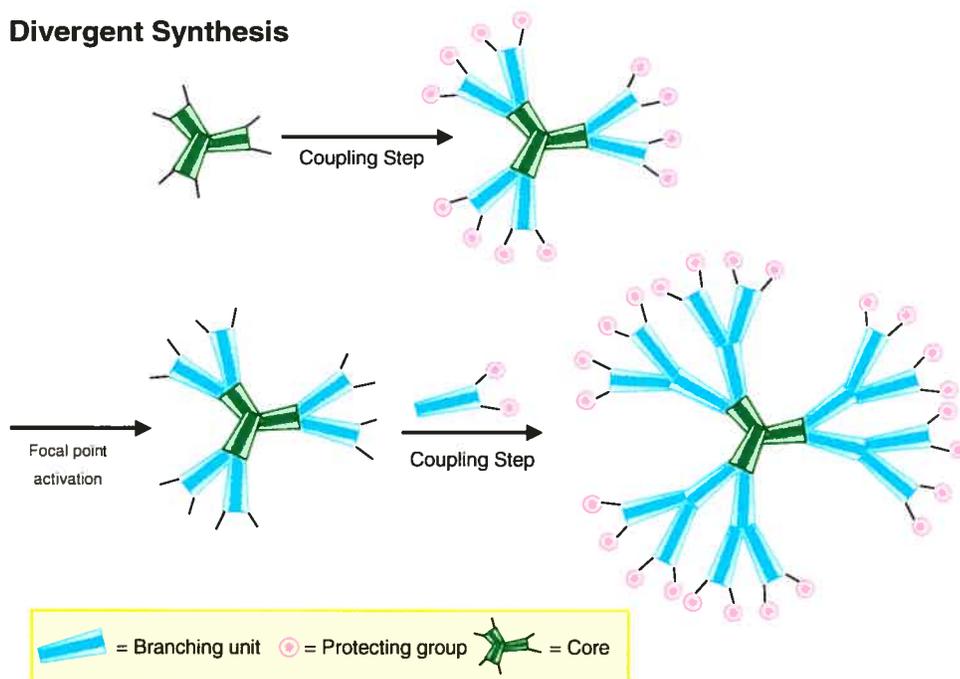
B). Synthesis of dendrimers

Dendrimers are synthesized by regular and repeated branching at each monomer group which provides globular and symmetric shape. They are generally prepared using either a divergent or convergent synthesis (Klajnert and Bryszewska, 2001; Boas and Heegaard, 2004):

a). Divergent Synthesis

In the divergent method of synthesis, dendrimer grows outwards from a multifunctional core (Figure 1.8a). The core molecule reacts with monomer molecules containing one reactive and two dormant groups giving the first generation dendrimer (Tomalia and Frechet, 2002). This new periphery of functional groups generated, later reacts with more monomers. This process is repeated for several generations and dendrimer is built layer after layer. The side reactions occurring during the synthesis and incomplete reaction of end groups lead to structure defects. To prevent side reactions and to force reactions to completion large excess of reagents is required. It causes difficulties in the purification of the final product.

a) Divergent Synthesis



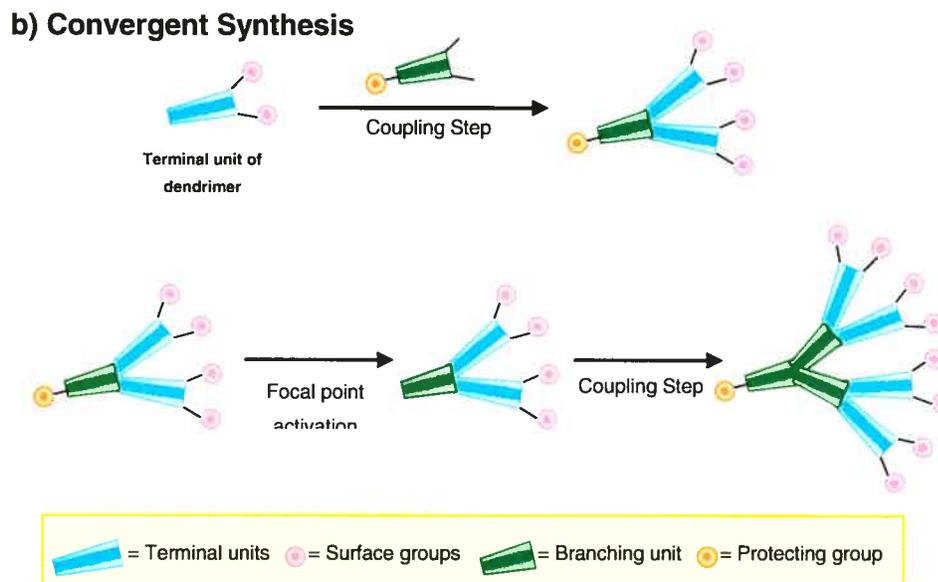


Figure 1.8. Divergent and convergent synthetic methods for dendrimer synthesis. Modified from (Klajnert and Bryszewska, 2001).

b). Convergent Synthesis

In the convergent approach, the dendrimer is constructed stepwise, starting from the end groups and progressing inwards (Pittelkow and Christensen, 2005). When the growing branched polymeric arms called dendrons, are large enough they are attached to a multifunctional core molecule (Figure 1.8b). The convergent approach was developed due to the weaknesses of the divergent method. Therefore, this synthesis method offers several advantages (Klajnert and Bryszewska, 2001). The purification of the final product is easier and the occurrence of defects in the final product is minimized. It becomes possible to introduce subtle engineering into the structure by placement of the functional groups at the periphery of the macromolecule. However, this approach does not allow the formation of high generations because of the steric hindrance during coupling of the dendrons to the core.

C). Properties of dendrimers

Dendrimers are monodisperse macromolecules unlike linear polymers. Linear polymers are generally random in nature and produce molecules of different size and molecular mass; whereas, size and molecular mass can be specifically controlled during dendrimer synthesis. Dendrimers of lower generation have highly asymmetric shape and possess more open structure as compared to higher generation dendrimers (Boas and Heegaard, 2004). As the generation of the dendrimer grows they become more globular in structure. The increasing branch density with generation is also believed to have striking effect on the structure of the dendrimers. These include presence of internal cavities and large number of surface groups (Aulenta et al., 2003). Because of their molecular architecture, dendrimers show some significantly improved physical and chemical properties as compared to traditional polymers. Dendrimer exist as tightly packed balls in solution unlike linear polymers which exist as random coils. This solution behaviour of dendrimers has great impact on their rheological properties; in particular, they have significantly lower viscosity than linear polymers. Another interesting property of the dendrimers is that their intrinsic viscosity goes through a maximum at the fourth generation and then begins to decline, unlike linear polymers which show increase in viscosity with increase in molecular mass. Due to the presence of many chain ends dendrimers have high solubility and miscibility and high reactivity (Klajnert and Bryszewska, 2001). Dendrimers' solubility is strongly influenced by the nature of surface groups. Dendrimers terminating in hydrophilic groups are soluble in polar solvents, while dendrimers terminating in hydrophobic groups are soluble in non-polar solvents. Further, due to their globular shape and the presence of internal cavities they have ability to encapsulate guest molecules. The unique advantage of the architecture of the dendrimers is that the core, shell and surface groups can be tailored specifically by choosing different monomers, core molecule and the surface groups to obtain dendrimer of desired properties and architecture (Boas and Heegaard, 2004). Thus, dendrimers can be easily designed according to the desired

application. This ability to control placement of functional groups and to obtain polymeric scaffolds with nanoscopic dimensions, has stimulated research into their application in diverse areas, such as drug delivery, diagnostic tools, rheology control, nanofabrication, and molecular electronics (Esfand and Tomalia, 2001; Cloninger, 2002; Aulenta et al., 2003).

D). Dendrimers in drug delivery

The therapeutic effectiveness of any drug is often diminished by its inability to gain access to the site of action in an appropriate dose. This is often due to the poor solubility of the drug in the bodies' aqueous environment. One method of aiding solubilization is to encapsulate the drug within the hydrophobic domains of a colloidal or surfactant based system (i.e. emulsions, micelles etc.). However, the unstable dynamics of emulsions and micelles systems, as well as their sensitivity to other functionality and pH, can lead to uncontrolled and premature release of the bound drug moieties rendering this approach ineffectual. Ideally a static or covalent micellar system is desirable. Dendrimer with charged or polar terminal groups are water soluble and can be considered as static, covalent micelles. Therefore, numerous studies have been initiated for evaluating application of dendrimers in drug delivery (Aulenta et al., 2003). Dendrimers can be used to deliver drug by encapsulating the drug in the internal cavity or by complexing or conjugating it to surface functionalities.

a). Dendrimer drug conjugates

The presence of abundant number of functional groups at the periphery of the dendrimers provides an advantage of linking large number of drug molecules or ligands to the surface of dendrimer (Tomalia and Frechet, 2002). This advantage has been enormously exploited for the delivery of various drugs by synthesizing dendrimer-drug conjugates that have the drug attached to the terminal functional groups. Because of its multiplicity one

dendrimer molecule can carry multiple drug molecules, and the number of drug molecules per conjugate can be varied by using different generations of dendrimers or by changing the coupling conditions. Though conjugation serves to increase the pay-load, it could also result in limited aqueous solubility of the drug-dendrimer conjugate, especially for hydrophobic drugs. In addition, the conjugation of drug to polymers is a time consuming process (requiring additional synthetic steps) and could sometimes result in decreased therapeutic benefit principally due to failure of conversion or slow conversion to the parent drug or decreased permeability of the conjugate. Conjugation of drugs to the dendrimers also generates the new chemical entity rather than a new delivery system for the drug requiring generation of extensive data on the safety, efficacy and toxicity of the dendrimer-drug conjugate. Nevertheless, various biologically active molecules like antibodies, sugars and drugs have been attached to the end groups of the dendrimers. However, most of these conjugates have lower or decreased activity relative to the free drug. Duncan and co-workers (Malik et al., 1999) have conjugated cis-platinum to the PAMAM dendrimers and found that the conjugates have activity in all tumor models including platinum-resistant. Kono et al., (Kono et al., 1999) synthesized a multifunctional dendrimer conjugate consisting of MTX and folic acid for targeted delivery to the tumor cells; however, this conjugate was water soluble only above pH 7.4. In another study, Baker and co-workers (Majoros et al., 2006) also conjugated MTX and folic acid to the PAMAM dendrimers. They found that ester linked MTX-dendrimer conjugate was more active than free MTX while amide-linked dendrimer conjugate was less active. Recently, Lee et al., (Lee et al., 2006) have shown that single dose of doxorubicin-functionalized bow-tie dendrimer cures mice bearing C-26 colon carcinomas.

b). Complexation of drugs with polar groups on the dendrimer surface

The multiplicity of the surface groups provides dendrimers with advantage of interacting with various charged molecules. This technique was given precedence in studies by Twyman and co-workers (Twyman et al., 1999) in which small acidic molecules, such

as benzoic acid, were encapsulated in TRIS terminated water-soluble PAMAM dendrimers. The acidic molecules were bound within the interior of the dendrimer, ion paired with the tertiary nitrogens, as evidenced by the precipitation of solubilized guests at pH 2 at which the interior nitrogen would be protonated. In another study (Kolhe et al., 2003), based on NMR and Fourier transform infrared spectroscopy it was reported that the carbonyl group of the nonsteroidal anti-inflammatory drug ibuprofen forms electrostatic interactions with the amine groups of PAMAM dendrimers,. A generation (G) 3 PAMAM dendrimer bound 32 molecules of ibuprofen (equal to the 32 surface amines) while the G4 dendrimer with 64 surface amines bound 78 molecules of ibuprofen (50% by weight), suggesting that there may also be some encapsulation in the interior of the larger dendrimer. The complex showed facilitated entry into A549 cells as well as more rapid suppression of cyclooxygenase-2 (COX-2) mRNA levels than the free drug. Ketoprofen has also been complexed to PAMAM through electrostatic interactions. The drug showed concentration and generation dependent solubilization with the dendrimer, increasing with dendrimer concentration. In pH studies ketoprofen was least soluble at pH 3 and most soluble at pH 6, supporting the conclusion that it is electrostatically complexed to the dendrimer, as the drug would not be fully ionized at low pH and would not interact as strongly with the dendrimer. *In vitro* release of ketoprofen from the complex into water was significantly slower than diffusion of free drug across the dialysis membrane, and the complex showed prolonged pharmacodynamic *in vivo* activity (Na et al., 2006). In spite of the ease of complexing the drug molecules with dendrimers, the unsuitable immediate release of the drug in release medium has weakened the interest in actively pursuing this approach for developing dendrimer based delivery systems.

c). Encapsulation of drugs within a dendrimer

The open nature of the dendritic architecture has led several groups to investigate the possibility of encapsulating drug molecules within the branches of a dendrimer. This offers the potential of dendrimers to interact with labile or poorly soluble drugs. Such

systems may enhance drug stability and bioavailability. Encapsulation of a drug within a dendrimer may also be used to provide a means of controlling its release. Maciejewski (Maciejewski, 1982) indeed suggested the use of egg shell-like architectures for the encapsulation of guest molecules in polymers. Studies in past have shown that they can act as potential carriers for small guest molecules (Kojima et al., 2000; Liu et al., 2000; Ooya et al., 2004; Namazi and Adeli, 2005). The nature of drug encapsulation within a dendrimer may be simple physical entrapment, or can involve non-bonding interactions with specific structures within the dendrimer. Several types of dendrimer have been investigated for the encapsulation of drugs, including systems designed for triggered release. Some of the dendrimers evaluated for this purpose are listed below;

i). Unimolecular micelles

Dendrimers consisting of an apolar core and polar shell have been referred to as “unimolecular micelles”. Unlike conventional micelles, however, the dendritic structure is independent of dendrimer concentration. The first such structure was ‘arborol’ reported by Newkome et al., (Newkome et al., 1991). The same group also synthesized a symmetrical, four directional saturated hydrocarbon cascade polymer containing 36 carboxylic acid moieties with a neopentyl core. It was shown that lipophilic probes were located within the lipophilic infrastructure of the dendritic structures and it was concluded that the polymers exist as single molecules capable of molecular inclusion and therefore act as unimolecular micelles. Hawker et al. (Hawker et al., 1993) described the synthesis of dendritic polyether unimolecular micelles based on an electron-rich 3,5-dihydroxybenzyl alcohol building block with carboxylate surface groups (Figure 1.9). The dendrimers were able to solubilize a range of polycyclic compounds in water due to π - π interactions, and a relationship was found between the solubilizing power of the dendrimer and electron density of the polycyclic aromatic region. The solubilizing power of the dendrimers was found to be similar in magnitude to that of sodium dodecyl sulfate, but unlike conventional micelles, increased with increase in the concentration of dendrimer at very low concentration, i.e. the

dendrimers did not have a critical micelle concentration (Newkome et al., 1991, Liu, 2000). One of the limitations in the solubilization capacity of unimolecular micelles is a consequence of conformational collapse of the hydrophobic core in water.

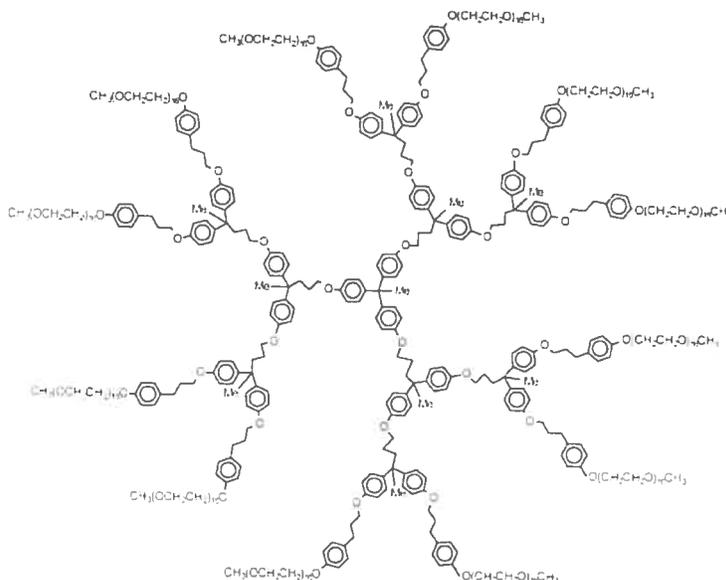


Figure 1.9. Unimolecular micelle consisting of 4, 4-bis(4'-hydroxyphenyl) pentanol core and PEG mesylate shell. Reproduced from (Liu et al., 2000).

ii). Cored dendrimers

Zimmerman and coworkers synthesized cored dendrimers that resemble hollow nanospheres and suggested their potential to encapsulate substances, making them candidates for delivery vehicles (Schultz et al., 2001). This architecture was achieved in dendrimers by post-synthetic modification of the dendritic structure. The core unit in a typical dendrimer is essential for interconnecting the dendrons, or branches of the structure. An alternative approach for maintaining the structural integrity of a dendrimer is to crosslink the peripheral surface groups. In the case of cored dendrimers, structural integrity

is maintained by the latter approach. For instance, Schultz et al., (Schultz et al., 2001) have achieved this by a ring-closing metathesis reaction of the terminal aromatic rings, followed by interlinking of the dendritic wedges and removal of the core by cleavage of ester bonds (Figure 1.10), leaving an empty core available for encapsulation. However, it is not clear, how guest molecules could be loaded into such structures.

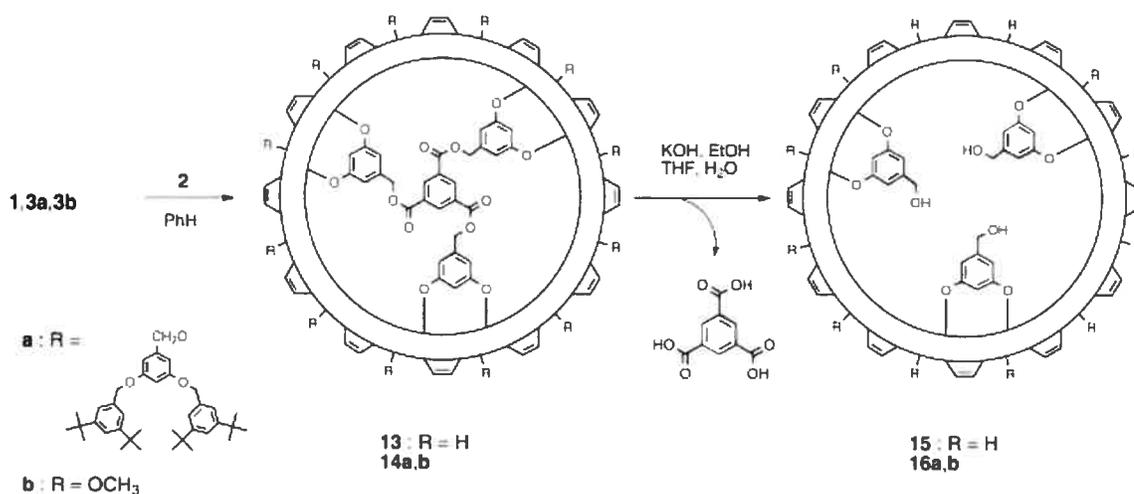


Figure 1.10. Synthesis of cored dendrimers. Reproduced from (Schultz et al., 2001).

iii). Dendritic box

Jansen et al. described the synthesis of dendritic boxes based on poly(propyleneimine) dendrimers (Figure 1.11) (Jansen et al., 1994). Guest molecules could be entrapped within the cavities of the dendritic boxes during the synthetic process, with a dense surface shell preventing diffusion from the structures, even after prolonged heating, solvent extraction or sonication. The rigid shell of these dendrimers can be obtained through end group modification with a bulky amino acid derivative to yield a dense and rigid chiral shell with solid-phase properties and a flexible core capable of entrapping molecules. A number of dye molecules were encapsulated in the dendritic box,

for example, up to 4 molecules of Bengal Rose could be encapsulated per dendrimer (Jansen et al., 1994). The shape selective liberation of guests from dendritic boxes was also described. Hydrolysis of the surface t-BOC groups (with formic acid) of a dendritic box containing 4 molecules of Bengal Rose and 8–10 molecules of 4-nitrobenzoic acid resulted in perforation of the dendrimer allowing the release of 4-nitrobenzoic acid. The larger Bengal Rose molecule remained entrapped and could only be liberated following hydrolysis of the outer shell by 12 mol dm^{-3} HCl under reflux for 2 h. In order to be feasible for drug delivery applications less harsh conditions would be required for cleavage of the dendrimer surface groups.

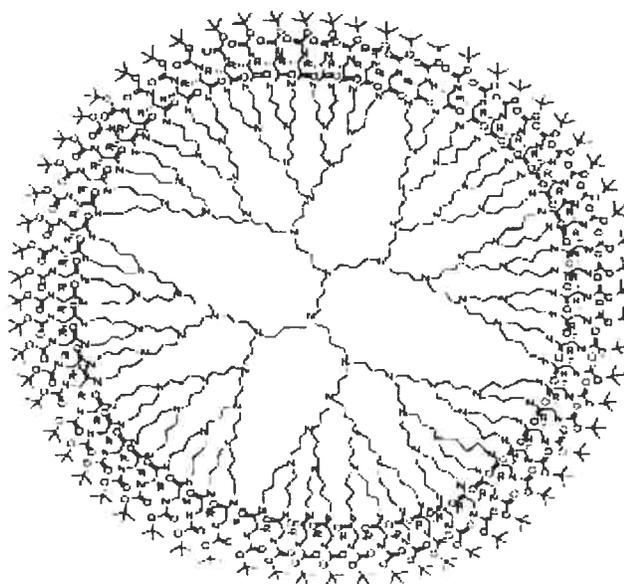


Figure 1.11. Dendritic box of poly(propyleneimine) dendrimers. Reproduced from (Jansen et al., 1994).

iv). Dendrimer-based block copolymers

Dendrimeric di- and tri-block copolymers with linear hydrophilic block(s) and a hydrophobic dendritic block have been synthesized by a number of groups. The self-

association of these amphiphilic molecules in aqueous solution and the ability of the aggregates to solubilize or complex poorly water-soluble molecules have been examined for several of these systems. The hydrophilic blocks of the majority of the dendrimeric block copolymers reported are linear polyethers, usually PEG. A feature of these micelles that distinguishes them from those of classical surfactants is their stability to dilution or prolonged heating, thought to be due in part to the slow establishment of an entanglement–disentanglement equilibrium in the dendritic cores of the micelles as a consequence of the highly branched structure of the end blocks (Choi et al., 1999). Chapman et al. (Chapman et al., 1994) synthesized diblock dendrimers with a PEG hydrophilic block and a hydrophobic block of poly(l-lysine), aqueous solutions of which formed micelles at well-defined critical concentrations (Figure 1.12). Di- and tri-block copolymers with PEG as the linear block and poly(benzyl ether) as the hydrophobic dendritic block have been designed and synthesized by Fréchet et al. (Fréchet et al., 1999). Examination of their solution behaviour by surface tension and light scattering techniques revealed the presence of micelles below a critical concentration and multimolecular micelles at higher concentrations, with the PEG chains forming a hydrophilic corona around the poly(benzyl ether). A series of G1-G5 PAMAM-*block*-PEG-*block*-PAMAM triblock copolymers were synthesized by Kim et al. (Kim et al., 2004) and investigated as potential polymeric gene carriers. The copolymers were shown to form highly water-soluble polyplexes with plasmid DNA, which had high transfection efficiencies. Their self-assembly with DNA was a function of the number of surface primary amines, the internal tertiary amines were unable to participate in complexation because of steric hindrance. However, sizes of micelles formed by these dendritic polymers are large and are typically in the range of 150-190 nm. This size limitation together with the complexity in their synthesis has limited the interest of pharmaceutical scientist in using them as drug delivery carriers.

in low ionic strength aqueous solutions, but rapidly in isotonic solutions. Similar characteristics were found in the release of MTX from non-PEGylated PAMAM dendrimers conjugated with folate. The anticancer drug 5-fluorouracil and the blood schizonticide chloroquine phosphate have been encapsulated in PEGylated PAMAM dendrimers, resulting in increased drug entrapment with an increase in molecular weight of PEG and *versus* non-PEGylated dendrimers.

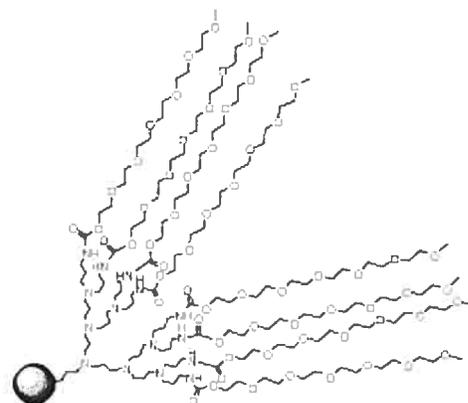


Figure 1.13. PEGylated PAMAM dendrimer with PEG chains attached to the terminal amino groups. Reproduced from (Kojima et al., 2000).

E). Limitations of dendrimers- toxicity and biodistribution

Of the many considerations for *in vivo* use of any molecule, cytotoxicity, hemocompatibility, immunogenicity, and organ accumulation are critical. The biocompatibility and cytotoxicity of these polymers are the major concerns for drug delivery application (Cloninger, 2002).

a). Toxicity

Dendrimer cytotoxicity has been related to generation, concentration, and the chemistry of surface groups. Most widely used dendrimers like PAMAM, poly(propylene imine) and polyaryl ether dendrimers are rendered cytotoxic by virtue of presence of aromatic rings or amino groups at periphery (Jevapresphant et al., 2003). The cytotoxicity and cell permeability of dendrimers has been found to augment with increasing generation and concentration. Malik et al. found that cationic PAMAM dendrimers showed increased toxicity compared to anionic dendrimers (Malik et al., 2000). These results were supported by a study by Chen et al. in which cationic melamine-based dendrimers were much more cytotoxic than PEGylated or anionic dendrimers (Chen et al., 2004). Recent studies have shown that generation 7 cationic PAMAM dendrimers interact with lipid bilayers comprising dimyristoylphosphatidylcholine, causing the formation of 15–40 nm wide holes in the bilayers. The formation of holes in cell membranes creates a disturbance in electrolyte flux, causing cell death. Although the cytotoxicity of a dendrimer is most strongly influenced by its surface charge, a computational study by Pricl and co-workers suggests that cytotoxicity may also be related to the radius of gyration, molecular shape, and dimensions of a dendrimer (Pricl et al., 2003). In a series of PMAM dendrimers, the non-cytotoxic dendrimers were characterized by a dense, globular shape and a smooth surface pattern. Cationic PAMAM dendrimers larger than first generation exhibited generation-dependent hemolysis above a concentration of 1mgmL^{-1} ; however, diaminobutane (DAB) and diaminoethane (DAE) dendrimers did not show generation-dependent hemolytic activity, and anionic PAMAM and DAB showed no hemolysis (Malik et al., 2000).

It has been proposed that the interaction of cationic and anionic dendrimers with serum albumin is the major cause of the immunogenicity. Dendrimers can also trigger the release of cytokines and chemokines, which can cause inflammation and cytotoxicity. Many of the adverse affects displayed by these polymeric macromolecules can be

attenuated through the conjugation of PEG to their surface. PEGylated materials have shown greatly reduced cyto- and hemotoxicity. These effects are attributed to the reduction or shielding of the positive charge on the dendrimer surface by the PEG chains.

b). Biodistribution

The biodistribution of parenterally administered dendrimers has been widely studied due in large part to the development of dendritic-based imaging agents. In general, smaller generation dendrimers show rapid renal elimination, while higher molecular weight dendrimers as well as those with charged or hydrophobic surfaces, are cleared by the liver. Dendrimers bearing hydrophilic or PEGylated surfaces are not cleared rapidly (Gillies et al., 2005). Selective accumulation of dendrimers in tumor tissue may be accomplished through the enhanced permeability and retention effect. The discontinuous endothelium of tumor vasculature (their so-called 'leaky' vasculature), which allows large molecules circulating in the bloodstream to pass through, and the lack of effective lymphatic drainage in tumors together cause the passive accumulation of macromolecules in solid tumor tissue. This may increase the tumor concentration of antitumor drugs up to 70-fold when drug delivery systems are injected i.v..

F). Designing dendrimers based on PEG

The application of polymeric building blocks like poly(caprolactone) and PEG to dendritic macromolecules has been explored (Trollsås and Hedrick, 1998; Trollsås et al., 2000). The concept was developed in order to obtain structures that combine classical properties of linear polymers such as entanglements and crystallinity with those of dendritic macromolecules and in particular, the high functionality resulting from many end groups.

The conventional dendritic polymers are synthesized from small molecular weight units and require a large number of careful synthetic steps and tedious purification methods

(Boas and Heegaard, 2004). The application of polymeric blocks like PEG in dendrimers and other dendritic polymers can offer lot of advantages over usage of small molecular weight monomer units. The use of PEG can facilitate tunability of dendritic properties due its higher molecular weight. Using this approach i.e. by successive grafting of polymeric building blocks, a high molecular weight dendritic polymer can be obtained in only a few steps. This approach is also reported to simplify purification of dendrimers which is otherwise a tedious process (Trollsås et al., 2000). Furthermore, this strategy allows the preparation of architectures with significant variation in polymer morphology by simply varying either the molecular weight (degree of polymerization) of PEG in the different generations, the number of branching points to which PEG chains attached or the generation. In addition, dendritic polymers of high molecular weight can be obtained by using PEG, which improves circulation half-life and drug loading as compared to conventional dendrimers (Gillies et al., 2005). Since, circulation half-life and drug loading are the major factors which can improve therapeutic efficacy of a drug they are of major concern to drug delivery scientist and by using PEG in dendritic structure these issues can be addressed.

1.7. Application of dendrimers in delivery to brain tumors

Although numerous attractive approaches are reported in literature, the clinically available treatments involve either surgical placement of wafers (Gliadel[®]) or pastes at the site of brain tumor (Boiardi et al., 1999). However, such strategies lack patient compliance and are invasive in nature. The challenge is to design therapeutic strategies that deliver drugs to brain without alteration of BBB properties i.e. in a more patient compliant, safe and effective manner. In this context colloidal carriers like liposomes, micelles, nanoparticles and dendrimers are very advantageous because they have small size and thus they can cross BBB (Garcia-Garcia et al., 2005). They can mask and protect the loaded drug from external environment in particular from metabolism and efflux transporters at BBB and also can be targeted to the desired region of brain (Misra et al., 2003). However,

the poor stability of liposome has limited their applicability and market potential. Micelles are generally unstable and dissociate into monomers after administration thus, resulting in loss of the encapsulated drug before reaching the target site. On the other hand, binding ligands to the surface of nanoparticles remains problematic due to the small number of available surface groups and steric hindrance. Dendrimers can provide an interesting alternative to these colloidal carriers for this purpose, because of their highly branched architecture, monodisperse size and presence of internal cavities (Kojima et al., 2000; Aulenta et al., 2003; Boas and Heegaard, 2004). By virtue of the presence of internal cavities they have ability to encapsulate guest molecules like dyes, drugs etc. While the presence of large number of surface groups, provides opportunity to conjugate not only ligands for transport across BBB but also, for specific delivery to the cancerous cells. Additionally, dendrimers have nanometric size, much smaller than nanoparticles and micelles and thus, can also enhance passive uptake of dendrimers across BBB. Because of their small size, they can also provide intracellular delivery of the chemotherapeutic agents and thus, can enhance the efficacy of the therapy. Another most lucrative property of the dendrimers as discussed before is the easy tunability of their properties like architecture, size, solubility etc. (Patri et al., 2002). It is well known that permeability of drug molecules across BBB is dependent on the lipophilicity as well as the size of the molecules. Keeping these factor in consideration, unlike any other drug carrier, dendrimers can be easily be modified to obtain desired lipophilicity and size which will also enhance their permeability across BBB. Thus, dendrimers can serve as a tailorable delivery system for enhanced delivery of drugs to brain.

1.8. Methotrexate

The first cure of metastatic cancer was obtained in 1956 when MTX was used to treat a rare tumor called choriocarcinoma. MTX is one of the oldest and effective chemotherapeutic drugs. It is a folate antagonist first developed for the treatment of

malignancies (Farber et al., 1956) and, subsequently, used in non-neoplastic diseases as an immunosuppressive drug. It acts by binding to enzyme dihydrofolate reductase and inhibiting the formation of tetrahydrofolate, which is required by cells for thymidine synthesis. It also inhibits enzymes for purine synthesis and thus indirectly results in inhibition of DNA synthesis. MTX is currently the most commonly used treatment of chronic inflammatory disorders (Weinblatt et al., 1985) and cancer chemotherapy (Jolivet et al., 1982; Jolivet and Chabner, 1983).

Various drugs are reported to be effective in the treatment of brain tumors like vincristine, cisplatin, BCNU, CCNU, procarbazine, methotrexate, doxorubicin, paclitaxel etc. (Castro et al., 2003). Of these agents cisplatin, BCNU, CCNU can easily penetrate brain (Figure 1.4), while vincristine, doxorubicin and paclitaxel are substrates of P-gp efflux mechanism. MTX is reported to have good activity against brain tumors (Abelson et al., 1981) in particular against metastatic brain cancer (Rustin et al., 1986; Newton, 2002) and lymphomas (Jellinger and Paulus, 1992). (Djerassi et al., 1977) have also reported that high dose of MTX with Citrovorum Factor Rescue is most effective treatment for brain gliomas (glioblastoma multiforme, anaplastic astrocytomas, oligodendroglioma and related tumors). Chemotherapy with this drug and method has treated over 200 patients with brain gliomas. It is noteworthy to mention that in this intensive therapy high dose of MTX is given due to its low permeability across BBB (Zunkeler et al., 1996; Muldoon et al., 2007), high doses result in high plasma concentration and consequently increase drug available in the brain but at the same time high dose also cause toxicity resulting in need for Citrovorum Factor Rescue. This highlights that efficacy of MTX in the treatment of brain cancer is limited due to its low permeability across BBB. The strategies that could increase the permeability of MTX can significantly increase its activity against various brain tumors.

HYPOTHESIS

Most of the systemically administered chemotherapeutic agents do not enter brain in adequate amounts. To counteract this limitation high doses of drugs are administered systemically, however, this causes systemic toxicity and there by compromises the quality of patient life. Other traditional methods like disruption of BBB by various agents also compromise quality of patient life and are highly invasive in nature (Misra et al., 2003). Thus, extensive efforts have been made to develop novel strategies to overcome the obstacles of brain drug delivery. Nevertheless, the delivery approaches available clinically or in clinical trials for delivery of drugs to brain include, intracerebral implantation of controlled release implants, intracerebroventricular infusion, CED, BBBD by osmotic agents etc. These strategies are either highly invasive and compromise BBB properties or have low efficacy due to limited diffusion of drug to the surrounding tissue. Due to these limitations of the conventional delivery methods, brain tumors remain as unsolved clinical problem in spite of decades of research. Thus, there is a need for multi-functional carrier that can be engineered into a single nano-platform such that it can carry drug, cross BBB and target the tumors. As discussed before, the delivery of various drugs by nanoparticles have shown promising results. The conjugation of vectors to nanoparticles would further facilitate and provide selective delivery of drugs to brain. However, binding of ligands/vectors to the surface of nanoparticles remains problematic. In this direction, dendrimers can serve as a versatile targeting platform due to their unique structural and functional advantages originating from the multiple surface groups that can be used for conjugating multi-functional ligands and from the presence of internal voids in which drugs can be easily encapsulated or complexed (Klajnert and Bryszewska, 2001; Boas and Heegaard, 2004). It has been reported that PEGylation increases permeability of nanoparticles into the brain (Calvo et al., 2001). Thus, dendrimers with PEG chains at the surface can aid in increasing the delivery to brain. Therefore, in this project the improved

delivery to brain would be obtained by synthesizing dendrimers with PEG chains on the surface. Additionally, in an attempt to maintain lipophilicity on the surface of dendrimer, PEG methacrylate with free allyl group (which is known to be hydrophobic) would be used as the surface group. The presence of PEG would also improve circulation half-life of dendrimers and hence, reduce the toxicity and immunogenicity of these dendrimers (Bhadra et al., 2002). One of the major limitations of dendrimers is low drug loading as compared to other delivery systems. Since, it is important to deliver high payloads of drug to the tumor, attempts would be made in this project to design the dendrimers with ability to encapsulate large amount of drug. Poly(ethylene oxide) (PEO) is a commonly utilized hydrotropic agent to enhance the solubility of poorly water soluble drugs. Based on this understanding, we hypothesized that introduction PEO in the cavity of the dendrimer would not only increase the size of the cavity but also provide hydrotropic interior with ability to solubilize both water soluble and poorly water soluble compounds. Thus, by using PEO in the dendrimer cavity we intended to enhance the encapsulation of drugs.

OX-26, an antibody to transferrin receptor is one of the most commonly used ligand for brain targeting. RME is one of the major ports of entry into the brain (Pardridge, 1999). However, it has been reported that immunoliposomes with OX-26 have poor permeability across BBB due to their large size. Therefore, small targeting ligands which target the receptors highly expressed on BBB would be an ideal targeting moiety. Glucose transporter GLUT-1, is one such transporter found in high density on BBB (Pardridge et al., 1990). GLUT-1 is also known to be over-expressed on the brain tumors. Targeting to various tumors by the glucose transporters has been successfully done for positron emission tomography, magnetic resonance contrast imaging (Luciani et al., 2004) and gene targeting. Glucose conjugation to the delivery system confers tumor-targeting property through facilitative glucose metabolism by the glucose transporters in the tumors. Thus, glucose can be used not only for enhanced delivery across BBB but also for targeting to the brain tumors. Considering the advantage of dual targeting using the same ligand and synthetic

simplicity of conjugating a single ligand, glucose was used as a targeting moiety in the present work.

MTX, a dihydro folate (DHF) reductase inhibitor was selected as a model anticancer agent because it has good activity against various brain tumors, additionally it has low BBB permeability (Zunkeler et al., 1996). The strategies that could increase the permeability of MTX can significantly increase its activity against various brain tumors and thus, would demonstrate the ability of the dendrimers to deliver drug across BBB into the brain tumors.

OBJECTIVE

The objective of this project was to design and evaluate dendritic nanocarriers for the delivery of MTX across the BBB. The ability of these MTX loaded dendrimers in the treatment of gliomas was also established. The specific aims of the project were:

- a) Synthesis and characterization of the dendrimers with different generation and surface groups
- b) Optimization of the loading method for encapsulation of drugs in these dendrimers
- c) Compare encapsulation efficiency and release characteristics of all the synthesized dendrimers
- d) Determine toxicity and plasma protein adsorption on dendrimers
- e) Conjugation of D-glucosamine ligand to the dendrimers
- f) Evaluation of the growth inhibition potential of MTX loaded dendrimers for MTX sensitive and resistant glioma cells
- g) Assessment of the ability of these dendrimers to cross BBB and deliver MTX using an *in vitro* BBB model
- h) Evaluation of the efficacy of MTX loaded dendrimers in inhibiting growth of avascular human glioma tumor spheroids
- i) Determining ability of dendrimers to deliver drug to the hypoxic regions of solid tumors by using avascular human glioma tumor spheroids

1.9. References

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CHAPTER ONE

RESEARCH PAPER

Synthesis and evaluation of novel dendrimers with hydrophilic interior as nanocarriers for drug delivery

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2.1. Abstract

Novel polyether-co-polyester dendrimers consisting of hydrophilic core were synthesized by the combination of convergent and divergent synthesis. The core was synthesized from biocompatible moieties, butane tetracarboxylic acid and aspartic acid, and the dendrons from PEO (poly (ethylene oxide)), dihydroxy benzoic acid or gallic acid and PEG (poly ethylene glycol) monomethacrylate. The dendrimers, den-1-(G2) (second generation dendrimer-1) and den-2-(G2) (second generation dendrimer-2) consisting of 16 and 24 allyl surface groups, respectively, were obtained by coupling the dendrons to the core. The dendrimer (Den-1-(G2)-OH) with hydroxyl groups at the surface was synthesized by the oxidation of allyl functional groups of den-1-(G2), which was divergently coupled to the dendrons to obtain third generation dendrimer den-1-(G3), consisting of 32 surface groups. The modifications in surface groups and generation of dendrimers were shown to influence the shape of dendrimers in the AFM studies. The aggregation as well as self assembly of dendrimers was observed at high concentration in water by light scattering studies, however, it was reduced on dilution and in the presence of sodium chloride. Dendrimers demonstrated good ability to encapsulate guest molecule, with loading of 15.80 and 6.47 % w/w for rhodamine and β -carotene, respectively. UV spectroscopy proved the absence of any π - π complexation between the dendrimer and encapsulated compounds. ^1H NMR and FTIR studies showed that the physical entrapment and/or hydrogen bonding by PEO in the interior and branch of the dendrimer are the mechanisms of encapsulation. The release of the encapsulated compounds was found to be slow and sustained, suggesting that these dendrimers can serve as potential drug delivery vehicles.

Keywords: dendrimer, synthesis, encapsulation, rhodamine, β -carotene, mechanism, release

2.2. Introduction

The discovery and development of new and potent drug molecules is a time consuming and costly process. It is estimated that every new drug takes 12 to 15 years to develop, at an approximate cost of \$ 800 million (Bolten and Degregorio, 2002; Dimasi et al., 2003). A more economical and viable strategy is to devise effective delivery systems for drugs that have failed to provide optimum therapeutic benefit. It is stated that controlled release of drug at specific target can significantly improve the effectiveness of drug and thereby increase therapeutic benefit (Langer, 1998). Two most common delivery systems that have been explored for this purpose are liposomal and polymeric systems. The poor stability of liposome based delivery systems has limited their applicability and market potential. On the other hand, polymeric systems remain one of the most widely sought delivery vehicles by virtue of their high stability. Furthermore, advances in the polymer chemistry have constantly provided avenues to the drug delivery scientists to explore these polymeric architectures for providing more efficient drug delivery. Various categories of polymeric materials that have been discovered in the past century include linear, cross-linked, branched and dendritic polymers (Tomalia and Frechet, 2002). Among these polymeric scaffolds, dendrimers have well defined nanoscopic dimensions, with highly branched structure and discrete number of end functional groups. These characteristics endow them with many intriguing properties like globular shape (resembling proteins), monodisperse size, and presence of internal cavities. By virtue of these properties, evaluation and application of dendrimers in drug delivery is becoming one of the most lucrative areas of drug research.

Dendrimers can be used to deliver drug either by encapsulating the drug in internal cavity or by conjugating it to surface functionalities (Esfand and Tomalia, 2001). Since, the conjugation of drug to polymers is time consuming process and could sometimes result in decreased therapeutic benefit (due to failure of conversion to parent drug or decreased permeability of the conjugate) (Fix et al., 1990; Denny, 2004) the encapsulation of drug by

dendrimers seems to be more attractive approach. Studies in past have shown that they can act as potential carriers for small guest molecules (Kojima et al., 2000; Liu et al., 2000; Ooya et al., 2004; Namazi and Adeli, 2005). However, the biocompatibility and cytotoxicity of these polymers are the major concerns for drug delivery application. Most widely used dendrimers like PAMAM, poly(propylene imine) and polyaryl ether dendrimers are rendered cytotoxic by virtue of presence of aromatic rings or amino groups at periphery. The covalent attachment of poly(ethylene glycol) (PEG) to the PAMAM dendrimers has shown to reduce the cytotoxicity (Jevapresphant et al., 2003). PEGylation of dendrimers can also significantly reduce immunogenicity and anti-genecity by shielding the system from recognition by the defense mechanism of the body. It also prevents sequestration and uptake by reticulo-endothelial system (Bhadra et al., 2002) and thereby prolongs circulation time in the body. Another major concern on application of these dendrimers to drug delivery is the presence of amidoamine and ether linkages that are inherently resistant to degradation and hence, limits their biodegradability. Incorporation of hydrolysable linkages like ester bond is of particular interest to increase biodegradability of these delivery vehicles (Jesus et al., 2002).

It is a rationale in drug delivery that presence of hydrophobic core is essential for encapsulating hydrophobic drug molecules and in general, polymeric micelles are tailored based on this concept. However, recently Ooya et al., synthesized polyglycerol dendritic polymers and reported three fold increase in the water solubility of paclitaxel (Ooya et al., 2004). Due to the absence of hydrophobic region in the dendrimer, hydrotropic solubilization was proposed to be the probable mechanism of solubilization of paclitaxel. PEG is a biocompatible polymer, utilized as a *cosolvent* or hydrotropic agent (Mora et al., 2003) to enhance the solubility of poorly water soluble drugs (Sugimoto et al., 1998). There are number of reports in literature on the use of PEG grafts of various dendrimers to increase the encapsulation of drugs (Liu et al., 1999; Kojima et al., 2000; Bhadra et al., 2003; Yang et al., 2004). It is also reported that PEG 400 increases the aqueous solubility of β -estradiol by 4 to 5 fold at high concentrations (Groves et al., 1984). Based on these

observations, we hypothesized that introduction PEO in the cavity of the dendrimer will not only increase the size of the cavity but also will provide dendrimers' ability to solublize both water soluble and poorly water soluble compounds, in contrast to drug carriers with hydrophobic core, which have preferential ability to solublize hydrophobic compounds. Thus, we intended a new approach to enhance solubility of compounds as well as the encapsulation of drugs by using PEO in the dendrimer cavity.

The aim of this study was to synthesize dendritic nanocarriers with hydrophilic interior/cavity composed of PEO as drug delivery vehicles. Dendrimers were synthesized using butane tetracarboxylic acid as core molecule and composed of allyl or hydroxyl groups as surface functionalities. The synthesized dendrimers were characterized for their chemical and physical properties. Their ability to act as drug carrier was evaluated by encapsulating rhodamine and β -carotene as model hydrophilic and hydrophobic compounds, respectively. Further, the mechanism of encapsulation of compounds by dendrimer was also studied.

2.3. Materials

Rhodamine, β -carotene, 1,2,3,4 butane tetracarboxylic acid, L-aspartic acid, PEG (M_n 400), PEG monomethacrylate (M_n 360), 3,5 dihydroxy benzoic acid (DHBA), 4-(dimethyl amino) pyridine (DMAP), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), gallic acid, hydroxy benztriazole (HOBT), Borane-tetrahydrofuran complex ($BH_3 \cdot THF$, IM), p-toluene sulfonic acid monohydrate (TsOH), chromium trioxide (CrO_3), hydrogen peroxide (H_2O_2), triethyl amine, sodium chloride, sodium hydroxide (NaOH), urea, activated carbon 4-14 mesh granular, and filter aid Celite[®]521 were purchased from Sigma-Aldrich Canada (Oakville, ON). Anhydrous pyridine, N,N-dimethyl formamide (DMF), dimethyl sulfoxide-d₆ (99.9 % D) (DMSO-d₆), and $CDCl_3$ (99.9% D) were supplied from Sigma-Aldrich Canada., (Oakville, ON). Acetone, dichloromethane,

methanol, chloroform, anhydrous diethyl ether, tetrahydrofuran (THF), sulfuric acid and silica gel (70-230) were purchased from Laboratoire Mat. (Montreal, PQ). And thionyl chloride was purchased from A & C chemicals Ltd., (Montreal, PQ). THF was dried over sodium while dichloromethane was dried with molecular sieves; all other solvents were used without purification unless specified otherwise.

2.4. Experimental procedures

2.4.1. Characterization techniques

^1H NMR and ^{13}C NMR spectra were recorded with Bruker ARX400 NMR spectrometer (Bruker Biospin, Billerica, MA) at 400 MHz using tetramethyl silane (TMS) as internal reference. FTIR spectroscopy was done using Bruker vector 22 spectrometer (Bruker Optik GmbH, Ettlingen, Germany) and the matrix associated laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy of dendrimers was done on Bruker Autoflex[®] (Bruker Daltonik GmbH, Bremen, Germany) in linear, positive ion mode using dithranol as matrix and sodium iodide as cationizing agent. The molecular weights of dendrimers were also determined by gel permeation chromatography (GPC) with Waters[®] system (Waters, Milford, MA) equipped with refractive index detector, using DMF as mobile phase at flow rate of 1 ml/min.

2.4.2. Synthesis of the core (3):

1,2,3,4 butane tetracarboxylic acid (**1**) (5.00 g, 0.021 mol) was dried in vacuum for 24 h. Thionyl chloride (5 mL) was added drop wise to the reactant over a period of 15 min, followed by reflux at 50°C for 6 h under nitrogen atmosphere. Subsequently, thionyl chloride was removed by evaporation under reduced pressure and the product was dried under vacuum for 48 h to obtain compound **2**. L-aspartic acid (7.71 g, 0.058 mol) was

added to compound **2** (4.50 g, 0.0144 mol), the flask was purged with nitrogen for 15 min to displace any moisture. Anhydrous pyridine (20 mL) was added dropwise at room temperature under nitrogen atmosphere. The above solution was refluxed at 60°C for 12 h. Pyridine was evaporated by rotaevaporation and the white product obtained was purified by column chromatography using dichloromethane: acetone: water (1:2:0.5). The solvents were evaporated under reduced pressure and product obtained was dried under vacuum for 48 h.

Yield: 90% ; ¹HNMR (δ, ppm, D₂O): 5.52 (q, 4H, -CH-NH-), 4.93 (d, 2H, -NH-CO-), 4.91 (d, 2H, -NH-CO-), 3.92 (q, 2H, -CH-CO-), 3.30 (d, 8H, -CH₂-COOH), 2.85 (d, 4H, -CH₂-CO); ¹³CNMR (δ, ppm, D₂O): 174.94, 174.11, 173.44, 42.83, 34.15, 33.87, 14.75; Elemental Analysis, calculated (%): C (41.51), H (4.35), N (8.07); observed (%): C (41.53), H (4.80), N (8.65).

2.4.3. Synthesis of poly(oxyethylene) monomethacrylate carboxylic acid (**4**):

PEG monomethacrylate (20 g, 0.055 mol) was dissolved in 200 mL of acetone at room temperature. The solution was cooled to 0°C by placing the flask in ice bath. Later, 9 mL Jones's reagent (containing 0.02M CrO₃) was added dropwise over a period of 15 min. The flask was removed from ice bath after 30 min and reaction was allowed to occur at room temperature for 18 h. The reaction was quenched by adding 5 mL of isopropyl alcohol. Subsequently, 2 g of finely powdered activated carbon (10% w/w) was added and stirred for 2 h. This was filtered over wet Celite[®] and the filtrate was evaporated on rotavapour. The viscous liquid obtained was dissolved in water and extracted with dichloromethane, the extracts were combined and evaporated to obtain the product which was dried under vacuum for 48 h.

Yield: 95%; ¹HNMR (δ, ppm, DMSO): 6.02 (s, 1H, *trans* CH₂=CH-), 5.64 (s, 1H, *cis* CH₂=CH-), 4.18 (t, 2H, -CH₂-), 3.62 (t, 2H, -CH₂-), 3.46 (m, 24H, -O-CH₂-CH₂-O-),

1.83 (s, 3H, CH₃); ¹³CNMR (δ, ppm, DMSO): 167.15, 159.40, 73.45, 70.80, 69.28, 64.70, 61.70, 18.18. Elemental Analysis, calculated (%): C (51.66), H (8.39); observed (%): C (51.65), H (8.88). FTIR (neat) ν_{max} cm⁻¹: 2883, 1753, 1467, 1358, 1279, 1239, 1145, 1101, 1062, 967, 842, 528.

2.4.4. Synthesis of POE-DHBA (6):

Compound 4 (11.00 g, 0.029 mol) was purged with nitrogen for approximately 15 min and thionyl chloride was added drop wise at room temperature. It was then refluxed at 50°C for 7 h. Thionyl chloride was evaporated under reduced pressure and viscous liquid obtained was dried under vacuum to obtain compound 5. Compound 5 (11.00 g, 0.028 mol) was dissolved in pyridine under nitrogen atmosphere and to this solution DHBA (1.96 g, 0.0127 mol) was added. Reactants were refluxed at 50°C for 18 h. Thereafter, pyridine was evaporated by rotavapour and product was precipitated in diethyl ether. It was further purified by gel chromatography (Sephacrose CL-4B) using water at pH 7.4 as eluent. The product was recovered as viscous liquid by freeze drying.

Yield: 93%; ¹HNMR (δ, ppm, DMSO): 6.77 (d, 2H, -CH-aromatic), 6.40 (t, 1H, -CH-aromatic), 6.00 (s, 2H, *trans* CH₂=CH-), 5.65 (s, 2H, *cis* CH₂=CH-), 4.15 (t, 4H, -CH₂-), 3.63 (t, 8H, -CH₂-), 3.50 (m, 54H, -O-CH₂-CH₂-O-), 3.42 (t, 4H, -CH₂-), 3.39 (t, 4H, -CH₂-), 3.37 (t, 4H, -CH₂-), 1.85 (s, 6H, CH₃); ¹³CNMR (δ, ppm, DMSO): 168.41, 167.55, 159.44, 147.13, 142.80, 136.80, 133.41, 128.29, 108.31, 73.33, 70.78, 69.26, 64.76, 61.19, 18.99. Elemental Analysis, calculated (%): C (53.75), H (7.51); observed (%): C (54.29), H (7.43). FTIR (neat) ν_{max} cm⁻¹: 2936, 2872, 1713, 1599, 1540, 1453, 1296, 1097, 1004, 944, 860, 772, 682, 608, 519.

2.4.5. Synthesis of dendron 1, POE-DHBA-PEG (7):

Compound **6** (5 g, 5.77 mmol) was dissolved in dichloromethane, followed by addition of PEG (400) (2.8 g, 7.00 mmol) and TsOH (1.14 g, 5.99 mmol). Dean-stark trap was placed on the mouth of the flask and it was connected to reflux condenser. The reaction was carried out at 50°C for 24 h. Solvent was evaporated under reduced pressure and the product was precipitated in diethyl ether. Subsequently, it was purified by column chromatography using mobile phase composed of dichloromethane, ethanol and acetic acid (2:1:0.05). The product was further purified by dialysis against deionized water for 72 h (Spectra/Por no.1 dialysis tubing, MWCO 1000 Da). The dialysate was freeze dried to obtain the product (Yield: 79%).

Another method was also employed to synthesize dendron 1. In this method, compound **6** (8 g, 0.0092 mol) was dissolved in dichloromethane, later, PEG (400) (4.4 g, 0.011 mol), EDC (2.76 g, 0.014 mol), DMAP (0.15 g, 0.0012 mol) and HOBT (0.2025 g, 0.0015 mol) were added to the flask. Reaction was allowed to occur for 24 h at room temperature. The precipitate of EDCU (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide urea) was removed by filtration and solvent was evaporated. The precipitate was purified by using the same procedure as mentioned in the previous method and was freeze dried to obtain the product (Yield: 88%). The later method was found to improve the product yield as well as purity, thus, it was used for other synthetic steps.

¹HNMR (δ , ppm, DMSO): 6.81 (d, 2H, -CH-aromatic), 6.48 (t, 1H, -CH-aromatic), 6.01 (s, 2H, *trans* CH₂=CH-), 5.67 (s, 2H, *cis* CH₂=CH-), 4.42 (s, 4H, -CH₂-), 4.15 (t, 4H, -CH₂-), 3.69 (t, 4H, -CH₂-), 3.65 (t, 8H, -CH₂-), 3.49 (m, 98H, -O-CH₂-CH₂-O-), 3.45 (t, 4H, -CH₂-), 3.43 (t, 4H, -CH₂-), 2.07 (t, 1H, OH), 1.85 (s, 6H, CH₃); ¹³CNMR (δ , ppm, DMSO): 168.41, 167.54, 159.45, 146.94, 142.92, 136.81, 133.41, 128.24, 108.31, 73.34, 70.79, 69.26, 64.76, 64.61, 62.99, 61.19, 53.95, 51.93, 18.19. Elemental Analysis, calculated (%): C (53.99), H (8.11); observed (%): C (53.09), H (8.39). FTIR (neat) ν_{\max}

cm⁻¹: 2933, 2873, 1716, 1598, 1540, 1453, 1295, 1185, 1128, 1003, 946, 862, 815, 771, 680, 597.

2.4.6. Synthesis of den-1-(G2) (8):

Den-1-(G2) was synthesized by coupling core with dendron 1. Briefly, compound 7 (2.02 g, 1.60 mmol) was dissolved in DMF, to this, compound 3 (0.124 g, 0.18 mmol), EDC (0.35 g, 1.83 mmol) and DMAP (0.18 g, 1.48 mmol) were added. The reaction was allowed to occur for 96 h at room temperature. The EDCU precipitate was filtered and the filtrate was precipitated in diethyl ether. The product obtained was dialyzed (Fisher Scientific dialysis tubing, MWCO 3500 Da) against deionized water for 72 h and the dialysate was freeze dried.

Yield: 75%; ¹HNMR (δ, ppm, DMSO): 8.27 (d, 16H, -CH-, aromatic), 7.02 (t, 8H, -CH-, aromatic), 6.03 (s, 16H, trans CH₂=CH-), 5.69 (s, 16H, cis CH₂=CH-), 4.36 (s, 2H, -NH-CO-, core), 4.34 (s, 2H, -NH-CO-, core), 4.19 (t, 32H, -CH₂-), 4.14 (t, 32H, -CH₂-), 4.07 (t, 64H, -CH₂-), 3.93 (s, 4H, -CH-NH-, core), 3.79 (t, 32H, -CH₂-), 3.65 (m, 32H, -CH₂-), 3.42 (m, 386H, -O-CH₂-CH₂-O-), 3.19 (t, 32H, -CH₂-), 2.95 (2H, -CH-CO-, core), 2.20 (s, 8H, -CH₂-CO-, core), 2.17 (s, 4H, -CH₂-, core), 1.88 (s, 6H, CH₃); ¹³CNMR (δ, ppm, DMSO): 165.39, 165.31, 164.64, 164.58, 159.76, 159.68, 152.60, 152.19, 131.69, 131.24, 126.85, 122.41, 115.83, 108.76, 71.57, 70.80, 69.21, 64.76, 61.91, 61.22, 44.61, 26.91, 19.01. Elemental Analysis, calculated (%): C (52.61), H (7.88), N (0.70); observed (%): C (51.97), H (8.34), N (0.80). FTIR (neat) ν_{max} cm⁻¹: 2935, 2870, 1728, 1649, 1540, 1454, 1350, 1100, 947, 878, 771.

2.4.7. Synthesis of den-1-(G2)-OH (9):

The allyl surface functional groups in dendrimer (Den-1-(G2)) were oxidized to hydroxyl group to obtain den-1-(G2)-OH. For this purpose, compound 8 (0.1 g, 12.9 μmol)

was dissolved in mixture of DMF and THF (1:20; dried over molecular sieves) at room temperature. Later, the solution was cooled to 0°C by placing the flask in ice bath. Thereafter, IM BH₃.THF (12.9 μmol) was added dropwise over a period of 5 min and contents were stirred at 0°C for another 15 min. The reaction was allowed to occur at room temperature for 18 h. Excess of reagent was hydrolyzed by addition of water, and 3N NaOH (11.6 μmol) was added. After 15 min 30% H₂O₂ (25.8 μmol) was added dropwise, and the solution was stirred for another 5 h. The product was purified by dialysis (MWCO 3500 Da) against deionized water for 72 h.

Yield: 85%; ¹HNMR (δ, ppm, DMSO): 7.67 (d, 16H, -CH-, *aromatic*), 7.18 (s, 8H, -CH-, *aromatic*), 4.47 (s, 16H, -OH), 3.90, 3.79 (m, 379H, -O-CH₂-CH₂-O-), 3.61 (s, 4H, -CH-NH-, *core*), 3.57 (t, 64H, -CH₂-), 2.91 (s, 2H, -CH-CO-, *core*), 2.90 (s, 8H, -CH₂-CO-, *core*), 2.13 (s, 4H, -CH₂-, *core*), 1.37 (d, 48H, CH₃); ¹³CNMR (δ, ppm, CD₃OD and pyridine-D5): 164.73, 160.23, 159.97, 153.05, 15.84, 145.14, 131.78, 114.91, 104.43, 79.87, 75.84, 70.92, 68.06, 65.08, 59.11, 54.65, 30.93, 30.11, 23.72. Elemental Analysis, calculated (%): C (50.78), H (7.99), N (0.67); observed (%): C (50.91), H (8.07), N (1.30). FTIR (neat) ν_{max} cm⁻¹: 3280, 2949, 2873, 2360, 1917, 1868, 1835, 1732, 1649, 1599, 1543, 1513, 1451, 1291, 1188, 1132, 1083, 1000, 947, 867, 807, 770, 673.

2.4.8. Synthesis of den-1-(G3) (10):

Compound **9** (0.10 g, 0.013 mmol) was dissolved in DMF, compound **6** (0.22 g, 0.28 mmol), EDC (0.07 g, 0.36 mmol) and DMAP (0.028 g, 0.23 mmol) were added to this solution. Reaction was allowed to occur for 96 h at room temperature. The EDCU precipitate was filtered and the filtrate was dialyzed (MWCO 3500 Da) against deionized water for 72 h. The product obtained by precipitation of dialysate in diethyl ether was dried under reduced pressure.

Yield: 81%; ^1H NMR (δ , ppm, DMSO): 6.97 (d, 32H, $-\text{CH}-$, aromatic), 6.51 (t, 32H, $-\text{CH}-$, aromatic), 6.00 (s, 64H, *trans* $\text{CH}_2=\text{CH}-$), 5.66 (s, 64H, *cis* $\text{CH}_2=\text{CH}-$), 4.35 (t, 64H, $-\text{CH}_2-$), 4.16 (t, 64H, $-\text{CH}_2-$), 3.61 (t, 128H, $-\text{CH}_2-$), 3.44 (m, 1239H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$), 3.01 (t, 64H, $-\text{CH}_2-$), 1.84 (s, 192H, CH_3); ^{13}C NMR (δ , ppm, DMSO): 163.18, 163.16, 162.15, 158.27, 158.25, 144.33, 130.18, 130.16, 121.91, 107.25, 89.43, 87.79, 75.30, 69.38, 67.76, 64.25, 49.98, 23.92, 17.52. Elemental Analysis, calculated (%): C (54.05), H (7.63), N (0.27); observed (%): C (55.47), H (6.15), N (0.60). FTIR (neat) ν_{max} cm^{-1} : 3334, 2949, 2874, 2361, 2335, 1916, 1867, 1835, 1792, 1738, 1648, 1598, 1549, 1514, 1452, 1341, 1289, 1187, 1130, 1082, 1000, 948, 863, 810, 770, 672, 585, 514.

2.4.9. Synthesis of POE-gallate (11):

Gallic acid (1.44 g, 0.0085 mol) was added to compound **5** (11 g, 0.028 mol) and flask was purged with nitrogen to remove any moisture. Pyridine was added dropwise to the flask under nitrogen atmosphere and contents were refluxed at 50°C for 18 h. Pyridine was evaporated under reduced pressure after completion of the reaction and product was precipitated in diethyl ether. Finally, the product was dried in vacuum for 48 h.

Yield: 92%; ^1H NMR (δ , ppm, DMSO): 6.92 (s, 2H, $-\text{CH}-$ aromatic), 6.02 (s, 3H, *trans* $\text{CH}_2=\text{CH}-$), 5.67 (s, 3H, *cis* $\text{CH}_2=\text{CH}-$), 4.18 (t, 6H, $-\text{CH}_2-$), 3.81 (t, 6H, $-\text{CH}_2-$), 3.62 (m, 70H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$), 1.86 (s, 9H, CH_3); ^{13}C NMR (δ , ppm, DMSO): 167.60, 146.39, 145.96, 143.98, 136.80, 128.75, 127.83, 126.87, 109.85, 71.55, 70.79, 69.26, 65.31, 64.76, 61.21, 18.96. Elemental Analysis, calculated (%): C (52.96), H (7.69); observed (%): C (51.23), H (7.12). FTIR (neat) ν_{max} cm^{-1} : 2871, 1714, 1634, 1488, 1451, 1349, 1318, 1297, 1247, 1171, 1097, 1037, 945, 851, 775, 684, 579, 523.

2.4.10. Synthesis of dendron 2, POE-gallate-PEG (12):

Compound **11** (5 g, 4.1 mmol) was dissolved in dichloromethane, followed by addition of PEG (400) (1.85 g, 4.6 mmol), EDC (1.33 g, 6.9 mmol), DMAP (0.55 g, 4.5 mmol) and HOBT (0.061 g, 0.45 mmol). Reaction was allowed to occur for 48 h at room temperature, afterward the EDCU precipitate was filtered and filtrate was concentrated by rotaevaporation. The product obtained was precipitated in hexane and diethyl ether mixture (1:1). Finally, it was subjected to dialysis against deionized water for 72 h (MWCO 1000 Da). The product was obtained by freeze drying of dialysate.

Yield: 86%; ^1H NMR (δ , ppm, DMSO): 6.89 (s, 2H, -CH-aromatic), 6.00 (s, 3H, *trans* $\text{CH}_2=\text{CH}$ -), 5.66 (s, 3H, *cis* $\text{CH}_2=\text{CH}$ -), 4.19 (t, 4H, - CH_2 -), 4.03 (t, 6H, - CH_2 -) 3.77 (t, 12H, - CH_2 -), 3.46 (m, 106H, -O- CH_2 - CH_2 -O-), 1.85 (s, 9H, CH_3); ^{13}C NMR (δ , ppm, DMSO): 167.36, 151.53, 149.53, 147.46, 146.51, 143.31, 136.55, 126.74, 110.13, 72.05, 70.62, 69.08, 64.92, 64.60, 64.51, 62.89, 61.08, 52.82, 50.80, 26.32. Elemental Analysis, calculated (%): C (52.34), H (7.43); observed (%): C (52.51), H (7.42). FTIR (neat) ν_{max} cm^{-1} : 2870, 1715, 1635, 1540, 1507, 1488, 1456, 1349, 1297, 1096, 1032, 944, 844, 757, 682, 567.

2.4.11. Synthesis of den-2-(G2) (13):

For synthesis of den-2-(G2), compound **12** (1.8 g, 1.11 mmol) was dissolved in DMF and compound **3** (0.083 g, 0.12 mmol) was added to it. Later, EDC (0.27 g, 1.41 mmol) and DMAP (0.12 g, 0.98 mmol) were added. The reaction was allowed to occur at room temperature for 96 h. Subsequently, EDCU precipitate was filtered and the filtrate was dried under reduced pressure. The product obtained was precipitated in diethyl ether and dried under reduced pressure. It was later dialyzed (MWCO 3500 Da) against deionized water for 72 h, the dialysate was freeze dried to obtain the dendrimer.

Yield: 73%; ^1H NMR (δ , ppm, DMSO): 7.29 (s, 16H, -CH-aromatic), 5.57 (s, 24H, *trans* CH₂=CH-), 5.52 (s, 24H, *cis* CH₂=CH-), 3.48 (t, 16H, -CH₂-), 3.33 (m, 755 H, -O-CH₂-CH₂-O-), 2.87 (s, 8H, -CH₂-CO-, *core*), 2.71 (s, 4H, -CH-CH₂-CO-, *core*), 1.85 (s, 4H, -CH₂-, *core*), 1.69 (s, 2H, -CH-, *core*), 1.47 (t, 72H, CH₃). ^{13}C NMR (δ , ppm, DMSO): 167.56, 165.32, 163.44, 163.65, 151.66, 149.48, 147.26, 146.31, 143.45, 136.61, 126.82, 72.25, 70.82, 69.11, 65.02, 64.73, 64.65, 63.00, 61.16, 53.05, 50.95, 26.75, 18.93. Elemental Analysis, calculated (%): C (52.34), H (7.17), N (0.55); observed (%): C (52.41), H (7.25), N (1.11). FTIR (neat) ν_{max} cm⁻¹: 2932, 2361, 1715, 1677, 1560, 1508, 1453, 1319, 1262, 1175, 1082, 1029, 947, 890, 754, 647, 540.

2.4.12. Determination of acid value of the core

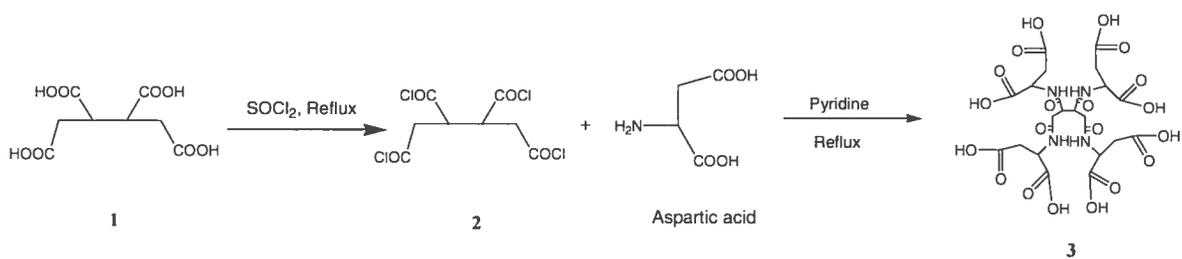
The acid value of core was determined by classical titration method. In brief, approximately 0.2 g of the compound was dissolved in 50 mL of water. The solution was titrated with 0.1N NaOH using phenolphthalein as an indicator. The method was standardized using monocarboxylic acid (hexanoic acid), dicarboxylic acid (oxalic acid, succinic acid) and tetracarboxylic acid (butane tetracarboxylic acid) prior to determination of acid value of the core. The theoretical acid value for a compound was obtained by normalizing molecular weight of compound by number of carboxylic groups.

Acid value represents milliequivalents of NaOH per mg of compound; it was calculated using the following equation;

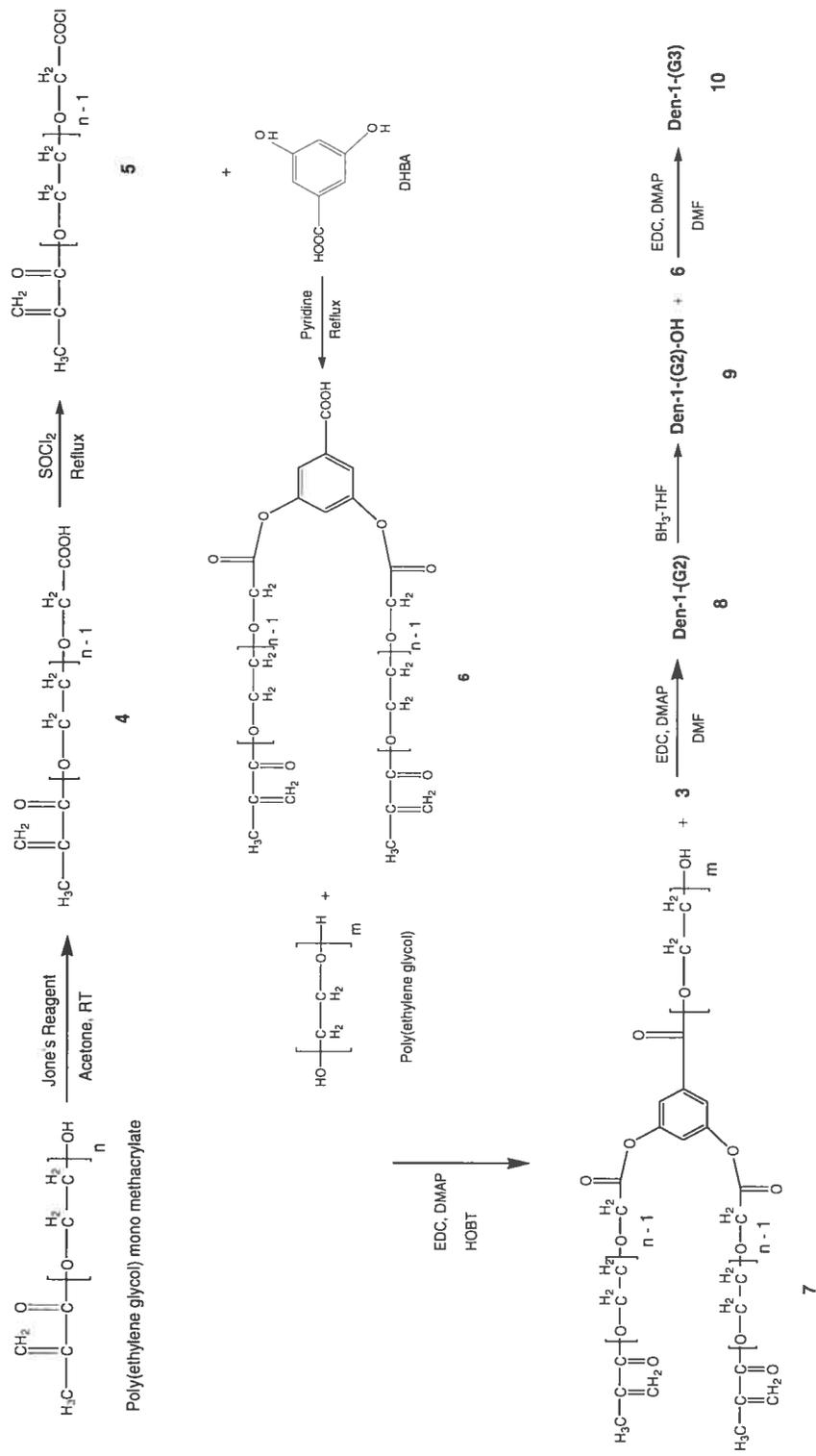
$$\text{Acid value} = (\text{Weight of acid}) / (\text{NaOH normality}) * (\text{Volume of NaOH}) \dots\dots\dots\text{Equation 1}$$

Where, weight of acid used in titration is expressed in mg and volume of NaOH used in titration is expressed in mL.

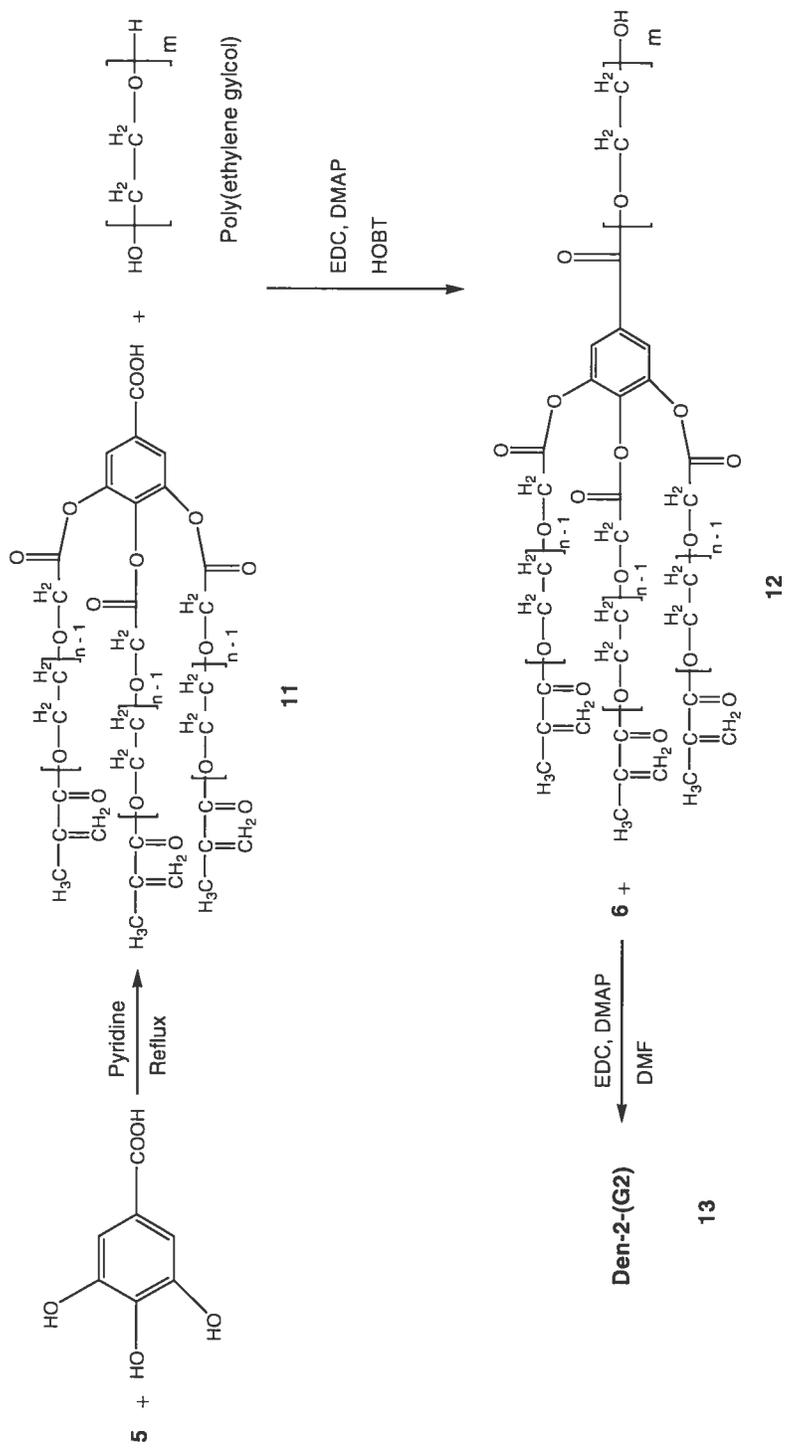
Scheme 2.1. Synthesis of core



Scheme 2.2. Synthesis of den-1-(G2), den-1-(G2)-OH and den-1-(G3)



Scheme 2.3. Synthesis of den-2-(G2)



2.4.13. Determination of hydroxyl value of den-1-(G2)-OH

Pyridine-catalyzed acetylation and phthalation are standard methods for the determination of hydroxyl value, which is indicator of number of hydroxyl groups in the compound. DMAP catalyzed acetylation with acetic anhydride reported to have specific catalytic activity 10^4 times greater than that of pyridine catalyzed reactions (Connors and Albert, 1973) was utilized in this study. Approximately, 5-6 mEq of the dendrimer (lyophilized to remove traces of moisture) was transferred to dried stoppered tube under nitrogen atmosphere and was dissolved in 1 mL pyridine (anhydrous). To this solution, 1 mL of freshly prepared acetic anhydride solution consisting of acetic anhydride and pyridine (1:4) was added. This was followed by the addition of 0.5 mL of catalyst solution (2% w/v of DMAP in anhydrous pyridine). The reaction was carried out in strictly anhydrous conditions. The stoppered tubes were maintained at 50°C for 18 h, because, shorter reaction times have yielded incomplete acetylation of the dendrimer. After 18 h, 5 mL of water was added to the tube and contents were brought to room temperature. Later, 25 mL of water was added to the solution (to ascertain complete hydrolysis of excess of acetic anhydride) and samples were incubated for approximately 15 min. All the samples were titrated with 0.1 N NaOH using phenolphthalein as indicator. A blank determination was carried out in exactly the same way. Hydroxyl value was calculated as follows:

$$\text{Hydroxylvalue} = ((V_{\text{sample}} - V_{\text{blank}}) \times N \times 1.701) / W_{\text{sample}} \dots\dots\dots \text{Equation 2}$$

Where, V_{sample} is volume of 0.1N NaOH used for sample; V_{blank} is volume of 0.1N NaOH used for blank; N is normality of NaOH and W_{sample} is the weight of sample used

2.4.14. Particle size measurements

The size of the dendrimers was determined by Coulter Nanosizer, N4 plus (Coulter Corporation, Hialeah, FL) and ALV/LSE-5003 (ALV-GmbH, Langen, Germany). Dendrimers were suspended in water (filtered through 0.22 μm) to obtain a concentration of 0.1 mg/mL. The samples were vigorously agitated and subsequently filtered through

0.22 μm or 0.1 μm filter (wherever specified) to remove dust particles. Each experiment was performed in triplicate at 25°C and at angle of 90°.

2.4.15. Differential scanning calorimetry (DSC)

The thermal properties of dendrimer were determined by employing DSC as a tool. The experiments were performed in triplicate on Metler TA 4000 (Metler Toledo, Switzerland) which was calibrated by using indium as a reference. Samples weighing between 3-5 mg were placed in a standard aluminum pan with a pin hole and were scanned in the temperature range of -70°C to 380°C at a heating rate of 10°C/min under nitrogen atmosphere.

2.4.15. Atomic force microscopy (AFM)

AFM was done with Nanoscope IIIa, Dimension™ 3100 (Digital Instruments, Santa Barbara, CA) in tapping mode. In this mode tip spends less time on the sample surface, as a result the lateral forces on the sample are reduced and thus, it provides opportunity to image delicate samples without severe distortion. Samples were prepared by dissolving the dendrimers in DMF at room temperature and later diluting with water. These were deposited on freshly cleaved mica surface by spin coating (WS,-400A-6NPP/LITE, Laurell Tech Corporation, North Wales, PA) at 2000 rpm for a period of 1 min. The samples were allowed to dry at room temperature. Subsequently, they were imaged in air at ambient conditions using etched silicon probes with tip radius of 5-10 nm and spring constant in the range of 20-100 N/m, oscillated at its fundamental resonant frequency (200-400 KHz).

2.4.16. Encapsulation studies

The ability of the dendrimers to act as host for the molecules was determined by encapsulating rhodamine and β -carotene as hydrophilic and hydrophobic model

compounds, respectively. The weighed amount of dendrimer was transferred to screw capped vials and was dissolved in DMF. Later, defined weight of compound to be encapsulated was added to the vials and stirred at room temperature. The unencapsulated drug was removed by dialysis of the solution against deionized water, till visibly no further diffusion of fluorescent compounds was observed (approximately 18 h). The sink conditions for removal of β -carotene were maintained by increasing volume of water and addition of ethanol (10% v/v) in the dialysis medium. The dialysate was lyophilized to obtain freeze dried dendrimer with encapsulated compounds. For the determination of the loading and encapsulation efficiency, weighed amount of freeze dried dendrimer was dissolved in DMF and samples were analyzed with appropriate blank corrections for dendrimer. Rhodamine was assayed fluorimetrically (excitation and emission wavelength 550 and 625 nm) using SAFIRE microplate reader (Tecan, Salzburg, Austria), while β -carotene was determined at 460 nm (λ_{\max}) using UV spectrophotometer U-2001 (Hitachi high technologies, Orlando, FL).

2.4.18. Release studies

The release of encapsulated compounds from the dendrimers was studied using vertical, jacketed Franz diffusion cells with an area of 0.79 cm² (Permgear, USA). The experiments were carried out at 37°C and phosphate buffer (0.1 M, pH 7.4) was used as release medium for studying rhodamine release while, ethanol and 0.1M phosphate buffer (2:1) was used to maintain the sink conditions for β -carotene release experiments. A definite amount of dendrimer with encapsulated compound was added to 800 μ L of respective release medium and was transferred into the donor compartment. It was separated from the receptor compartment containing 5 mL of release medium by dialysis membrane MWCO of 3500 Da (equilibrated overnight with release medium at 37°C). The samples were taken with replacement from the receptor compartment at each

predetermined time point (1, 2, 3, 4, 6 h and later, after every 24 h) and assayed as mentioned in the previous section.

2.5. Results and discussion

Dendrimers can be synthesized by iterative sequence of steps using either divergent or convergent procedure (Klajnert and Bryszewska, 2001). The divergent approach is reported to lead to structural defects and some difficulties in purification of the final product. However, the convergent approach is stated to minimize these synthetic problems, but, it seems that steric hindrance in coupling of dendron to core molecule limits the synthesis of higher generation of dendrimer by this approach. Thus, in the present work, dendrimers were synthesized using a strategic combination of both the approaches, i.e. the core molecule was grown divergently to obtain multifunctional core and this was then coupled to the dendron synthesized convergently, to obtain the final dendrimer. The higher generation dendrimer was synthesized by divergently coupling lower generation dendrimer to the dendrons. To the best of our knowledge, this is the first report on the combination of these two synthetic approaches for synthesis of the dendrimers.

2.5.1. *Synthesis and characterization of dendrimers*

The polyether-co-polyester dendrimers containing PEO in the cavity were synthesized using simple synthetic scheme by which den-1-(G2) (Figure 2.1) could be obtained in seven reaction steps (Scheme 2.1-2.2). The use of PEO as major building block helped in obtaining the desired molecular architecture in less synthetic steps. Trollsås et al., have also reported similar strategy to obtain high molecular weight dendrimers of specific architecture by using poly(ϵ caprolactone) as the building block (Trollsås et al., 2000). The synthesis was initiated by esterification of butane tetracarboxylic acid with an amino acid, aspartic acid to obtain the core. The ^1H NMR, ^{13}C NMR and elemental analysis showed

successful synthesis of the core. To estimate, the number of carboxylic acid groups in the core, the acid value/neutralization equivalent was determined (Table 2.1). The acid value for core obtained by titration method was within the experimental errors of the method. Since, acid value is specific for each compound; it can be used as derivative to identify it. This parameter can also be used to back calculate the number of carboxyl group if the molecular weight is known. For the core acid value of 7.8 was obtained, substantiating, that synthesized core has eight carboxylic groups at the surface.

The dendron was synthesized convergently by esterification of compound **5** with DHBA for synthesis of den-1-(G2) and to gallic acid for synthesis of den-2-(G2), followed by esterification with PEG 400. The esterification was monitored by FTIR spectroscopy, the appearance of ester C=O peak in the FTIR spectra of compound **6** (Figure 2.2) gave the evidence about the formation of an ester bond. In addition, the characteristic stretch of alkene group at 1540 cm^{-1} , PEO between 1300 and 1500 cm^{-1} and C-O-C stretch at 1000 - 1200 cm^{-1} proved the incorporation of PEG monomethacrylate in compound **6**. Further esterification of compound **6** by PEG 400 was supported by the splitting of ester -C=O and -C-O ether peaks in spectra of compound **7**, which confirmed the synthesis of a new ester bond as well as introduction of new ether bonds in the dendron.

It is reasonable to speculate that the methacrylate ester present in PEG monomethacrylate could cleave during reaction synthesis. To circumvent this probability, the reactions were carried out in mildest possible conditions and the peaks due to allyl group of PEG methacrylate were followed through out the synthesis as the marker. The existence of peaks due to cis and trans protons of allyl group of PEG methacrylate in FTIR and ^1H NMR spectra (Figure 2.3 and 2.4) of compound **5**, **6**, **7**, **8** and den-1-(G2) indicated

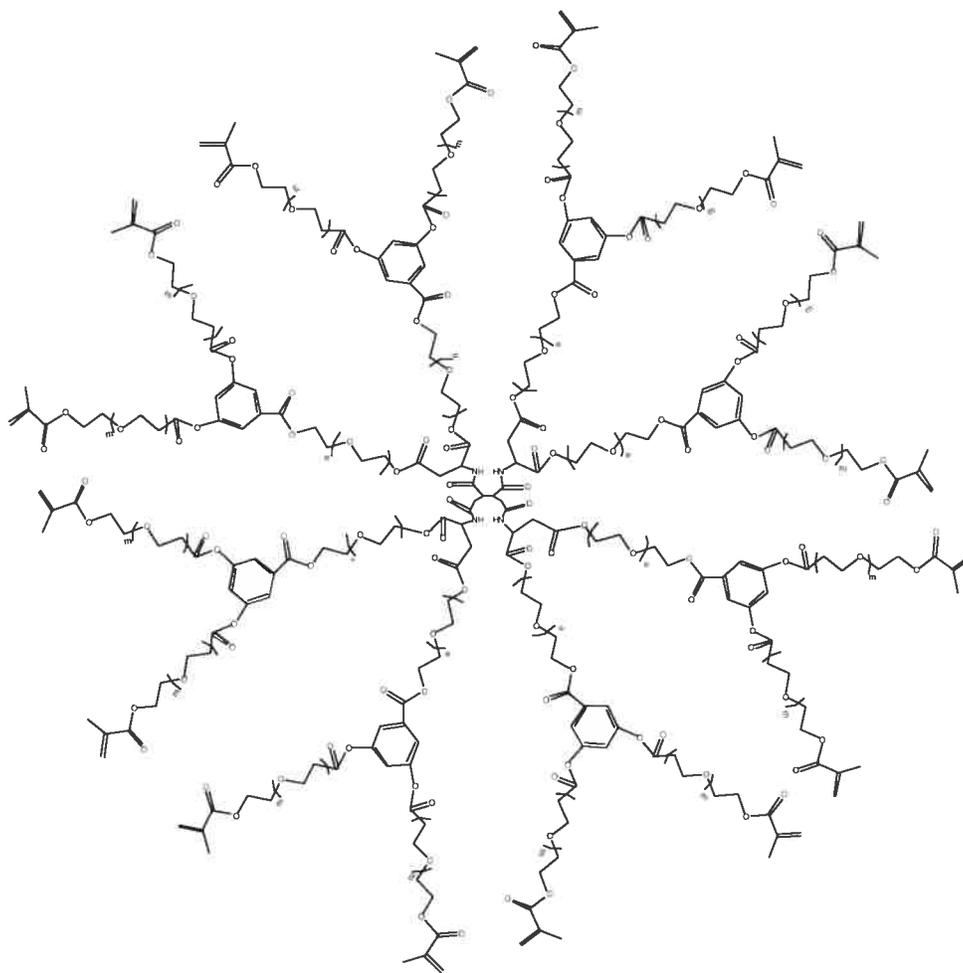


Figure 2.1. Structure of den-1-(G2).

Table 2.1. Acid value of the core determined by titration method

<i>Sample</i>	<i>Theoretical acid value</i>	<i>Observed acid value^a</i>	<i>Calculated no. of carboxylic groups^b</i>
Butane tetra carboxylic acid	58.50	60.60 ± 0.26	3.86
Core	86.75	88.58 ± 0.58	7.83

^a data are mean ± SD (n=4)

^b average acid value was used for calculation

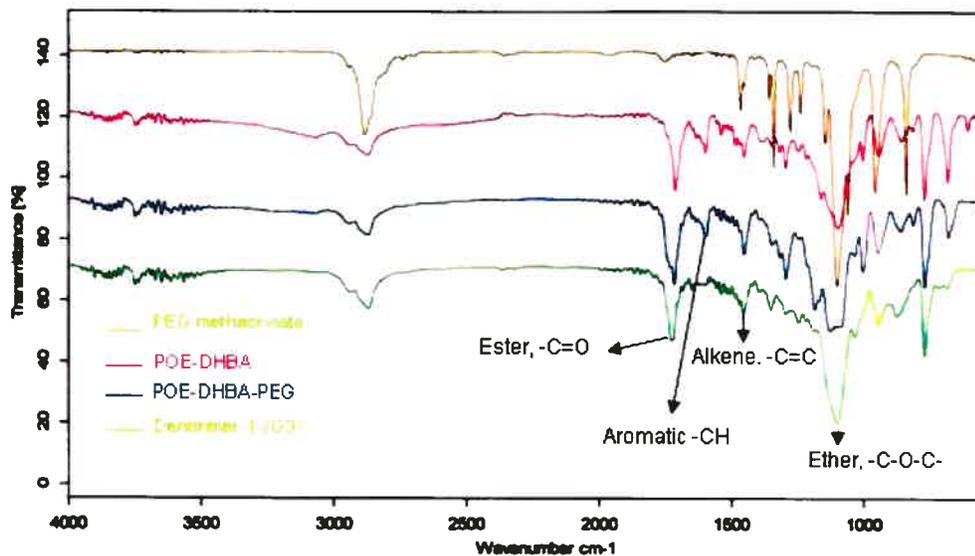


Figure 2.2. FTIR spectra's of PEG monomethacrylate, POE-DHBA, dendron 1 and dendrimer (den-1-(G2)).

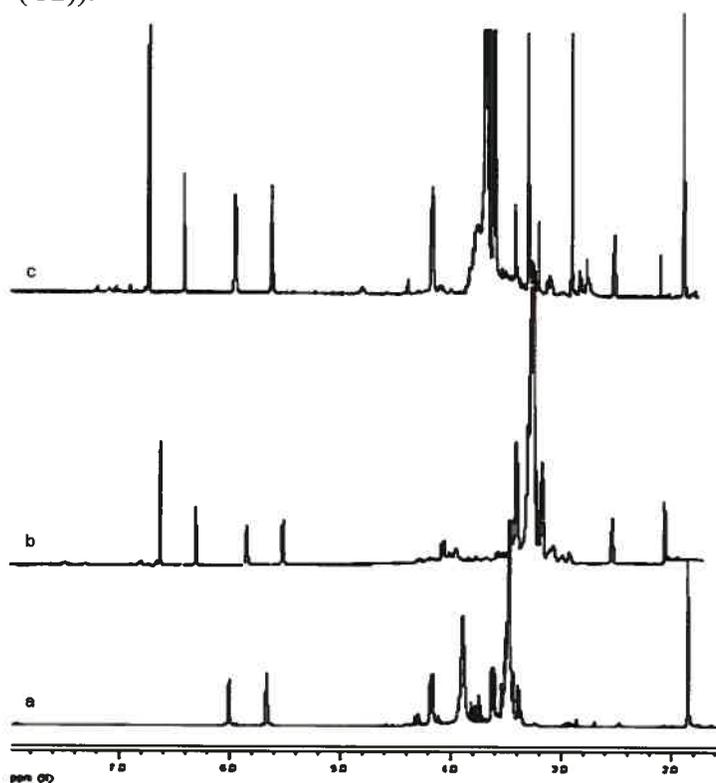


Figure 2.3. ^1H NMR spectra of (a) PEG-methacrylate, (b) POE-DHBA and (c) POE-DHBA-PEG in DMSO at 400 MHz.

that methacrylate moiety did not cleave throughout the synthesis. Additionally, the relative integration area of allyl proton and benzyl protons showed presence of two PEG methacrylate units per dendron in case of compound **6**, **7**, **8** and three units in case of **11**, **12** and **13**, respectively. Summarizing these observations, it was concluded that polyester dendrons with two and three allyl surface functionalities have been synthesized. Later, dendrons **7** and **12** were coupled to the multifunctional core to build dendrimers den-1-(G2) and den-2-(G2), respectively.

The dendrimer den-1-(G2)-OH was synthesized by hydroboration of dendrimer den-1-(G2) (Scheme 2.2). The conversion of allyl functional group to hydroxyl was monitored by disappearance of peak due to allylic protons in ^1H NMR spectra (Figure 2.5) as well as by determination of hydroxyl value of the product. Initial incubation of 18 h was allowed to assure complete addition of BH_3 to allyl groups, and further reaction time of 5 h was found to be adequate to convert all allyl groups to hydroxyl groups.

It was previously reported that steric hindrance offered by hyperbranched polymers could lead to incomplete acetylation of hydroxyl groups and thus, results in lower than expected hydroxyl values (Sunder et al., 2000). Thus, to minimize the possibility of this experimental limitation, acetylation was performed at higher temperature and for longer duration of time. Further, it was also reported that presence ethylene oxide units in the polymer could lead to erroneous results and often hydroxyl values obtained are over estimated (Yamamoto et al., 2003). Keeping this factor in consideration, dendrimer without any hydroxyl groups (i.e. den-1-(G2)) was always titrated in similar conditions and was used to correct the final values obtained. The experimental hydroxyl value determined by titration was found to be 4.12 ± 0.57 , which is in close agreement with the calculated value of 3.63 (calculated on the basis of molecular weight (M_n) of the dendrimer), thus, confirming that all the allyl groups have been oxidized.

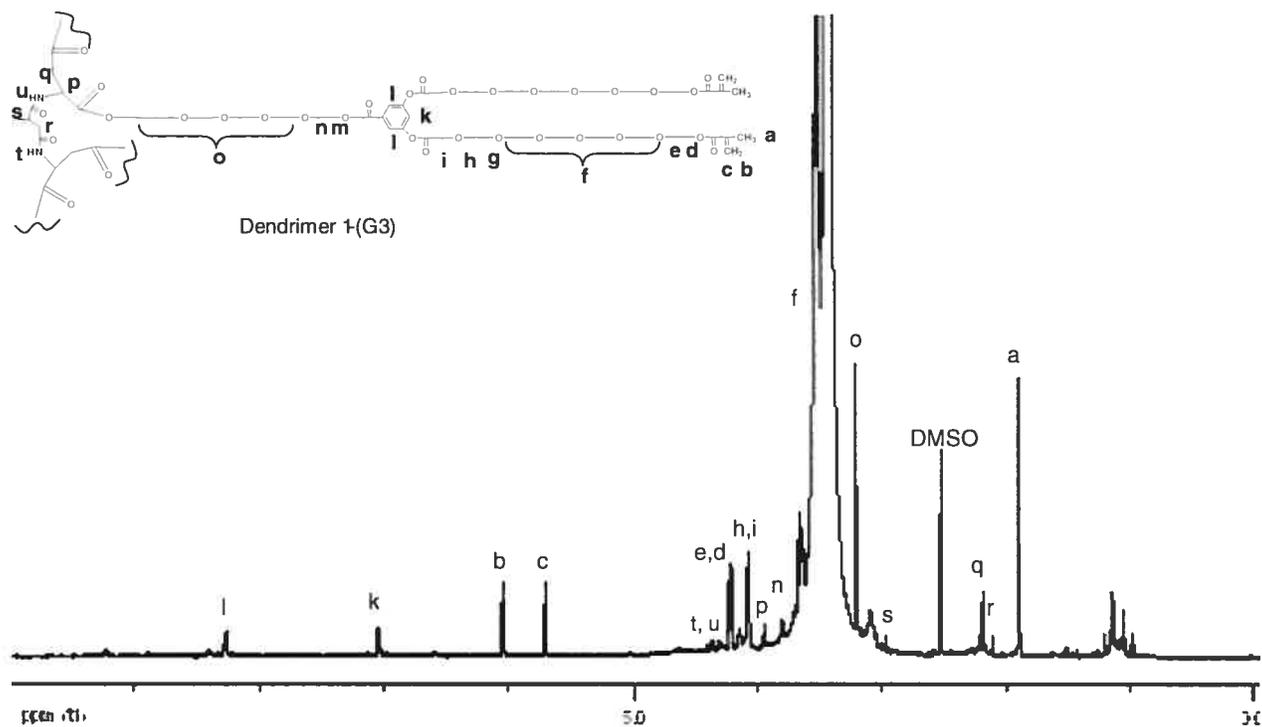


Figure 2.4 ^1H NMR spectrum of dendrimer (Den-1-(G2)) in DMSO at 400 MHz.

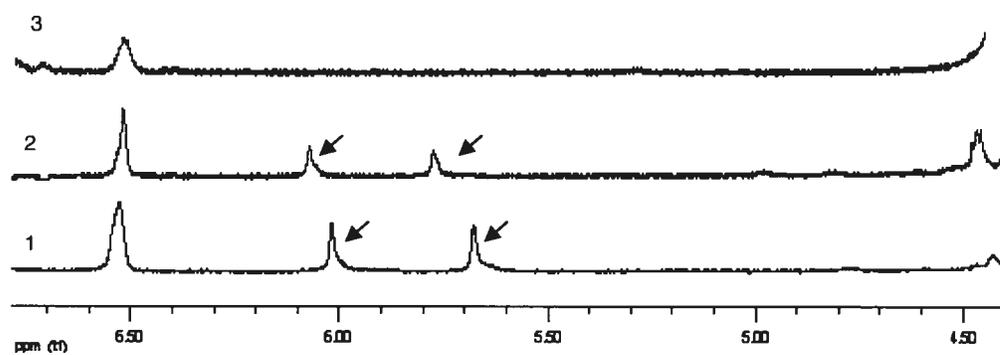


Figure 2.5. ^1H NMR spectra showing disappearance of allylic cis and trans protons during oxidation to hydroxyl group, (1) without any oxidation, (2) after approximately 50% oxidation, (3) after complete oxidation.

The higher generation dendrimer, den-1-(G3) was synthesized by divergently coupling den-1-(G2)-OH to POE-DHBA (**6**) in the presence of EDC and DMAP (Scheme 2.2). Characterization of den-1-(G3) by routine instrumental techniques showed successful synthesis.

The characteristics of all the synthesized dendrimers are presented in Table 2.2. The M_n of dendrimers was determined by ^1H NMR, GPC and MADLI-TOF. The M_n values determined by GPC are less than the expected theoretical values, this deviation is due to difference in hydrodynamic volume between dendrimers and linear PEG standards.

2.5.2. Particle size measurements

The size of the dendrimers was determined in water at various concentrations (data not shown). At concentration of 1 mg/mL, size of about 200 nm was observed which indicated high aggregation of the dendrimers. At lower concentrations (0.5 mg/mL) bimodal size distribution with average size of 200 nm and small population with approximate size between 10-30 nm was observed for all dendrimers. It was also found that even after filtration through 0.10 μm membrane dendrimer population with size around 200 nm was obtained (data not shown), which indicated self-assembly of dendrimer after filtration of the sample. Percec et al., and Wang et al., have also reported self assembly of dendrimers, which they explained to be mediated by various non-directional forces like hydrophobic, electrostatic interactions, and coulombic forces etc. (Percec et al., 2002; Wang et al., 2004). To overcome this problem, experiments were performed at lower concentration (0.1 mg/mL) and effect of various additives like triethyl amine, NaOH, HCl, DMF, methanol, acetone, urea and sodium chloride was examined. Although, triethyl amine and methanol were found to reduce aggregation of dendrimers, they had no significant influence on self assembly. Sodium chloride (0.25-0.35 M) inhibited not only aggregation but also self assembly phenomenon. Thus, hydrodynamic sizes of all the

Table 2.2. Characteristics of dendrimers

<i>Dendrimer</i>	<i>Surface functional group</i>	<i>Number of functional group</i>	M_n (NMR) ^a	M_n (GPC) ^b	M_n (MALDI-TOF)	<i>Particle size (nm) (AFM)^c</i>	<i>Particle size (nm) (DLS)</i>
Den-1-(G2)	-CH=CH ₂	16	8130	6909	7743	5.39	4.7 ± 0.4
Den1-(G3)	-CH=CH ₂	32	16978	-	15072	11.13	8.5 ± 0.3
Den-2-(G2)	-CH=CH ₂	24	10096	-	11922	9.85	7.2 ± 0.4
Den-1-(G2)-OH	-OH	16	8024	6795	7325	9.05	5.3 ± 0.6

^a Calculated based on relative integration ratio of peaks with respect to peak of -CH=CH₂ cis /trans proton or aromatic proton (in case of den-1-(G2)-OH)

^b Determined using PEG standards and DMF as mobile phase at flow rate of 1 mL/min, values in bracket are polydispersity index

^c Data are mean values, determined by ≥ 30 measurements

^d Data are mean ± SD (n=3)

Table 2.3. Details of thermal events for various dendrons and dendrimers obtained by DSC

<i>Sample</i>	<i>T_g</i> (°C)	<i>T_m</i> (°C)
PEG methacrylate	-67.50	-
POE-DHBA	-31.10	234.60
POE-DHBA-PEG	-21.45	293.20
Den-1-(G2)	-12.52	311.90
Den-1-(G3)	-10.89	308.30
Den1-(G2)-OH	-13.50	300.60
POE-gallate	-45.50	218.50
POE-gallate-PEG	-41.70	231.60
Den-2-(G2)	-21.37	298.20

* Mean ± S.D. (n=3)

Mean ± S.D. (n=2)

dendrimers were determined in sodium chloride solution and results are shown in Table 2.2. Even though dendrimers showed aggregation under *in vitro* conditions, it is speculated that after *in vivo* administration these aggregates will breakdown due to dilution as well as presence of various biological substances (Allen et al., 1999). The absence of aggregates is significantly important for improved *in vivo* efficacy because, the size of the drug carrier influences not only the circulation time in blood but also the endocytosis or uptake by the cells. The smaller particles are rapidly taken up by cells, thus they can provide intracellular delivery and there by increase the therapeutic efficacy.

2.5.3. Thermal properties

It is reported that polymeric carriers undergo glass-rubber transition during the penetration of water and this influences the drug release profile (Omelczuk and McGinity, 1992). Thus, thermal properties of polymers not only influence the stability but also the release of drug (Liggins and Burt, 2004). All the synthesized dendrimers were investigated for their thermal properties and in particular, glass transition temperature (T_g) by DSC. The T_g for PEG-methacrylate (1) was observed to be -67.50°C which is close to the reported value of -65°C . The T_g values for POE-DHBA and POE-DHBA-PEG were found to be -31.10°C and -21.45°C , respectively (Table 2.3), indicating increase in the transition temperature. Further, the transition temperature for den-1-(G2) (-12.52°C) is higher than that of POE-DHBA-PEG. These increases in transition temperature could be explained by increase in molecular weight as well as by the decrease in chain flexibility as the branching increases (Tadlaoui et al., 1991; Stevens, 1999). Similar trend was observed for in POE-gallate, POE-gallate-PEG and den-2-(G2), however, the T_g values were always less than that for den-1-(G2) series. This is probably due to higher number of PEG chains present per dendron (POE-DHBA has 2 PEG chains while POE-gallate has 3 PEG chains) in the later case. Interestingly, only one T_g was observed for all the polymers analyzed, which suggests that all the polymers had no unreacted monomers and also there was no nanophase

separation due to clustering of the polar groups as reported by Sunder et al., for hyperbranched polymers (Sunder et al., 2000). Small melting peaks were also observed for all the polymers, which indicate the existence of amorphous as well as crystalline nature.

2.5.4. Atomic force microscopy

AFM is a versatile technique which offers opportunity to obtain high resolution topographic images of nanoscale polymeric materials. All the dendrimers were imaged in tapping mode after depositing the sample by spin coating on freshly cleaved mica surface. The spin coating results in the formation of monolayer of polymer at low concentrations, in addition, the centrifugal forces operating during the process aids in breaking the aggregates.

The dendrimers height between 1.39 and 3.55 nm was observed on the mica surface, which indicates the absence of pillar like aggregation. The scope trace of the surface as well as the topographical view showed the presence of few aggregates but majority of the dendrimers were present as isolated molecules. All the dendrimers appeared flattened on the substrate in surface plot as has been reported for other hydrophilic dendrimers (Betley et al., 2001). However, as seen in Figure 2.6, den-1-(G2) and den-2-(G2) were approximately spherical in shape, while den-1-(G2)-OH were slightly oval in shape and den-1-(G3) showed a spherical shape with floral pattern, which indicates that the shape of dendrimer is influenced not only by surface groups but also by the generation (each scan was repeated 3-4 times to ascertain the reproducibility). This observation is in agreement with various simulation and molecular modeling reports of dendrimers (Ballauff and Likos, 2004; Rathgeber et al., 2004; Maiti et al., 2005) where the shape of dendrimer was found to be influenced by surface groups, solvents and generation.

AFM was also exploited as a tool to determine the size of the dendrimers. For this purpose, all the images were treated offline by using a particle size analysis function, which is designed for analyzing the lateral and vertical dimensions of isolated particles on the substrate surface based on height of pixel data. The dimensions obtained slightly deviate

from that observed by DLS (Table 2.2). Müller et al., found that residual water in the sample left after drying procedure, can lead to overestimation of size of dendrimers (Müller et al., 2002). Further, it is also reported (Hierlemann et al., 1998; Lackowski et al., 1999; Li et al., 2000; Betley et al., 2001) that hydrophilic and flexible dendrimers (like PAMAM) flatten and laterally expand on hydrophilic substrates like mica. This flattening and lateral expansion is enhanced by the large forces exerted by residual water on absorbed species (Müller et al., 2002). All these factors could contribute to overestimation of diameter of these hydrophilic dendrimers.

2.5.5. Encapsulation and release studies

Rhodamine, a hydrophilic fluorescent compound and β -carotene, a highly hydrophobic compound were encapsulated in dendrimer (den-1-(G2)). As shown in Table 2.4, at the drug/dendrimer feed ratio of 1:4.5, loading of 10.76% w/w was achieved for rhodamine and 5.86% w/w for β -carotene. It was observed that on increasing the drug/dendrimer feed ratio to 1:3.6, rhodamine loading increased to 15.80% w/w and that of β -carotene was increased to 6.47% w/w. Further increase in drug/dendrimer feed ratio had no increase in loading for either of the compounds. Kojima et al., also reported similar observation where an increase in drug/dendrimer feed ratio resulted in increase in the drug loading (mol/mol), which reached a maximum value at certain feed ratio (Kojima et al., 2000). The lower loading of β -carotene could be due to the highly hydrophobic nature as well as linear shape of the molecule. It was reported by Namazi and Adeli, that size and polarity of the guest molecule affect the ability of dendrimers to encapsulate drug (Namazi and Adeli, 2005). They also reported that the encapsulation of pyridine, mefenamic acid and acetyl salicylic acid in second generation citric acid dendrimers, on dendrimer/ drug ratio basis was 20, 32 and 49 mol% (i.e. 0.8, 3.8 and 4.4% w/w), respectively. In another study, PMAM dendrimers with PEG grafts had shown maximum encapsulation of

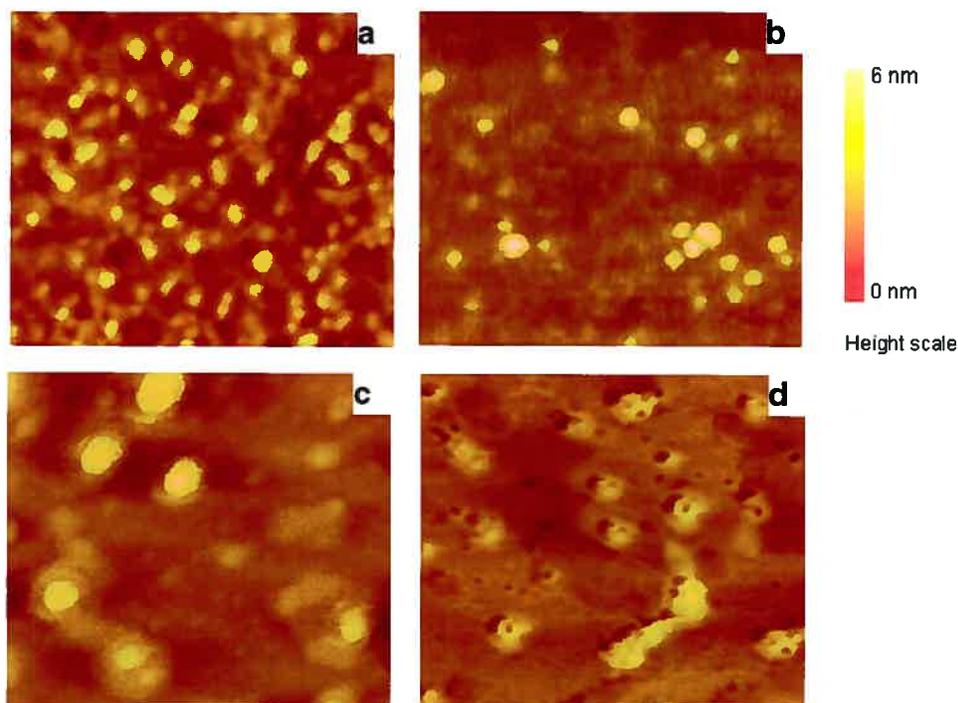


Figure 2.6. Topographical tapping mode AFM images of (a) den-1-(G2), (b) den-2-(G2), (c) den-1-(G2)-OH, and (d) den-1-(G3), spin coated on freshly cleaved mica surface. The scan area is 200×200 nm.

Table 2.4. Encapsulation of rhodamine and β -Carotene in den-1-(G2)

<i>Compound</i>	<i>Drug:dendrimer feed ratio (1:4.5)*</i>		<i>Drug:dendrimer feed ratio (1:3.6)#</i>	
	<i>Loading (% (w/w))</i>	<i>Encapsulation efficiency (%)</i>	<i>Loading (% (w/w))</i>	<i>Encapsulation efficiency (%)</i>
1 Rhodamine	10.76 ± 1.06	63.27 ± 0.75	15.80 ± 0.14	64.52 ± 0.58
2 β -Carotene	5.86 ± 0.24	33.49 ± 0.68	6.47 ± 0.02	33.66 ± 1.08

* Mean \pm S.D. (n=3)

Mean \pm S.D. (n=2)

6.5 mol/mol (drug/dendrimer mole ratio) i.e. 2.7% w/w for doxorubicin and 26 mol/mol (10.9% w/w) for methotrexate (Kojima et al., 2000). As seen above, drug loading is interplay of the size and solubility of the molecule, lower solubility results in lower loading and smaller size increases the loading for molecules with comparable polarity. Although, pyridine (79 Da), mefenamic acid (241 Da), acetyl salicylic acid (180 Da) are smaller and more soluble than β -carotene (537 Da), yet the loading obtained for β -carotene in this study is much higher. Further, comparatively the loading of rhodamine (479 Da) is higher than that of methotrexate (454 Da). This clearly indicates significantly improved drug loading by these dendrimers and thus, substantiates that incorporation of PEO in the interior of dendrimer increased the size of the internal cavity and thereby increased the encapsulation. Furthermore, it also provided favorable environment to encapsulate hydrophilic as well as large and hydrophobic molecules. Thus, incorporation of PEO in the core of drug carriers can provide an attractive approach to deliver both water soluble and poorly water soluble compounds.

Since, the dendrimer employed for encapsulation studies had no ionizable surface group, it was speculated that encapsulation is largely due to hydrogen bonding by PEO in the cavity and branch (Li et al., 2000) and/or physical entrapment of molecule in the cavity, rather than being a complexation phenomenon as has been reported for PAMAM dendrimers (Kohle et al., 2003). To provide evidence to this hypothesis, the mechanism involved the encapsulation of these compounds in dendrimer was investigated. The absorbance spectra of both the compounds before and after encapsulation in dendrimer were recorded and are presented in Figure 2.7. No shift in absorption maxima was observed for either of the compound. The absence of shift in absorption maxima indicates absence of any π - π group complexation, because such complex formation is evidenced by formation of a new chromophore (Coffman and Kildsig, 1996; Agrawal et al., 2004).

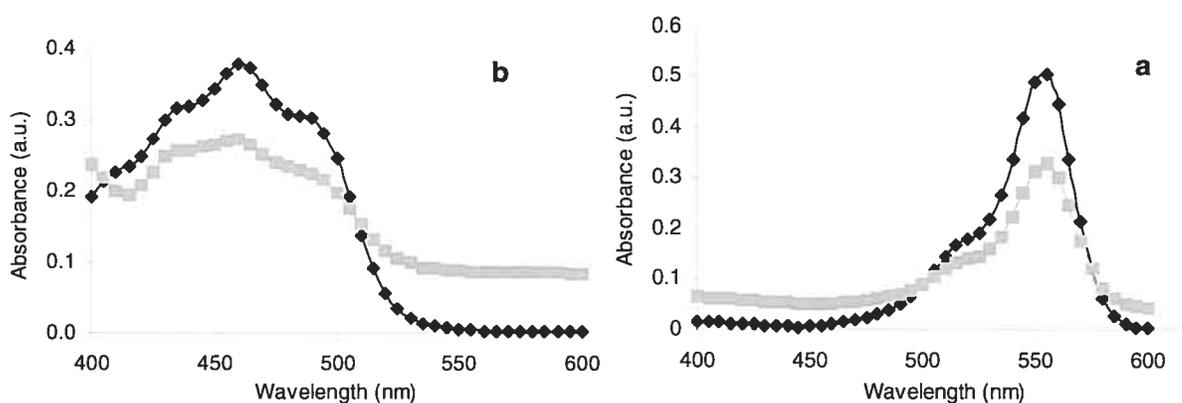


Figure 2.7 Absorbance spectra of (a) rhodamine and, (b) β -carotene before and after encapsulation in dendrimer, determined to study the effect of encapsulation on fluorophore and chromophore. Key: \blacklozenge before encapsulation; \blacksquare after encapsulation.

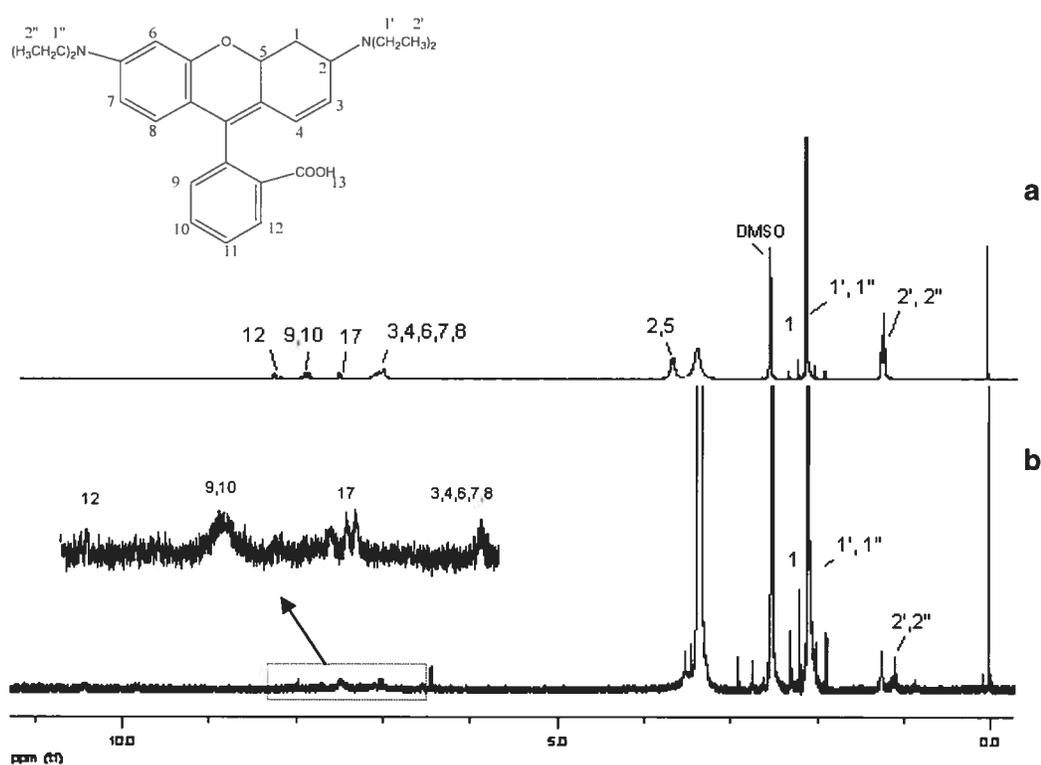


Figure 2.8. ^1H NMR spectra of (a) rhodamine ($10\ \mu\text{M}$) and (b) rhodamine encapsulated in dendrimer (Den-1-(G2)) ($4\ \mu\text{M}$) measured in DMSO at 400MHz.

NMR spectroscopy is reported to furnish important information on direct interaction between compound and dendrimers (Morgan et al., 2003; Ooya et al., 2004; Kohle et al., 2003). Thus, ^1H NMR of rhodamine and β -carotene encapsulated in dendrimer were acquired and the chemical shifts of rhodamine as well as β -carotene in DMSO before and after encapsulation in dendrimer were determined. No significant shift in the peaks of aromatic rings as well as ethyl amino was observed in spectra of rhodamine encapsulated in dendrimer but few peaks were suppressed (Figure 2.8), which implies that the compound is physically encapsulated. To further investigate into the mechanism of encapsulation and interactions between encapsulated compound and dendrimer, FTIR spectroscopy was employed as a tool. The FTIR spectra of rhodamine alone, den-1-(G2) and rhodamine encapsulated in den-1-(G 2) were acquired as neat samples. The spectrum of den-1-(G2) was subtracted from that of rhodamine encapsulated in den-1-(G2) to obtain the spectral features of rhodamine after encapsulation in dendrimer. It was observed that, the carboxylic -OH which appears in the region of $3300\text{-}2500\text{ cm}^{-1}$ and is overlapped by C-H stretch (Coates, 2000), had increased in intensity as well as broadened after encapsulation in the dendrimer. The absence of peak splitting around $1372\text{-}1344\text{ cm}^{-1}$ was also seen (Figure 2.9). These peaks in the range of $1370\text{-}1310\text{ cm}^{-1}$ are assigned to aromatic tertiary amine C-N stretch. Further, the shift in peak at 1223 cm^{-1} associated to aromatic C-O stretch was also seen. Since, these changes are associated with OH, C-N and C-O stretching frequencies, which are generally attributed to hydrogen bonding interactions. Thus, weak hydrogen bonding between dendrimer and rhodamine is postulated to exist. In addition, the significant decrease in intensity of peaks in the region of $1650\text{-}1500\text{ cm}^{-1}$ (aromatic ring stretch), was observed indicating either that aromatic region is surrounded by the PEO chain of dendrimer or there are dipole-dipole/ induced dipole interactions. The changes in FTIR spectral features of rhodamine after encapsulation indicated presence of weak hydrogen bonding or dipole-dipole/ induced dipole interactions. These results suggest that hydrogen bonding of molecule by PEO together with physical entrapment of molecule in the dendrimer is the probable mechanism of encapsulation. It is previously reported that at

high concentration PEG 400 self associate by hydrogen bonding and disrupts the water structure to certain extent (Sato et al., 1998). Since, these dendrimers have high density of PEO it is possible that self association of PEO chains could also occur resulting in hydrotropic solubilization of the compounds.

The release of drug molecules from polymeric carriers occurs by either diffusion and/or degradation of the polymer. Since, approximately 90% of both the encapsulated compounds were released in 172 h i.e. 7 days (Figure 2.10) and the preliminary *in vitro* biodegradation studies have shown that degradation of den-1-(G2) ensues after 10 days of incubation. Therefore, *in vitro* release of both the compounds will be mainly governed by diffusion. The release of rhodamine was slow and almost linear till 120 h, after which it reached a plateau phase. Approximately, 82% of rhodamine was released from dendrimer in 120 h and 90% was released after 172 h. Since, rhodamine is a water soluble compound, the slow release profile demonstrates the ability of these dendrimers to provide sustained release of even water soluble drugs.

β -carotene is practically insoluble in water and hence, ethanol was added to the release medium to obtain necessary sink conditions. In the absence of necessary sink conditions the hydrophobic compounds exhibit a slow release profile. Thus, the slow release of the drug from the carrier could be due to absence of sink conditions and not due to the influence of drug delivery vehicle. To over rule the factor that slow release of β -carotene is due to absence of sink condition or slow dissolution of drug, release of β -carotene from suspension was performed under same conditions but at 10 time higher amount. It also allowed comparison of the release characteristics of drug from dendrimer and suspension. Even though drug concentration in suspension was much higher, approximately 100% of the drug was released from suspension in 96 h as compared to 70% from dendrimers, indicating sustained release of drug encapsulated from the dendritic

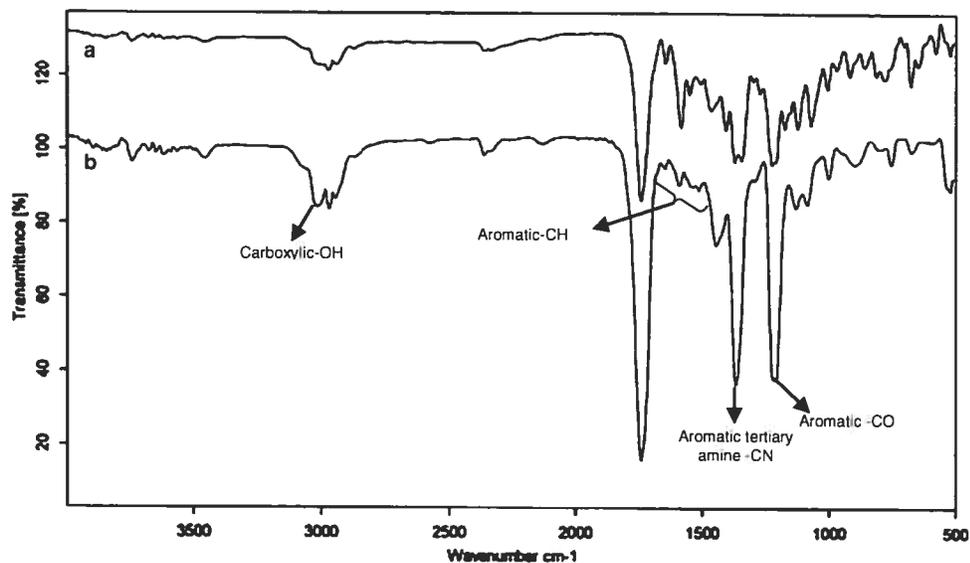


Figure 2.9. FTIR spectra of (a) free rhodamine and (b) encapsulated rhodamine (obtained by subtracting spectrum of den-1-(G2) from spectrum of rhodamine encapsulated in den-1-(G2)).

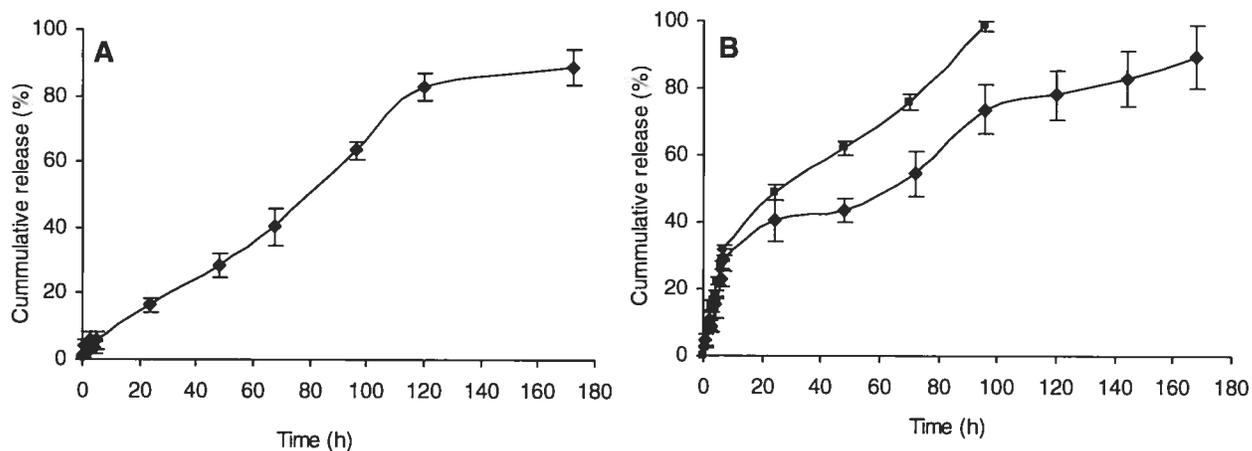


Figure 2.10. Cumulative release profile of (A) rhodamine encapsulated in den-1-(G2); (B) β -carotene encapsulated in den-1-(G2) (diamonds), and β -carotene suspension (squares). Data are mean \pm SD.

architecture. A triphasic release profile was observed in case of dendrimer, with an initial rapid release phase during which 30% of drug was released in 7 h, followed a slower release with drug release up to 70% in 96 h and another slow release with 90% of encapsulated drug being released in 168 h. The absence of burst release in case of both rhodamine and β -carotene also ascertains non-existence of surface bound drug. Comparatively, release of rhodamine was slower than that of β -carotene; this is in contrast to the general trend where hydrophobic compounds exhibit slower release as compared to hydrophilic compounds. This slower release of rhodamine is due to the hydrogen bonding interaction of the former with the PEO, which retards the diffusion and hence release of the drug. This interesting observation highlights the ability of these dendrimers to prolong the release of encapsulated drugs and thus, demonstrates their potential to act as carriers for sustained release of both hydrophilic and hydrophobic drugs. The ability to encapsulate both hydrophilic and hydrophobic drug is advantageous because, it eliminates the need to tailor polymeric architectures specifically for hydrophilic or hydrophobic drugs. Further, it provides an opportunity for combination therapy, wherein drugs with different solubilities can also be encapsulated simultaneously and delivered to the target site.

These dendrimers have hydrolysable ester linkages and are composed of mainly PEO/PEG, a biocompatible polymer that has shown to reduce the cytotoxicity, immunogenicity and anti-genecity of various dendrimers as well as other drug delivery vehicles (Bhadra et al., 2002; Jevapresphant et al., 2003). Further, due to the absence of cationic groups at the surface it is speculated that the synthesized dendrimers will be biodegradable and biocompatible.

2.6. Conclusion

Novel polyether-co-polyester second and third generation dendrimers were synthesized by simple synthetic schemes using butane tetracarboxylic acid as the core

molecule by combination of divergent and convergent synthesis. PEO was incorporated in the interior of the dendrimers to increase the size of the cavity as well as to provide hydrophilic interior region. The dendrimers demonstrated ability to encapsulate both hydrophilic and hydrophobic model compounds, with sufficiently high drug loading. Further, the release of both rhodamine and β -carotene was found to be slow and sustained, with approximately 90% of drug being released in 170 h. The study indicates that polymeric architectures with PEO interior can be potentially used for delivery of hydrophobic and hydrophilic drugs, which provides an advantage over drug carriers with hydrophobic core that have preferentially ability to deliver hydrophobic compounds. It is postulated that by virtue of their small size and slow release characteristics these dendrimers can be used as nanocarriers for intracellular drug delivery.

2.7. Acknowledgements

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CHAPTER THREE

RESEARCH PAPER

Polyether-co-polyester dendrimers for delivery across the blood brain barrier

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3.1. Abstract

In the present study, effects of alterations in the chemical structure of polyether-copolyester (PEPE) dendrimers on the encapsulation and release of methotrexate (MTX) was investigated. A series of PEPE dendrimers of different architecture were synthesized. Biocompatibility of the resulting dendrimers was evaluated *in vitro* by assessing their cytotoxicity on RAW 264.7 cells using lactate dehydrogenase (LDH) assay. Dendrimers caused no cell death even at the concentration of 250 $\mu\text{g/mL}$, suggesting that they are acceptable for pharmaceutical applications. They also showed good capacity to encapsulate MTX, with loading as high as 24.5% w/w. Increase in the number of branches and the size of internal voids were shown to enhance the encapsulation. On the other hand, absence of aromatic rings as branching units drastically reduced the loading capacity. Physical entrapment, weak hydrogen bonding and hydrophobic interactions were established to be the mechanisms of encapsulation. Release of MTX was biphasic, which included a burst release in 6 h followed by a slower release over a period of 50 or 168 h. Increase in the number of branches profoundly decreased this initial burst release; in contrast, absence of aromatic rings in the dendritic structure resulted in a very rapid release.

Keywords: dendrimer, methotrexate, release, encapsulation, architecture

3.2. Introduction

The effective delivery of drugs for the treatment of diseases has been the pursuit of pharmaceutical scientists for decades. This has led to the exploration of innumerable natural as well as synthetic materials, and as a consequence drug delivery has evolved from mere dispensing of elixirs and solutions to the state-of-the art technology with exquisitely tailored nano-delivery systems. In turn, this has resulted in the meticulous development of advanced nanoscale materials such as cross-linked nanoparticles (Butun et al., 1998; Sanji et al., 2000), hyperbranched polymers (Sunder et al., 1999; Thompson et al., 2000), dendrimers (Thompson et al., 2000; Gillies and Fréchet, 2005) etc. with the properties desirable for drug therapy. Amongst these nanocarriers, dendrimers represent relatively new class of macromolecules, which have been investigated for various therapeutic applications (Esfand and Tomalia, 2001; Cloninger, 2002). The unique properties of dendrimers, primarily their controlled branched architecture, nanometric size, monodispersity and host-guest chemistry (Tomalia et al., 1990; Patri et al., 2002) have spurred impetus in the dendrimer based drug delivery research. Dendrimers can be used to deliver drugs either by encapsulating them in the internal cavities or by conjugating to the surface functionalities (Esfand and Tomalia, 2001; Aulenta et al., 2003; Lee et al., 2005). Though conjugation serves to increase the pay-load, it could also result in limited aqueous solubility of the drug-dendrimer conjugate, especially for hydrophobic drugs (Kono et al., 1999). In addition, the conjugation of drug to polymers is a time consuming process, requiring additional synthetic steps and could sometimes result in decreased therapeutic benefit principally due to the failure of conversion or slow conversion to the parent drug or decreased permeability of the conjugate (Fix et al., 1990; Denny, 2004). Thus, physical encapsulation seems to be a more attractive approach for delivery of small guest molecules (Kojima et al., 2000; Liu et al., 2000; Ooya et al., 2004; Namazi and Adeli, 2005). The encapsulation of drug in the dendritic voids protects labile molecules from degradation and also eliminates solubility issue associated with poorly soluble compounds. However, it is

reported that drugs loaded non-covalently into dendrimers are released rapidly (Kojima et al., 2000; Liu et al., 2000; Kolhe et al., 2003; Patri et al., 2005), rendering them ineffective for targeted delivery. Hence presently, improvement in the encapsulation efficacy and release properties remain as the major challenges in dendrimer based delivery systems. To this end, we have recently reported polyether-co-polyester (PEPE) dendrimers with polyethylene oxide (PEO) in the interior cavity (Dhanikula and Hildgen, 2006). PEO was incorporated in the interior not only to increase the size of the cavity but also to enhance dendrimers ability to encapsulate drug by virtue of its solublizing properties (Sugimoto et al., 1998).

Since the inception of dendritic structure, numerous classes dendrimers have been extensively synthesized, yet fewer efforts have been made to physically encapsulate drug molecules in them. As a consequence, there is a paucity of information on chemical features desirable for controlling release of guest molecules from dendrimers. The macromolecular architecture of dendrimer governs the physical and chemical properties (Piotti et al., 1999; Sendijarevic and McHugh, 2000; Moschogianni et al., 2001), therefore it can have a significant influence on their performance as drug carriers and in particular on encapsulation efficiency and release. The comparative studies on different architectures with the perspective of understanding the influence on drug loading and release characteristics can provide knowledge that can be integrated into the dendritic technology to obtain the dendrimers with sustained release property. With this premise, a series of PEPE dendrimers were synthesized. The structure of dendrimers was tailored by varying the number of branches, branching units, terminal functional groups, generation and chain length of PEO in the interior cavity. Methotrexate (MTX) was employed as a guest molecule and its interaction with dendrimers was characterized by UV, 1D ¹HNMR, and FTIR spectroscopy. In addition, pharmaceutical acceptability of these carriers was evaluated by studying their *in vitro* cytotoxicity.

3.3. Experimental

3.3.1. Materials

1,2,3,4 butane tetracarboxylic acid, L-aspartic acid, PEG (M_n 200, 300 and 400 Da), PEG monomethacrylate (M_n 360 Da), 3,5 dihydroxy benzoic acid (DHBA), 2,2-bis(hydroxyl methyl) butyric acid (BHBA), 4-(dimethyl amino) pyridine (DMAP), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), gallic acid, hydroxy benzotriazole (HOBT), borane-tetrahydrofuran complex ($BH_3 \cdot THF$, 1M), hydrogen peroxide (H_2O_2), triethyl amine, sodium chloride, sodium hydroxide (NaOH), activated carbon 4-14 mesh granular, and filter aid Celite[®]521 were purchased from Sigma-Aldrich Canada, (Oakville, ON). Anhydrous pyridine, N, N-dimethyl formamide (DMF), and dimethyl sulfoxide-d6 (99.9% D) (DMSO-d6) were supplied from Sigma-Aldrich Canada, (Oakville, ON). Acetone, dichloromethane, methanol, chloroform, anhydrous diethyl ether, tetrahydrofuran (THF) and silica gel (70-230) were purchased from Laboratoire Mat. (Montreal, PQ). And thionyl chloride was purchased from A & C chemicals Ltd., (Montreal, PQ). Dialysis tubing (MWCO 1000, 3500 Da) were obtained from Fisher Scientific Co. (Ottawa, ON). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Invitrogen Corp. (Burlington, ON). LDH assay kit was purchased from Sigma-Aldrich Canada, (Oakville, ON). All other chemicals and solvents were of reagent grade. Water was deionized with a MilliQ purification system (Millipore, Bedford, MA) before use.

3.3.2. Synthesis of dendrimers

The PEPE dendrimers were synthesized as reported previously (Dhanikula and Hildgen, 2006). In order to obtain dendrimers of different architecture, three modifications were carried out (i) gallic acid, DHBA or BHBA was used as the branching molecule in the

dendron; (ii) molecular weight of PEO in the interior cavity was varied from 200, 300 to 400 Da; (iii) generation or terminal functional groups were varied. Dendrimers are referred as den-1-(Gn)-M, den-2-(Gn)-M or den-3-(Gn)-M in the manuscript, where 1, 2 and 3 represent dendrimers containing DHBA, gallic acid and BHBA, respectively; while, Gn represents the generation of the dendrimer and M denotes the molecular weight of PEO used in the interior cavity of the dendrimers.

Synthesis was initiated by esterification of butane tetracarboxylic acid with aspartic acid to obtain the core (scheme 3.1s, supporting information). Dendrons were synthesized as mentioned in scheme 3.2s (Supporting information). For the synthesis of den-1-(G2)-400, den-2-(G2)-400 and den-3-(G2)-400, PEG 400 was used in the synthesis of **6**, **7**, and **8** (scheme 3.2s supporting information). While, for synthesizing den-1-(G2)-200, den-2-(G2)-200, den-3-(G2)-200 and den-1-(G2)-300, den-2-(G2)-300, den-3-(G2)-300; PEG 200 and PEG 300 were used, respectively. Compound **6**, **7**, or **8** were coupled with the core to obtain the dendrimers. Den-1-(G2)-OH, containing hydroxyl groups as terminal functional groups was synthesized by hydroboration of den-1-(G2)-400. The higher generation dendrimer, den-1-(G3) was synthesized by divergently coupling den-1-(G2)-OH to compound **3**. The detailed synthetic procedure is mentioned in the supporting information.

3.3.3. Characterization of dendrimers

^1H NMR and ^{13}C NMR spectra were recorded with Bruker ARX400 NMR spectrometer (Bruker Biospin, Billerica, MA) at room temperature and resonance frequency of 300 or 400 MHz. IR spectra of the products were acquired on Bruker Vector 22 spectrometer (Bruker Optik GmbH, Ettlingen, Germany).

Mass spectroscopy was done using matrix associated laser desorption ionization-time of flight (MALDI-TOF) on Bruker Autoflex[®] (Bruker Daltonik GmbH, Bremen, Germany) in linear, positive ion mode using dithranol as matrix and sodium iodide as cationizing agent. The matrix, sample and cationizing agent were mixed in a ratio of 10:6:1

using THF as a solvent. These mixtures were deposited on the target plate (MTP 384) and dried at room temperature. Instrument was calibrated with PEG standards as well as protein calibrants (MW 2000-25000 Da).

3.3.4. Hydrodynamic size measurements

The hydrodynamic size of dendrimers was determined by dynamic light scattering (DLS) using ALV/LSE-5003 (ALV-GmbH, Langen, Germany). Samples were prepared in 0.15 M NaCl at 0.1 mg/mL and were filtered through 0.1 μm membrane prior to analysis. The measurements were carried out at 25°C and at an angle of 60°.

3.3.5. Cytotoxicity Studies

Cytotoxicity of dendrimers was determined by lactate dehydrogenase (LDH) assay. RAW 264.7 cells (American Type Culture Collection, Rockville, MA) were cultured in DMEM supplemented with 10 % FBS and penicillin/streptomycin at 37°C in a 5% carbon dioxide atmosphere. Cells were plated at the density of 5×10^5 cells/mL (100 μL /well) in 96 well plates. Later they were incubated with different concentrations of dendrimers (in PBS) for 24 h. After this incubation, plates were centrifuged for 5 min at 200 g, 5 μL of supernatant from each well was transferred to a new microplate and analyzed using LDH assay kit at 450 nm using Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria). Results were plotted in reference to 100% lysed cells. Changes in morphology and growth pattern of RAW 264.7 cells were also monitored after incubation with dendrimers.

3.3.6. DSC studies

DSC studies were conducted in triplicate on DSC Q1000 V9.0 (Universal 4.1 D, TA Instruments, USA) calibrated with indium (Goodfellow, 99.99 % Pure), and tin (NIST

SRM 2000). Samples weighing between 3-5 mg were placed in a standard aluminum pans and were scanned in the temperature range of 0 to 280°C at a heating rate of 10°C/min under nitrogen atmosphere. The DSC thermograms were analyzed using Universal analysis 2000 software (TA instruments, USA).

3.3.7. Drug loading

MTX was encapsulated in these nanocarriers by incubating them in DMF. Briefly, accurately weighed amount of dendrimer was dissolved in 5 mL of DMF in a screw capped vials. Subsequently, MTX was added to the vials and samples were stirred at room temperature for 48 h. To remove the unencapsulated drug, samples were dialyzed (MWCO 3500 Da) for 4 h against 4 L deionized water. The dialysate was lyophilized to obtain freeze dried dendrimer loaded with MTX. For the determination of the loading and encapsulation efficiency, DMF was added to weighed amount of freeze dried product. Samples were sonicated (550 Sonic dismembrator, Fisher Scientific, Ottawa, ON) for 1 min and later agitated for 24 h at 200 rpm to liberate MTX. MTX was analyzed using UV spectrophotometer U-2001 (Hitachi high technologies, Orlando, FL) at 376 nm with appropriate blank corrections to account for the interference due to absorbance by dendrimers.

3.3.8. Mechanism of drug encapsulation

The absorbance spectra of MTX, dendrimer and MTX loaded dendrimer in water were recorded on UV spectrophotometer U-2001 (Hitachi high technologies, Orlando, FL) from 200 to 500 nm. IR spectra of blank dendrimer and MTX loaded dendrimers were obtained without any sample preparation on Bruker vector 22 spectrometer (Bruker Optik GmbH, Ettlingen, Germany). Attenuated total internal reflectance accessory having a germanium crystal was used for this purpose. For each sample, 64 scans acquired at an angle of 45° were coadded with a spectral resolution of 2 cm⁻¹ in the region of 500-4000

cm^{-1} . ^1H NMR spectra of MTX ($50\ \mu\text{M}$) and MTX loaded dendrimer ($5\ \mu\text{M}$) in DMSO were recorded on Bruker ARX400 NMR spectrometer (Bruker Biospin, Billerica, MA) at room temperature and resonance frequency of 400 MHz.

3.3.9. Release studies

Release of MTX from dendrimers was studied in phosphate buffer (0.1 M, pH 7.4) at $37\pm 0.5^\circ\text{C}$ using dialysis membrane of MWCO 3500 Da. Dendrimers were weighed and transferred to the dialysis membranes which were placed in tubes containing 5 mL of release medium to maintain the sink conditions. Tubes were placed in a rotatory shaker and rotated at 50 rpm. Samples (1 mL) were collected at predetermined time-points with replacement. Amount of MTX released was quantified by UV spectrophotometer U-2001 (Hitachi high technologies, Orlando, FL) at 300 nm using a validated analytical method.

3.4. Results and Discussion

Synthetic control over the branched architecture of dendrimers places them apart from other polymeric scaffolds. Utilization of the structural attributes of dendritic constructs to develop nanocarriers for the improved containment of drug molecules depends on the better understanding of chemical features suitable for guest inclusion. Thus, in the present study correlation between the architecture of PEPE dendrimers and their drug loading as well as release properties was investigated.

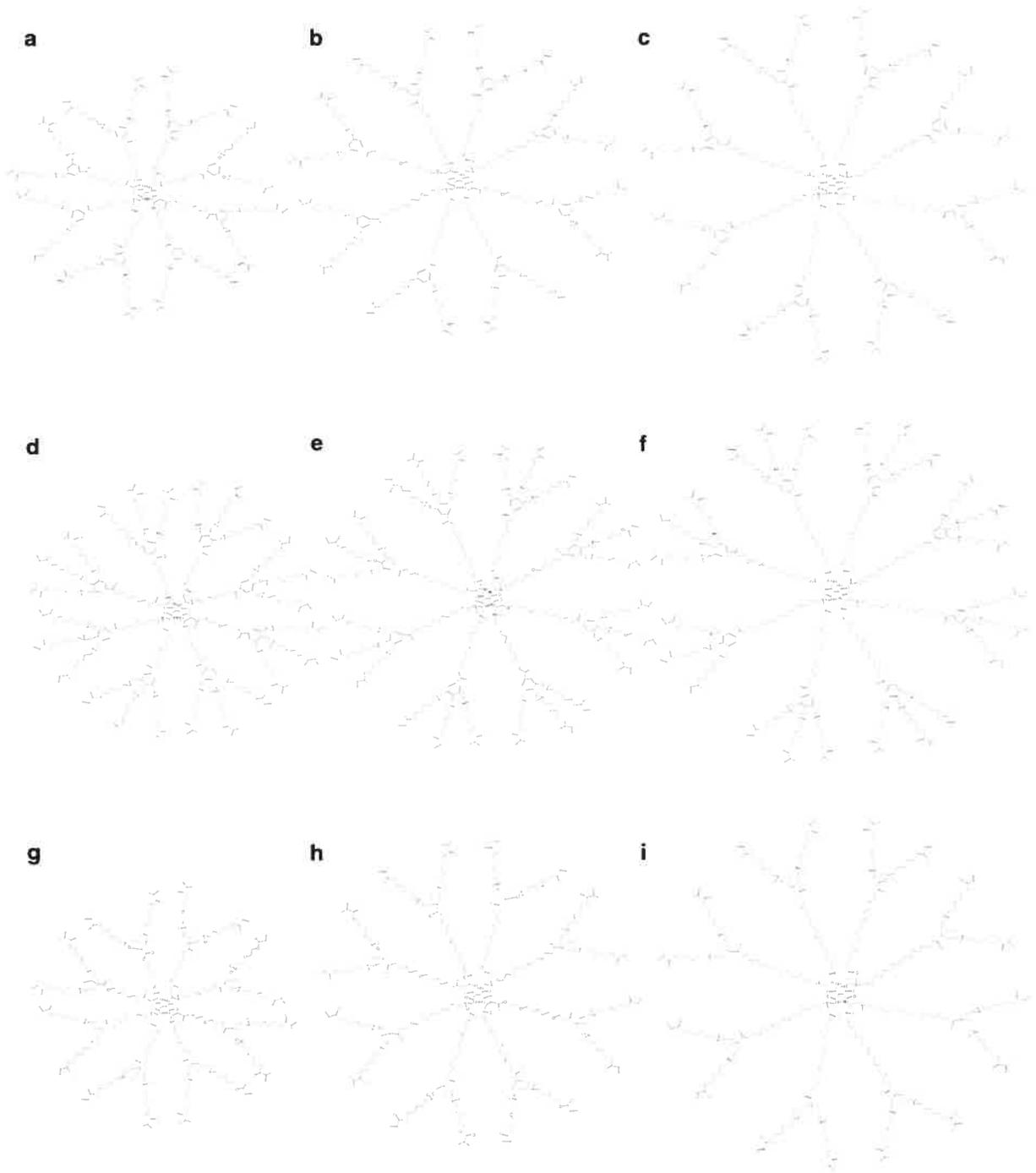


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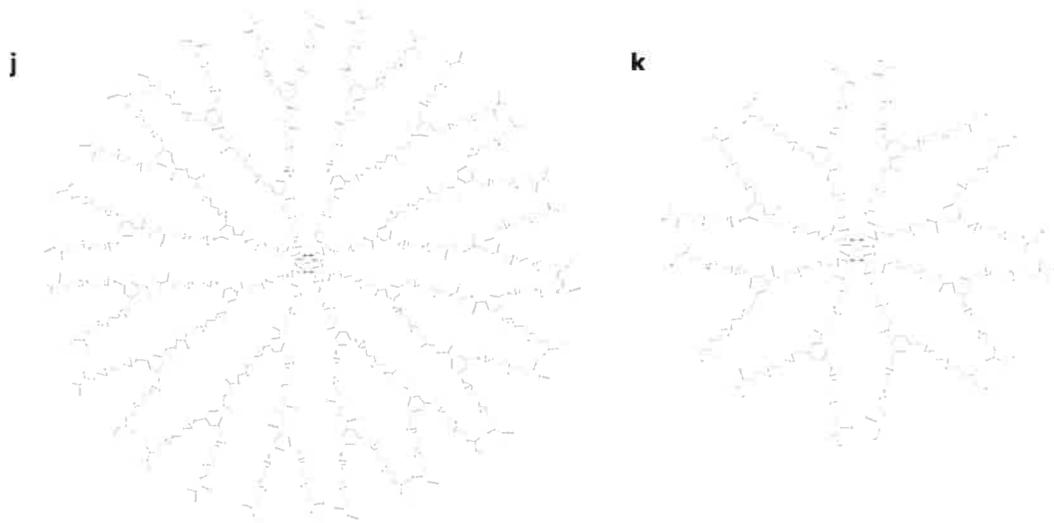


Figure 3.1. Structures of various dendrimers showing their architecture (a) den-1-(G2)-200, (b) den-1-(G2)-300, (c) den-1-(G2)-400, (d) den-2-(G2)-200, (e) den-2-(G2)-300, (f) den-2-(G2)-400, (g) den-3-(G2)-200, (h) den-3-(G2)-300, (i) den-3-(G2)-400, (j) den-1-(G3)-400, and (k) den-1-(G2)-OH-400.

3.4.1. Synthesis and characterization of dendrimers

Dendrimers consist of 1,2,3,4 butane tetracarboxylic acid as the core molecule, PEO in the interior cavity, DHBA, gallic acid or BHBA at the branching points and PEG methacrylate in terminal branches (Figure 3.1). Allyl group was selected as the pendant functional group because it is non-ionizable and hence, the resulting dendrimers would have reduced toxicity. In addition, it can be easily modified to hydroxyl, or carboxyl group (Nadeau et al., 2005) for conjugating ligands and other bioactive molecules. DHBA, gallic acid and BHBA were used as the branching units in den-1-series (Figure 3.1a-c), den-2-series (Figure 3.1d-f) and den-3-series (Figure 3.1g-i), respectively. The former two branching units yielded dendrimers having hydrophobic region in the structure; while, the incorporation of BHBA at branching points resulted in macromolecules devoid of hydrophobicity. Utilization of gallic acid as the branching unit in den-2-series also generated dendrimers with 24 terminal branches, as compared to den-1-series and den-3-series which have 16 terminal branches. The chain length of PEO (200, 300 and 400 Da) in the interior cavity of dendrimers was varied to achieve variation in the size of dendritic voids. All these modifications produced dendrimers with different architecture (Figure 3.1). The characterization of dendrimers by ^1H NMR and FTIR proved the successful synthesis (Supporting information). Further, molecular weight of dendrimers determined by MALDI-TOF and ^1H NMR indicated low polydispersity index ($M_n/M_w \leq 1.02$) and a good correlation with the expected values (Table 3.1). ^1H NMR spectra of the dendrimers of three different series shown in figure 3.2 demonstrate the differences in their structures.

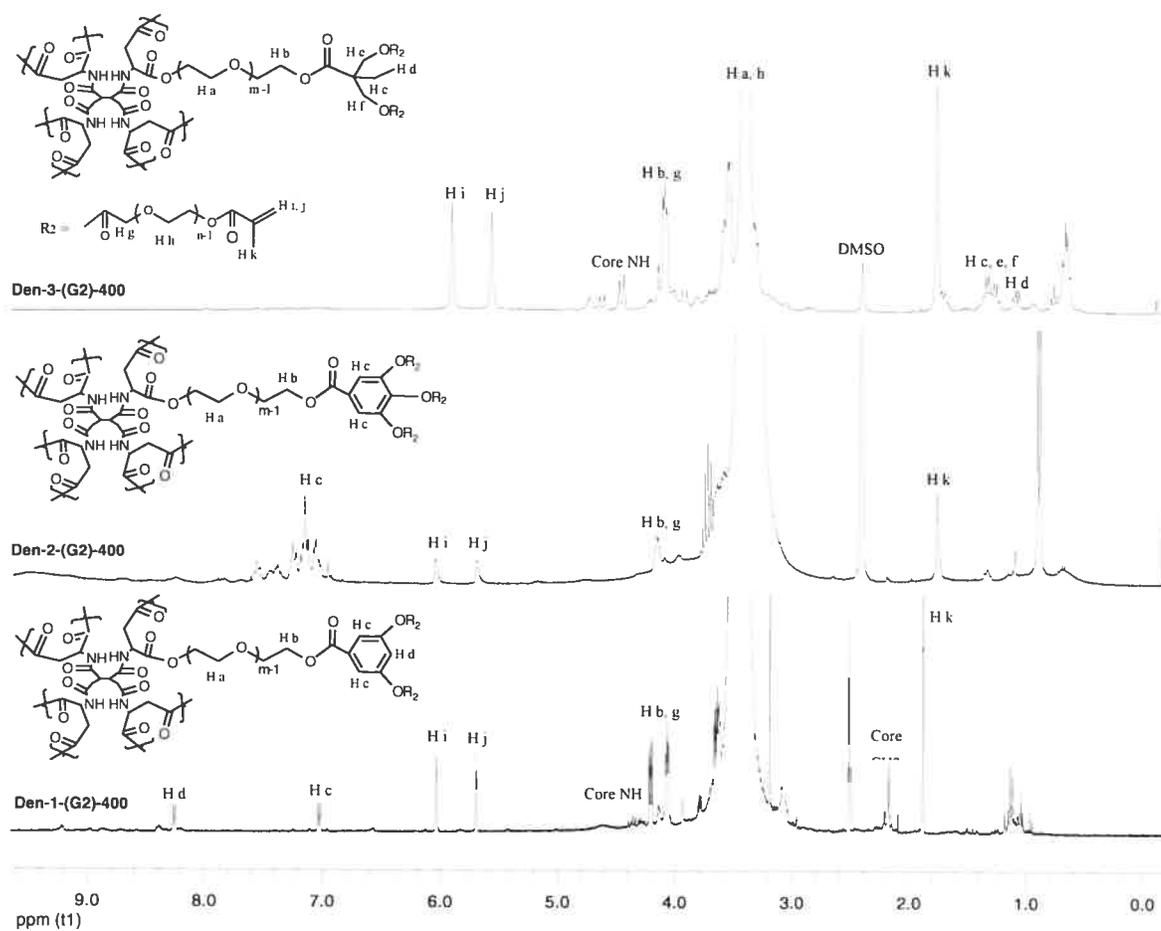


Figure 3.2. ^1H NMR spectra of dendrimers recorded at 400 MHz in DMSO.

Table 3.1. Characteristics of dendrimers

<i>Dendrimer</i>	<i>Generation</i>	<i>Branching molecule</i>	<i>Terminal functional group</i>	<i>Molecular weight (NMR)^a</i>	<i>Molecular weight (MALDI-TOF)</i>
Den-1-(G2)-200	2	DHBA	-COCH(CH ₃)CH=CH ₂	5142	3661
Den-1-(G2)-300	2	DHBA	-COCH(CH ₃)CH=CH ₂	7128	5550
Den-1-(G2)-400	2	DHBA	-COCH(CH ₃)CH=CH ₂	8130	7743
Den-2-(G2)-200	2	Gallic acid	-COCH(CH ₃)CH=CH ₂	8436	7134
Den-2-(G2)-300	2	Gallic acid	-COCH(CH ₃)CH=CH ₂	9212	10736
Den-2-(G2)-400	2	Gallic acid	-COCH(CH ₃)CH=CH ₂	10096	11922
Den-3-(G2)-200	2	BHBA	-COCH(CH ₃)CH=CH ₂	5502	-
Den-3-(G2)-300	2	BHBA	-COCH(CH ₃)CH=CH ₂	6474	-
Den-3-(G2)-400	2	BHBA	-COCH(CH ₃)CH=CH ₂	7026	-
Den-1-(G2)-OH	2	DHBA	-CO CH(CH ₃)CH ₂ CH ₂ OH	8024	7325
Den-1-(G3)-400	3	DHBA	-COCH(CH ₃)CH=CH ₂	16978	15072

^a Calculated based on relative integration ratio of peaks with respect to peak of -CH=CH₂ cis /trans proton or aromatic proton (in case of den-1-(G2)-OH)

3.4.2. Hydrodynamic size

Hydrodynamic size measurements were performed at concentration of 0.1 mg/mL in 0.15 M sodium chloride solution; since, aggregation was detected for all dendrimers in water. The size of dendrimers ranged between 2.45 to 8.24 nm (Table 3.2). On increasing the PEO molecular weight from 200 to 400 Da, size increased from 3.58 to 5.41 nm in den-1-series, from 4.19 to 7.41 nm in den-2-series and from 2.45 to 4.94 nm in den-3-series. Enhancement in the size was found to be statistically different at $p < 0.05$ for all series (One way RM ANOVA, *post-hoc* Tukey's test). This substantiates augmentation in the size of dendritic voids with molecular weight of PEO incorporated in the interior cavity.

3.4.3. Cytotoxicity of dendrimers

Dendrimers were synthesized largely from PEG and have hydrolysable ester linkages which will render them biodegradable and biocompatible (Bhadra et al., 2002; Jevapresphant et al., 2003). Dendrimers with the highest chain length of PEO were selected from each series for cytotoxicity studies because, higher molecular weight polymers are more toxic at same concentrations (Fischer et al., 2003). As seen in figure 3.3, no significant cell death was observed at concentrations as high as 250 $\mu\text{g/mL}$ for all the dendrimers. In addition, no changes in cell morphology and growth pattern were observed on microscopic examination of cells during the period of study. Even though these results are preliminary and more extensive toxicity studies in animal models are needed to ascertain their safety, yet they are satisfactorily encouraging to support the application of these dendrimers in drug delivery.

Table 3.2. Hydrodynamic size and loading of MTX in various dendrimers

	<i>Dendrimer</i>	<i>Hydrodynamic size (nm)^a</i>	<i>Drug loading (w/w)^b</i>	<i>Encapsulation efficiency (%)^b</i>
1	Den-1-(G2)-200	3.58 (0.11)	17.2 ± 1.8	62.8 ± 8.4
2	Den-1-(G2)-300	4.74 (0.13)	19.1 ± 1.1	65.5 ± 6.5
3	Den-1-(G2)-400	5.41 (0.27)	21.9 ± 1.7	71.9 ± 8.2
4	Den-2-(G2)-200	4.19 (0.11)	20.3 ± 1.0	68.8 ± 6.3
5	Den-2-(G2)-300	4.83 (0.19)	22.9 ± 1.2	73.3 ± 6.9
6	Den-2-(G2)-400	7.41 (0.14)	24.5 ± 1.1	78.4 ± 6.6
7	Den-3-(G2)-200	2.45 (0.12)	6.1 ± 1.6	27.1 ± 7.8
8	Den-3-(G2)-300	3.34 (0.06)	8.9 ± 1.4	31.2 ± 7.3
9	Den-3-(G2)-400	4.94 (0.22)	10.7 ± 1.7	35.6 ± 8.1
10	Den-1-(G2)-OH	5.11 (0.17)	21.4 ± 0.9	70.1 ± 4.9
11	Den-1-(G3)-400	8.24 (0.25)	22.3 ± 1.8	72.7 ± 8.6

^a Data are mean(PI) (n= 5)

^b Data are mean ± SD (n= 4-6)

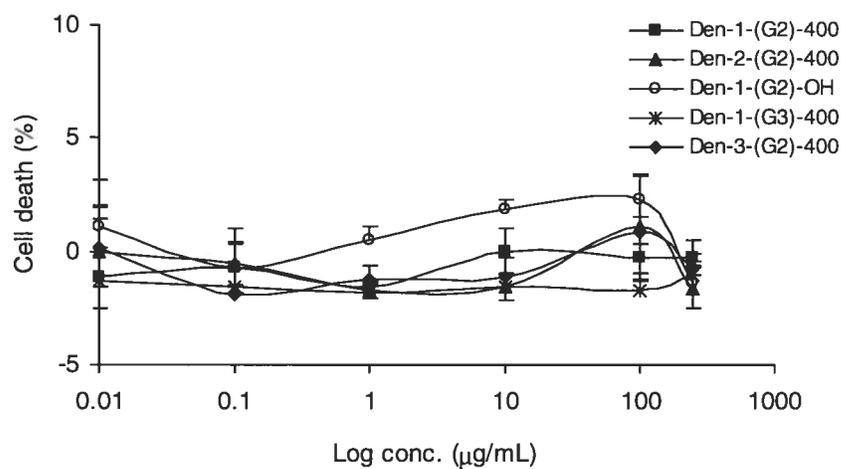


Figure 3.3. Cytotoxicity of dendrimers on murine macrophage cells, RAW 264.7. Data are mean ± SD (n=3).

3.4.4. Encapsulation studies

To evaluate the influence of architecture on drug loading capacity, MTX was selected as a guest molecule. Depending on the architecture, dendrimers showed encapsulation efficiency between 27-78% and drug loading between 6.1 to 24.5% w/w (Table 3.2). MTX loading increased with molecular weight of PEO in the interior cavity of the dendrimers for all three series. In den-1-series, it increased from 17.2 to 21.9 % w/w; in den-2-series, it increased from 20.3 to 24.5% w/w and in den-3-series from 6.1 to 10.7% w/w (Table 3.2). This is either due to increased solubilization of MTX by PEO chains and/or due to increase in the size of interior cavity with increase in the chain length of PEO. Though the former cannot be ruled, the latter effect seems to be pronounced as supported by DLS studies which revealed a PEO chain length dependent increase in dendrimer size. Several studies in literature have shown that increase in the molecular weight of PEG grafted on dendrimer surface enhances encapsulation capacity (Kojima et al., 2000; Yang et al., 2004). For instance, Kojima et al., (Kojima et al., 2000) reported increase in loading from 10 to 13 mol of MTX/mol of third generation PAMAM dendrimer on increasing PEG molecular weight from 550 to 2000 Da. But in these reports, PEG was typically grafted on the surface of dendrimers to provide a shell which is freely available to encapsulate the drug. In contrast, in the present study, the molecular weight of PEO incorporated in the interior cavity was varied. Thus, these results demonstrate the influence of the size of internal voids on the loading capacity. To the best of our knowledge, this is the first report in the literature where an attempt was made to alter the size of the interior cavity of dendrimer and observe its influence on drug loading.

MTX loading in den-3-series, i.e. dendrimers with BHBA as branching unit was low and ranged between 6.1 to 10.7% w/w. However, it was significantly higher ($p < 0.001$, One way RM ANOVA, *post-hoc* Tukey's test) in dendrimers of den-1-series and den-2-series (Table 3.2) which have DHBA and gallic acid (aromatic rings) as branching units. It is hypothesized that this occurs due to hydrophobic interaction of MTX with

aromatic rings which results in greater retention inside these carriers; demonstrating that incorporation of hydrophobic region in the dendritic structure could considerably improve loading capacity. MTX loading in den-2-series was relatively higher than in den-1-series ($p < 0.05$, one way RM ANOVA). Den-2-series has higher number of terminal PEG methacrylate branches as compared to den-1-series, hence it is postulated that improvement in loading capacity occurs due to higher density of PEG at the periphery. Maximum amount of drug (24.5% w/w) was encapsulated by den-2-(G2)-400, a second generation dendrimer containing PEO 400. MTX loading in den-2-(G2)-400 is higher than that reported by Pan et al., (Pan et al., 2005) for PEG (550 and 2000 Da) conjugated third generation PAMAM dendrimers and is comparable to that reported in PEG (550 and 2000 Da) conjugated fourth generation PAMAM dendrimers (Kojima et al., 2000).

Den-1-(G3)-400, a third generation dendrimer encapsulated 22.3% w/w of MTX which is comparable to den-1-(G2)-400, a second generation dendrimer. It is well documented in literature that increasing the generation of dendrimers improves drug loading (Kolhe et al., 2003; Ooya et al., 2004). On the contrary, in this study increase in generation did not significantly increase the inclusion of MTX in dendrimers, probably due to hindered diffusion of drug into the interior voids of dendrimers during the encapsulation process. Alteration in the allyl unit of terminal functional groups to hydroxyl also did not significantly change the loading efficiency (Table 3.2) signifying that MTX was not complexing with these terminal groups during encapsulation.

3.4.5. DSC studies

DSC thermogram of MTX showed three endotherms (Figure 3.4). The first two endotherms appearing at 95 °C and 120 °C are associated with loss of free and bound water, respectively and the third endotherm at 212 °C corresponds to the melting peak of MTX. These thermal characteristics of MTX implied that commercially obtained sample was a

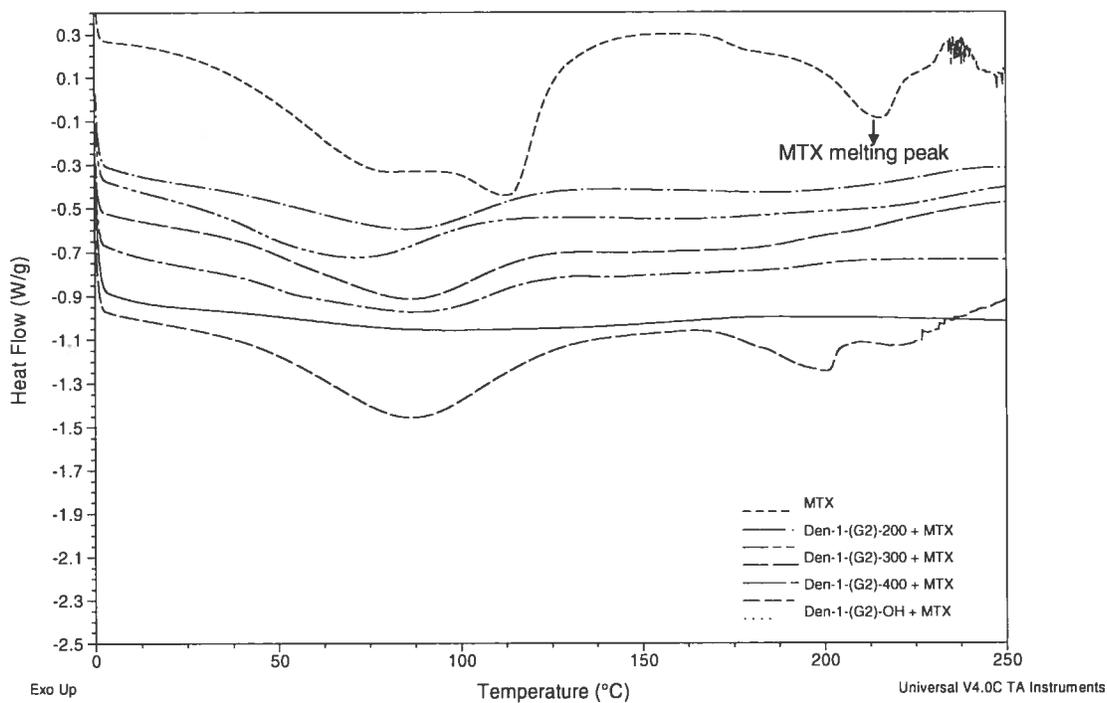


Figure 3.4. DSC thermograms of MTX and dendrimers loaded with MTX. Samples were scanned in a standard aluminum pan in the temperature range of 0 to 250°C at a heating rate of 10°C/min under nitrogen atmosphere.

hydrate form (Chan and Gonda, 1991). Thermograms of blank dendrimers exhibited no endotherm near the melting peak of MTX (Figure 3.1s, Supporting information). Encapsulation of MTX resulted in no apparent change in the thermal characteristics of dendrimers, moreover, the endotherm corresponding its melting was not observed in all cases. The absence of MTX melting endotherm suggests that drug is either molecularly dispersed or present in amorphous form inside the carriers.

3.4.6. Mechanism of encapsulation

In order to understand the chemical interaction between dendrimer and MTX, UV spectroscopy, FTIR and ^1H NMR studies were performed. The absorbance spectra of MTX before and after loading in dendrimer showed no shift in the absorption maxima (Figure 3.5). However, peak broadening was observed in den-1-(G3) and den-1-(G2)-OH. This advocates the absence of π - π complexation or interaction of dendrimer with the chromophore of MTX (Coffman and Kildsig, 1996; Agrawal et al., 2004). ^1H NMR spectra of MTX and MTX loaded den-1-(G2)-400 were acquired to probe further into the mechanism of encapsulation (Morgan et al., 2003; Ooya et al., 2004). Only the peaks corresponding to the aromatic protons could be identified in the spectrum of drug loaded dendrimer (Figure 3.6) and the estimation of ^1H NMR chemical shifts of un-encapsulated and encapsulated MTX exhibited no significant differences, demonstrating insignificant aromatic interactions. Absence of peaks designated to N-CH₃ (3.2 ppm) and methylene groups (2.0, 2.3 ppm) present in the side chain of MTX structure could be attributed to their involvement in interaction with dendrimers. Low peak intensities in ^1H NMR spectrum of MTX loaded dendrimer also proved that MTX is encapsulated inside the dendrimer and is not surface bound which is in agreement with the DSC data.

In the IR spectra of drug loaded dendrimers minor peaks appeared in the region of 1610-1500 cm^{-1} and 1250-1350 cm^{-1} (Figure 3.7). In the case of den-1-(G2)-400 loaded with MTX, peaks emerged at 1596 and 1508 cm^{-1} (Figure 3.7). Similar differences were

observed in the spectra of MTX-loaded den-1-(G2)-300 and den-1-(G2)-200 (data not shown). IR spectrum of MTX showed peaks at 1604 and 1625 cm^{-1} associated to carboxylate and amide C=O stretch. Thus, it is anticipated that peaks appearing in the region of 1610-1500 cm^{-1} in the spectra of MTX-loaded dendrimers correspond to amide carboxylic peaks of MTX which have been shifted due to the interaction of these groups with the ethylene oxide groups of dendrimer. Pan et al., (Pan et al., 2005) also reported similar interaction of MTX with PEGylated PAMAM dendrimers. However, it is interesting to note that no peak due to hydrogen bonding was detected in the spectra of drug loaded dendrimers pointing to negligible or very weak hydrogen bonding. The peaks at 1508 and 1600 cm^{-1} were not apparent in the case of den-2-(G2)-400, signifying minimal interaction between MTX and den-2-(G2)-400. The spectral changes appearing in den-1-(G2)-OH were strikingly different from other dendrimers, namely, broadening and decrease in the intensity of peak ascribed to primary or secondary hydroxyl in-plane bending at 1363 cm^{-1} , and shift in peaks due to asymmetric CH_2 vibrations at 2965 cm^{-1} (Coates, 2000). These changes in the spectral features of den-1-(G2)-OH loaded with MTX were due to substantial interaction between hydroxyl groups of dendrimer and amine or carboxylic groups of MTX. Higher drug loading in den-1-series and den-2-series (having aromatic rings in structure) as compared to den-3-series indicated involvement of hydrophobic interactions in the encapsulation of MTX. From these observations, it is comprehensible that the extent and type of interaction between dendrimer and MTX varies with the chemical structure. However, on the whole, weak hydrogen bonding, hydrophobic interactions and physical entrapment of drug are the major mechanisms of encapsulation.

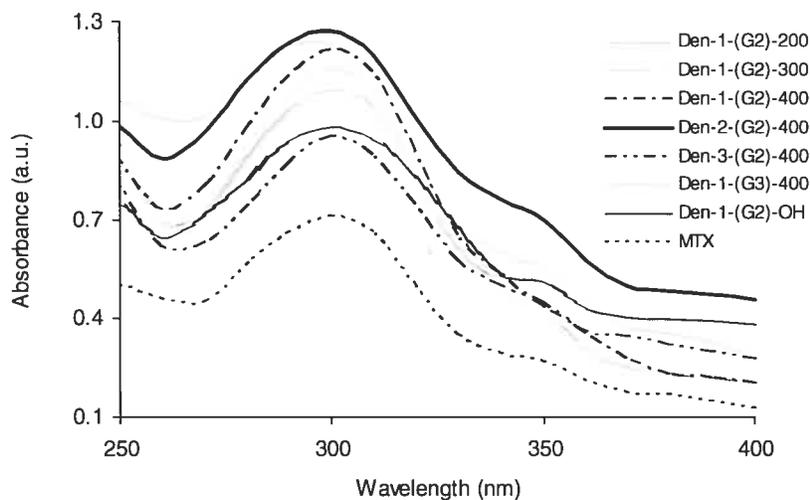


Figure 3.5. UV spectra of MTX and dendrimers loaded with MTX in water. Concentration of MTX in water was 0.026 mg/mL and that of dendrimers loaded with MTX was approximately 0.25 mg/mL.

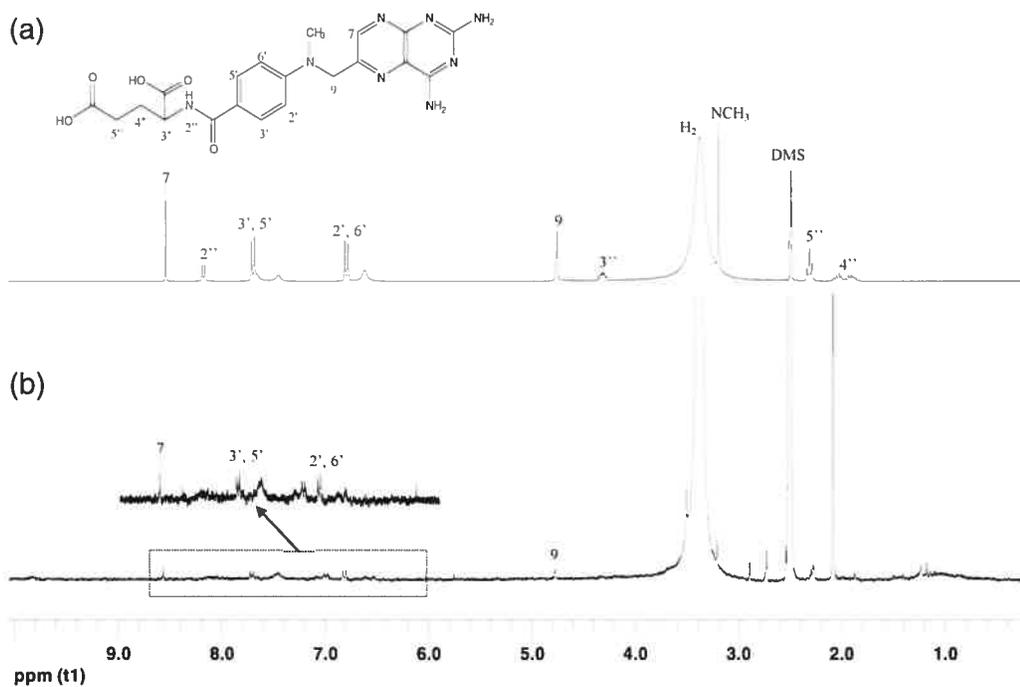


Figure 3.6. ^1H NMR spectra of (a) MTX (50 μM) and (b) MTX encapsulated in den-1-(G2)-400 (5 μM) measured in DMSO at 400MHz.

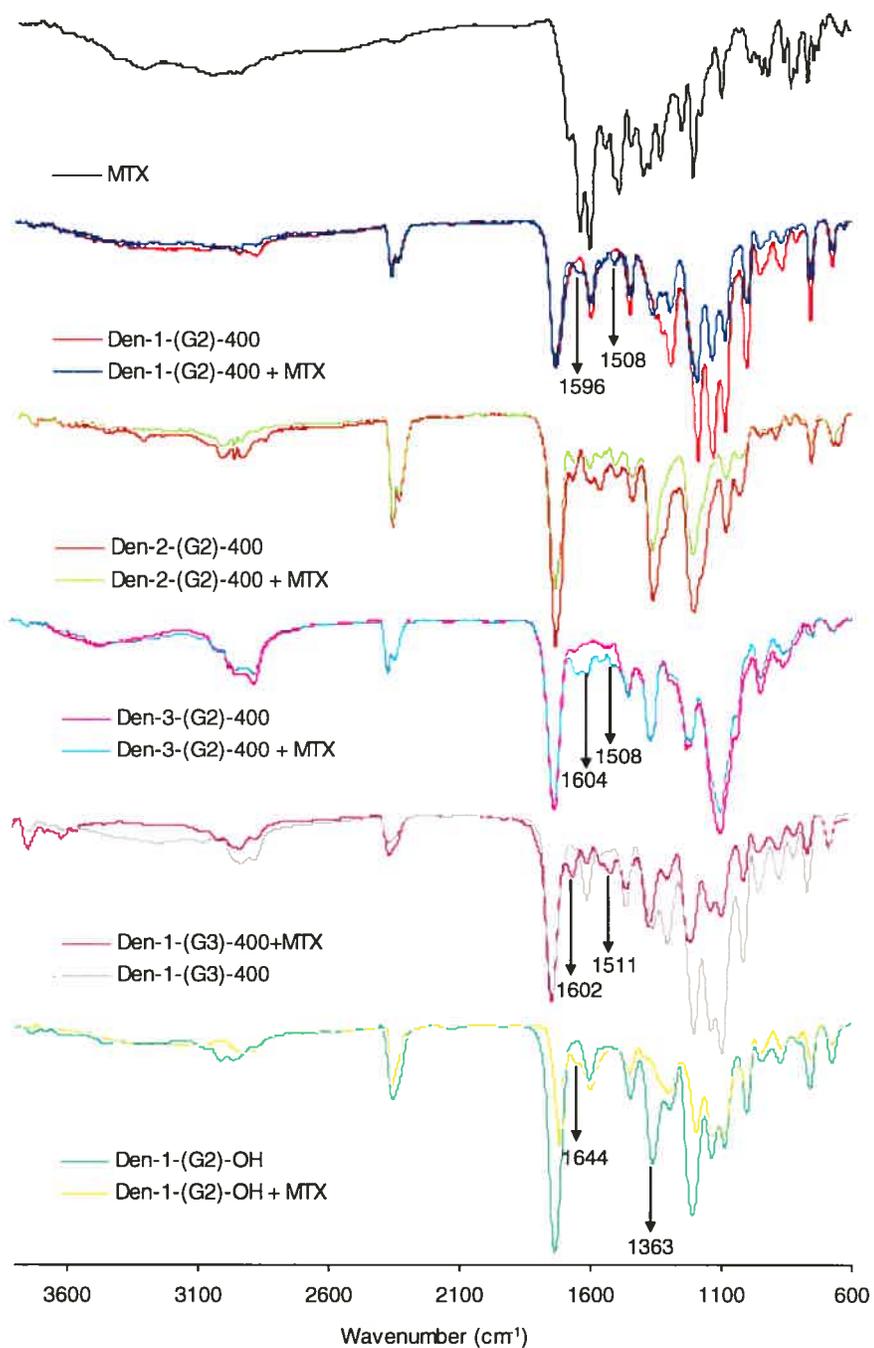


Figure 3.7. FTIR spectra of blank dendrimers and dendrimers loaded with MTX showing the changes in the IR spectral features of dendrimers in the presence of MTX. Samples were scanned at room temperature after lyophilization.

3.4.7. MTX release

Figure 3.8 shows the *in vitro* release of MTX from various dendrimers. A control experiment using free MTX was also carried out under similar conditions and complete diffusion across dialysis membrane was found to occur within 3 h (Figure 3.2s, Supporting information). Release of MTX from dendrimers was biphasic in all cases, with initial burst release followed by a slow release. Nevertheless, it is appreciable to mention that release of MTX in the present study was slower than PEG-conjugated PAMAM dendrimers wherein, all the encapsulated drug was released in 5 h in 150 mM NaCl (Kojima et al., 2000). Burst release of MTX has also been reported for various other delivery systems like, microspheres (Modi et al., 2006) (Liang et al., 2004), nanoparticles (Reddy and Murthy, 2004) in 0.01 M phosphate buffer. This rapid initial release of MTX is largely attributed to higher solubility of MTX in PBS at pH 7.4.

By and large, the release of MTX appeared to be profoundly influenced by the architecture of dendrimer. In den-3-series, release was very rapid with 70-90% of drug being released in 6 h and 100 % of drug being released in approximately 50 h (Figure 3.8c). Release of MTX was significantly slowed by the incorporation of aromatic rings at the branching points, for instance, in den-1-series, 64-70% of drug was released in 6 h and 100% of drug was released over a period of 168 h (Figure 3.8a). This probably happens due to the easy access of release medium to the encapsulated molecules in the absence of hydrophobic region in the structure of den-3-series. Interestingly, burst release was reduced to 43 to 61 % in den-2-series (Figure 3.8b). Most likely, the reduction in burst release is due to increase in the number of branches which results in condensed layer of PEG methacrylate at the outer surface and thus reduces the diffusion of drug, resulting in slower release. This 10-20% reduction in the burst release of drug like MTX, which has shown to exhibit rapid release from various delivery systems (Liang et al., 2004; Reddy and Murthy, 2004; Modi et al., 2006) indicates the potential of these dendrimers to control the release of

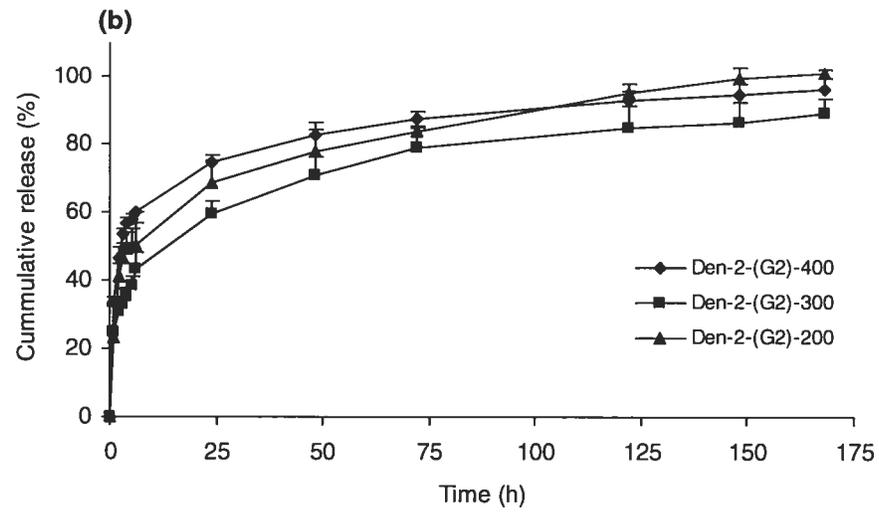
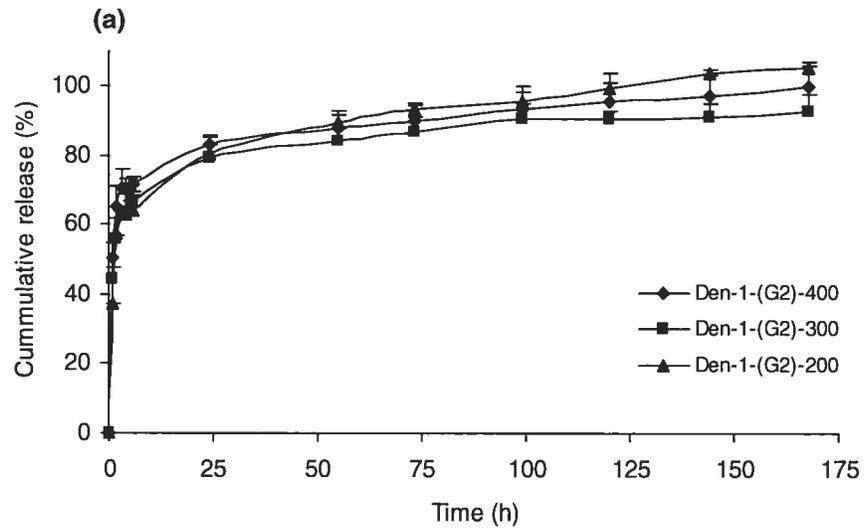


Figure continued.....

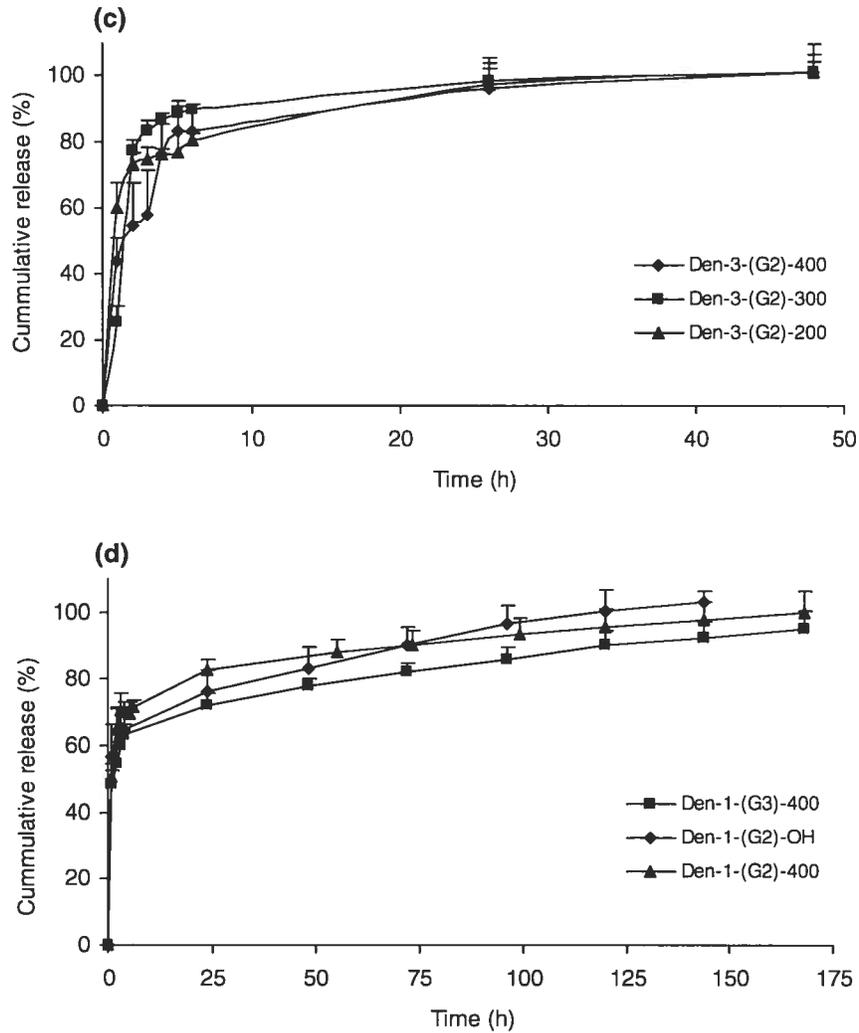


Figure 3.8. Comparative release profiles of MTX from (a) den-1-(G2)-400, den-1-(G2)-300 and den-1-(G2)-200; (b) den-2-(G2)-400, den-2-(G2)-300 and den-2-(G2)-200; (c) den-3-(G2)-400, den-3-(G2)-300 and den-3-(G2)-200; (d) den-1-(G3)-400, den-1-(G2)-OH and den-1-(G2)-400. Data are mean + SD (n=3).

other drugs with lower water solubility. No relationship between release rate and molecular weight of PEO in the interior cavity was observed (Figure 3.8a-c), highlighting that alteration in the size of the interior cavity of dendrimers does not significantly affect the release of drug. Such a characteristic of dendrimers with greater interior void could be advantageous since, it enhances drug loading but does not alter the release and hence, higher payloads of drug would be delivered in slow and sustained manner.

Increase in generation also reduced the burst release and overall release of MTX (Figure 3.8d), however, its influence was not as profound as observed by increase in the number of branches. MTX release from den-1-(G2)-OH was relatively rapid release in later stages (Figure 3.8d); even though it had shown greater interaction with MTX in FTIR studies (Figure 3.7). On the contrary, dendrimers of den-2-series which showed minimal interaction in FTIR exhibited slower release. This observation suggests that extent of interaction of encapsulated drug with dendrimer has no significant effect on its release properties. Dendrimers of den-1-series and den-2-series showed prolonged release of MTX, suggesting their usefulness in controlled release applications.

3.5. Conclusion

A series of PEPE dendrimers of different molecular architectures were synthesized. Encapsulation of MTX inside these macromolecules was profoundly influenced by the chemical structure of dendrimer. Spectroscopic studies illustrated that encapsulation occurs by weak hydrogen bonding and physical entrapment. Incorporation of aromatic rings in the structure of dendrimers, increase in the size of internal cavity and the number of PEG branches were found to be more attractive avenues for enhancing drug loading as compared to increasing the generation of dendrimer or changing the type of functional group. In addition, it was established that increase in the number of PEG branches and presence of hydrophobic region in dendritic architecture can be potentially employed for reducing the

burst release of drug from dendrimers. Dendrimers of den-2-series showed improved drug loading as well as slow release characteristics, indicating their potential application as prolonged release vehicles for drug targeting. It is contemplated that results described in this study will provide inputs into design and optimization of chemical structure of dendritic nanocarriers for sustained delivery applications.

3.6. Acknowledgements

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3.8. Supporting information

3.8.1. Synthesis of dendrimers

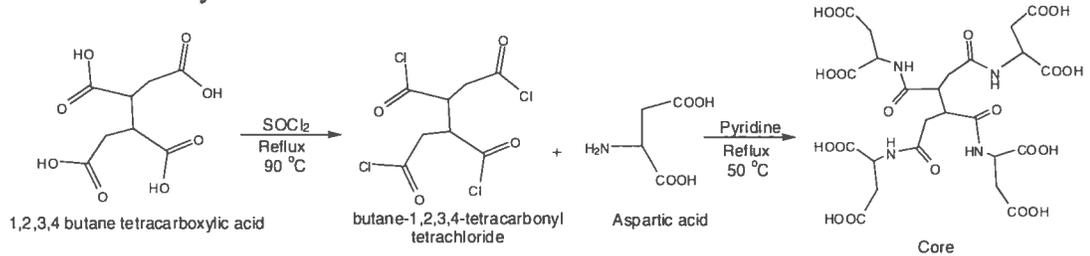
Dendrons were synthesized as mentioned in scheme 3.2s. For this purpose, PEG monomethacrylate (0.055 mol) was dissolved in 200 mL of acetone at room temperature. This solution was cooled to 0°C by placing the flask in ice bath. Later, 9 mL Jones' reagent (containing 0.02M CrO₃) was added dropwise over a period of 15 min. The flask was removed from ice bath after 30 min and reaction was allowed to occur at room temperature for 18 h. The reaction was quenched by adding 5 mL of isopropyl alcohol. Subsequently, 2 g of finely powdered activated carbon (10% w/w) was added and stirred for 2 h. This was filtered over wet Celite[®] and the filtrate was evaporated on rotatory evaporator. The viscous liquid obtained was dissolved in water and extracted with dichloromethane, the extracts were combined and evaporated to obtain the product (compound **1**) which was dried under vacuum for 48 h (Yield: 95 %).

Compound **1** (0.029 mol) was purged with nitrogen for approximately 15 min and thionyl chloride was added drop wise at room temperature. It was then refluxed at 50°C for 7 h. Thionyl chloride was evaporated under reduced pressure and viscous liquid obtained was dried under vacuum to obtain compound **2**. This step was followed by esterification of compound **2** with DHBA for den-1-(G 2) series, gallic acid for den-2-(G 2) series and BHBA for the synthesis of den-3-(G 2) series. In brief, compound **2** (0.028 mol) was dissolved in pyridine under nitrogen atmosphere and to this solution DHBA (0.013 mol) or gallic acid (0.0091 mol) or BHBA (0.013 mol) was added. Reactants were refluxed at 50°C for 18 h. Thereafter, pyridine was evaporated by rotavapour and product was precipitated in diethyl ether. It was further purified by gel chromatography (Sephacrose CL-4B) using water at pH 7.4 as eluent. The product was recovered as viscous liquid by freeze drying (Yields: 85-92 %). Subsequent to this step compound **3**, or **4**, or **5** (4.1 mmol) was

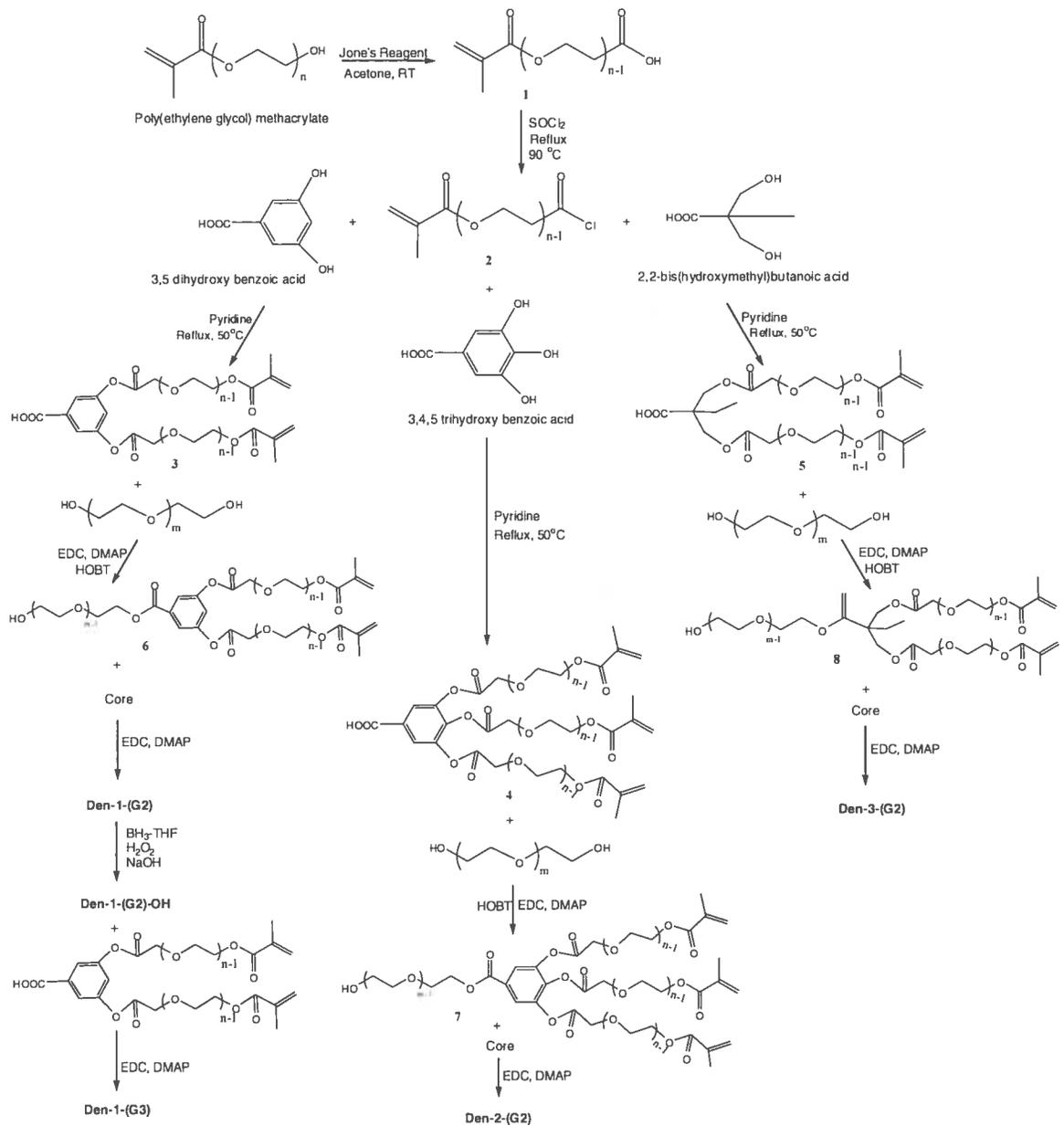
dissolved in dichloromethane, and esterified with PEG (400) (4.6 mmol) utilizing EDC (6.9 mmol), DMAP (4.5 mmol) and HOBT (0.45 mmol) for 48 h at room temperature. Afterwards the EDCU precipitate was filtered and filtrate was concentrated by rotatory evaporator. The product obtained was precipitated in hexane and diethyl ether mixture (1:1) and dialyzed against deionized water for 72 h (MWCO 1000 Da) (Yields: 79-88 %).

Dendrimer-1-(G 2)-400 was obtained by coupling core with compound **6**, dendrimer-2-(G 2)-400 with compound **7**, and for dendrimer-3-(G 2)-400 to compound **8**. Briefly, compound **6**, or **7**, or **8** (1.60 mmol) was dissolved in DMF, to this core molecule (0.18 mmol), EDC (1.83 mmol) and DMAP (1.48 mmol) were added. The reaction was allowed to occur for 96 h at room temperature. The EDCU precipitate was filtered and the filtrate was precipitated in diethyl ether. The product obtained was dialyzed (MWCO 3500 Da) against deionized water for 72 h and the dialysate was freeze dried (Yields: 70-75 %). For the synthesis of den-1-(G2)-200, den-2-(G2)-200, den-3-(G2)-200 and den-1-(G2)-300, den-2-(G2)-300, den-3-(G2)-300 similar reaction scheme were used except that instead of PEG 400, PEG 200 and PEG 300 were used, respectively. Den-1-(G2)-OH, containing hydroxyl groups as terminal functional groups was synthesized by hydroboration of den-1-(G2)-400. The higher generation dendrimer, den-1-(G 3) was synthesized by divergently coupling den-1-(G2)-OH to compound **3** in the presence of EDC and DMAP.

Scheme 3.1s. Synthesis of core



Scheme 3.2s. Synthesis of dendrimers



3.8.2. Characterization of dendrimers by $^1\text{HNMR}$ and FTIR

a) Den-1-(G2)-400: $^1\text{HNMR}$ (δ , ppm, DMSO): 8.27 (d, 16H, -CH-, *aromatic*), 7.02 (t, 8H, -CH-, *aromatic*), 6.03 (s, 16H, *trans* CH₂=CH-), 5.69 (s, 16H, *cis* CH₂=CH-), 4.36 (s, 2H, -NH-CO-, *core*), 4.34 (s, 2H, -NH-CO-, *core*), 4.19 (t, 32H, -CH₂-), 4.14 (t, 32H, -CH₂-), 4.07 (t, 64H, -CH₂-), 3.93 (s, 4H, -CH-NH-, *core*), 3.79 (t, 32H, -CH₂-), 3.65 (m, 32H, -CH₂-), 3.42 (m, 386H, -O-CH₂-CH₂-O-), 3.19 (t, 32H, -CH₂-), 2.95 (2H, -CH-CO-, *core*), 2.20 (s, 8H, -CH₂-CO-, *core*), 2.17 (s, 4H, -CH₂-, *core*), 1.88 (s, 48H, CH₃); FTIR (neat) ν_{max} cm⁻¹: 2935, 2870, 1728, 1649, 1540, 1454, 1350, 1100, 947, 878, 771.

b) Den-1-(G2)-300: $^1\text{HNMR}$ (δ , ppm, DMSO): 8.31 (d, 16H, -CH-, *aromatic*), 7.11 (t, 8H, -CH-, *aromatic*), 6.14 (s, 16H, *trans* CH₂=CH-), 5.81 (s, 16H, *cis* CH₂=CH-), 4.47 (s, 2H, -NH-CO-, *core*), 4.46 (s, 2H, -NH-CO-, *core*), 4.31 (t, 32H, -CH₂-), 4.24 (t, 32H, -CH₂-), 4.19 (t, 64H, -CH₂-), 4.05 (s, 4H, -CH-NH-, *core*), 3.92 (t, 32H, -CH₂-), 3.77 (m, 32H, -CH₂-), 3.54 (m, 338H, -O-CH₂-CH₂-O-), 3.21 (t, 32H, -CH₂-), 3.07 (2H, -CH-CO-, *core*), 2.32 (s, 8H, -CH₂-CO-, *core*), 2.29 (s, 4H, -CH₂-, *core*), 2.01 (s, 48H, CH₃); FTIR (neat) ν_{max} cm⁻¹: 3319, 2955, 2881, 2356, 1734, 1599, 1449, 1354, 1290, 1192, 1129, 1082, 999, 940, 864, 754.

c) Den-1-(G2)-200: $^1\text{HNMR}$ (δ , ppm, DMSO): 8.33 (d, 16H, -CH-, *aromatic*), 7.08 (t, 8H, -CH-, *aromatic*), 6.09 (s, 16H, *trans* CH₂=CH-), 5.79 (s, 16H, *cis* CH₂=CH-), 4.42 (s, 2H, -NH-CO-, *core*), 4.41 (s, 2H, -NH-CO-, *core*), 4.25 (t, 32H, -CH₂-), 4.20 (t, 32H, -CH₂-), 4.14 (t, 64H, -CH₂-), 3.98 (s, 4H, -CH-NH-, *core*), 3.86 (t, 32H, -CH₂-), 3.70 (m, 32H, -CH₂-), 3.48 (m, 224H, -O-CH₂-CH₂-O-), 3.25 (t, 32H, -CH₂-), 3.01 (2H, -CH-CO-, *core*), 2.26 (s, 8H, -CH₂-CO-, *core*), 2.23 (s, 4H, -CH₂-, *core*), 1.95 (s, 48H, CH₃); FTIR (neat) ν_{max} cm⁻¹: 3288, 2938, 2357, 1732, 1600, 1449, 1357, 1290, 1198, 1124, 1085, 1001, 945, 862, 757.

d) Den-2-(G2)-400: $^1\text{H NMR}$ (δ , ppm, DMSO): 7.29 (s, 16H, -CH-aromatic), 5.57 (s, 24H, *trans* $\text{CH}_2=\text{CH}$ -), 5.52 (s, 24H, *cis* $\text{CH}_2=\text{CH}$ -), 3.48 (t, 16H, $-\text{CH}_2$ -), 3.33 (m, 755H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}$ -), 2.87 (s, 8H, $-\text{CH}_2-\text{CO}$ -, *core*), 2.71 (s, 4H, $-\text{CH}-\text{CH}_2-\text{CO}$ -, *core*), 1.85 (s, 4H, $-\text{CH}_2$ -, *core*), 1.69 (s, 2H, $-\text{CH}$ -, *core*), 1.47 (t, 72H, CH_3). FTIR (neat) ν_{max} cm^{-1} : 2932, 2361, 1731, 1677, 1560, 1508, 1453, 1319, 1262, 1175, 1082, 1029, 947, 890, 754.

e) Den-2-(G2)-300: $^1\text{H NMR}$ (δ , ppm, DMSO): 7.43 (s, 16H, -CH-aromatic), 6.01 (s, 24H, *trans* $\text{CH}_2=\text{CH}$ -), 5.75 (s, 24H, *cis* $\text{CH}_2=\text{CH}$ -), 3.53 (t, 16H, $-\text{CH}_2$ -), 3.42 (m, 688H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}$ -), 2.98 (s, 8H, $-\text{CH}_2-\text{CO}$ -, *core*), 2.86 (s, 4H, $-\text{CH}-\text{CH}_2-\text{CO}$ -, *core*), 2.21 (s, 4H, $-\text{CH}_2$ -, *core*), 1.99 (s, 2H, $-\text{CH}$ -, *core*), 1.72 (t, 72H, CH_3). FTIR (neat) ν_{max} cm^{-1} : 3352, 2921, 2845, 2357, 1732, 1546, 1450, 1359, 1267, 1112, 1056, 982, 865, 756.

f) Den-2-(G2)-200: $^1\text{H NMR}$ (δ , ppm, DMSO): 7.45 (s, 16H, -CH-aromatic), 6.03 (s, 24H, *trans* $\text{CH}_2=\text{CH}$ -), 5.78 (s, 24H, *cis* $\text{CH}_2=\text{CH}$ -), 3.58 (t, 16H, $-\text{CH}_2$ -), 3.46 (m, 630H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}$ -), 3.02 (s, 8H, $-\text{CH}_2-\text{CO}$ -, *core*), 2.90 (s, 4H, $-\text{CH}-\text{CH}_2-\text{CO}$ -, *core*), 2.26 (s, 4H, $-\text{CH}_2$ -, *core*), 2.04 (s, 2H, $-\text{CH}$ -, *core*), 1.76 (t, 72H, CH_3). FTIR (neat) ν_{max} cm^{-1} : 3330, 2933, 2358, 1732, 1576, 1455, 1354, 1275, 1123, 1002, 865, 755.

g) Den-3-(G2)-400: 6.01 (s, 16H, *trans* $\text{CH}_2=\text{CH}$ -), 5.67 (s, 16H, *cis* $\text{CH}_2=\text{CH}$ -), 3.49 (t, 16H, $-\text{CH}_2$ -), 3.40 (m, 390H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}$ -), 2.74 (s, 8H, $-\text{CH}_2-\text{CO}$ -, *core*), 2.05 (s, 4H, $-\text{CH}_2$ -, *core*), 1.86 (t, 48H, CH_3), 1.45 (m, 144H, CH_2), 1.18 (m, 24H, CH_3). FTIR (neat) ν_{max} cm^{-1} : 3458, 2873, 2356, 1731, 1533, 1452, 1363, 1219, 1102, 944, 861, 751, 672.

h) Den-3-(G2)-300: 6.03 (s, 16H, *trans* $\text{CH}_2=\text{CH}$ -), 5.70 (s, 16H, *cis* $\text{CH}_2=\text{CH}$ -), 3.51 (t, 16H, $-\text{CH}_2$ -), 3.43 (m, 358H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}$ -), 2.77 (s, 8H, $-\text{CH}_2-\text{CO}$ -, *core*), 2.08 (s, 4H, $-\text{CH}_2$ -, *core*), 1.89 (t, 48H, CH_3), 1.48 (m, 144H, CH_2), 1.22 (m, 24H, CH_3). FTIR (neat) ν_{max} cm^{-1} : 3323, 2956, 2344, 1730, 1545, 1466, 1372, 1231, 1121, 1001, 927, 765, 686.

i) Den-3-(G2)-200: 5.99 (s, 16H, *trans* $\text{CH}_2=\text{CH}$ -), 5.64 (s, 16H, *cis* $\text{CH}_2=\text{CH}$ -), 3.47 (t, 16H, $-\text{CH}_2$ -), 3.37 (m, 305H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}$ -), 2.70 (s, 8H, $-\text{CH}_2-\text{CO}$ -, *core*), 2.01 (s, 4H,

-CH₂-, *core*), 1.82 (t, 48H, CH₃), 1.41 (m, 144H, CH₂), 1.15 (m, 24H, CH₃). FTIR (neat) ν_{\max} cm⁻¹: 3422, 2943, 2363, 1732, 1675, 1561, 1452, 1317, 1260, 1174, 1081, 1025, 940, 895, 753.

j) Den-1-(G2)-OH: ¹HNMR (δ , ppm, DMSO): 7.67 (d, 16H, -CH-, *aromatic*), 7.18 (s, 8H, -CH-, *aromatic*), 4.47 (s, 16H, -OH), 3.90, 3.79 (m, 379H, -O-CH₂-CH₂-O-), 3.61 (s, 4H, -CH-NH-, *core*), 3.57 (t, 64H, -CH₂-), 2.91 (s, 2H, -CH-CO-, *core*), 2.90 (s, 8H, -CH₂-CO-, *core*), 2.13 (s, 4H, -CH₂-, *core*), 1.37 (d, 48H, CH₃); FTIR (neat) ν_{\max} cm⁻¹: 3280, 2949, 2873, 2360, 1917, 1868, 1835, 1732, 1649, 1599, 1543, 1513, 1451, 1291, 1188, 1132, 1083, 1000, 947, 867, 807, 770, 673.

k) Den-1-(G3)-400: ¹HNMR (δ , ppm, DMSO): 6.97 (d, 32H, -CH-, *aromatic*), 6.51 (t, 32H, -CH-, *aromatic*), 6.00 (s, 64H, *trans* CH₂=CH-), 5.66 (s, 64H, *cis* CH₂=CH-), 4.35 (t, 64H, -CH₂-), 4.16 (t, 64H, -CH₂-), 3.61 (t, 128H, -CH₂-), 3.44 (m, 1239H, -O-CH₂-CH₂-O-), 3.01 (t, 64H, -CH₂-), 1.84 (s, 192H, CH₃); FTIR (neat) ν_{\max} cm⁻¹: 3334, 2949, 2874, 2361, 2335, 1916, 1867, 1835, 1792, 1738, 1648, 1598, 1549, 1514, 1452, 1341, 1289, 1187, 1130, 1082, 1000, 948, 863, 810, 770, 672, 585, 514.

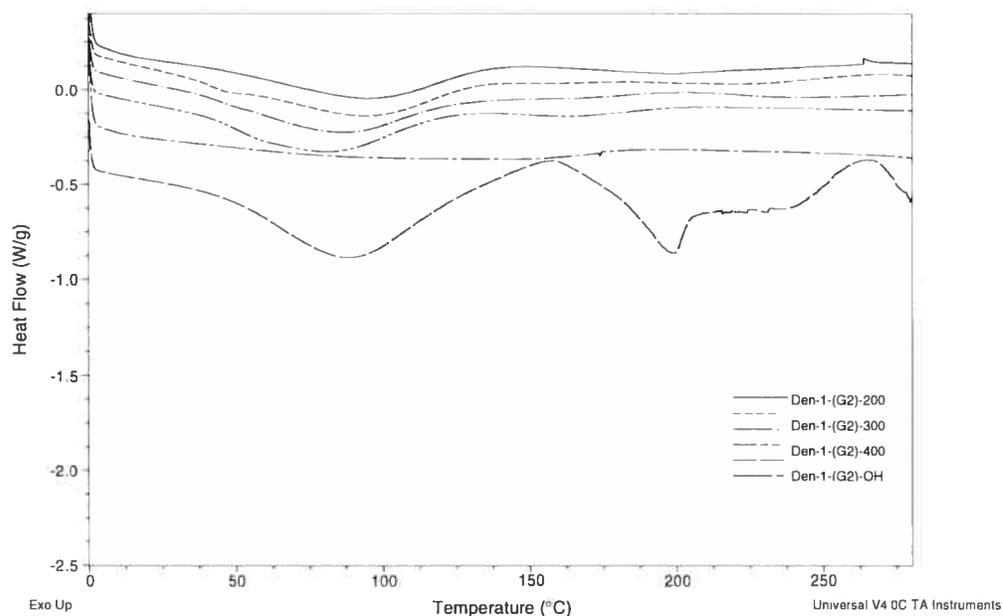


Figure 3.1s. DSC thermograms of blank dendrimers. Samples were scanned in a standard aluminum pan, in the temperature range of 0 to 250°C at a heating rate of 10°C/min under nitrogen atmosphere.

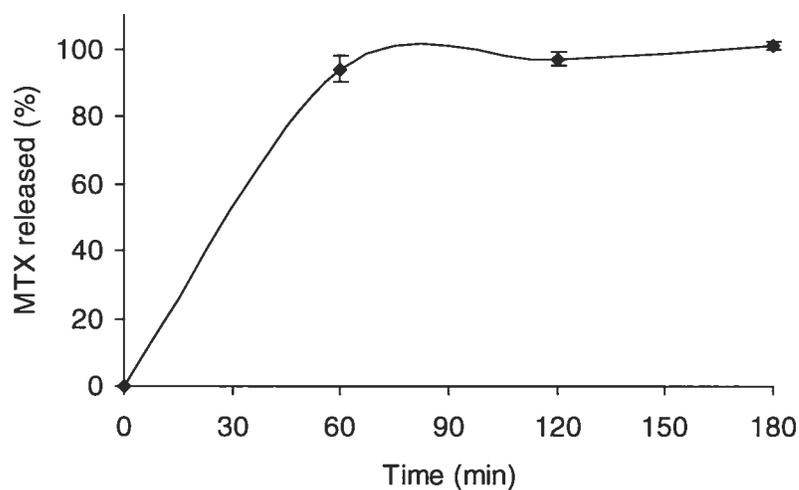


Figure 3.2s. Release of MTX from dialysis membrane (MWCO 3500 Da) in phosphate buffer (pH 7.4) at 37°C.

CHAPTER THREE

RESEARCH PAPER

Polyether-co-polyester dendrimers for delivery across the blood brain barrier

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4.1. Abstract

The present study was aimed at exploring the application of polyether-co-polyester (PEPE) dendrimers for delivery across the blood brain barrier (BBB). The cytotoxicity of a series of PEPE dendrimers was evaluated on brain endothelial cells (bEnd.3) and their hemolytic potential was determined by using rat red blood cells (RBCs). Plasma protein adsorption on the surface of dendrimers was also evaluated. Dendrimers were labeled with rhodamine as a fluorescent probe and their internalization into bEnd.3 cells was studied to comprehend the mechanism of uptake. Ability of these dendrimers to cross BBB was ascertained by using an *in vitro* model consisting of co-culture of bEnd.3 and U373 MG cells. Dendrimers produced no significant reduction in cellular proliferation even at concentration of 5 mg/mL and induced hemolysis less than 10% at concentration as high as 5 mg/mL. Opsonic proteins like immunoglobulin and complement factors were not adsorbed on their surface, additionally, total amount of protein adsorbed was also low. Internalization of dendrimers into bEnd.3 cells was inhibited by low temperature demonstrating energy dependent endocytosis. Clathrin and caveolin inhibitors produced partial inhibition of the uptake, signifying contribution of both pathways in the internalization process. Further, dendrimers were able to cross BBB model in high amounts with P_{app} of $19.7 \pm 1.9 \times 10^{-6}$ cm/sec and only 14% reduction in transendothelial electrical resistance (TEER) during initial 4 h. In a nutshell, these studies demonstrate the suitability of PEPE dendrimers for safe and enhanced delivery of therapeutics to the brain.

Keywords: dendrimers, toxicity, brain endothelial cells, uptake, BBB model, permeability, P-gp

4.2. Introduction

Delivery of drugs to the brain has always remained a formidable task for pharmaceutical scientists. This has been not only due to the inability of drugs to cross the blood brain barrier (BBB), but also owing to the shortcomings in the methods by which drugs are delivered to the brain (Pardridge, 2002a). The poor permeability of various drugs as well as delivery systems across BBB are primarily because of its anatomical characteristics, namely, the presence of tight junctions, lack of capillary fenestrations and presence of efflux transporters (Brightman, 1977; Bar, 1980; Tsuji and Tamai, 1999). It is reported that BBB blocks delivery of more than 98% of the central nervous system (CNS) drugs (Pardridge, 2002b). As a consequence, many potentially effective therapeutics for the treatment of CNS diseases have failed to reach the clinics. Due to the inefficiency of conventional drug delivery systems, extensive efforts have been made to develop novel strategies to overcome the obstacles of brain drug delivery (Fishman and Christy, 1965; Alyautidin et al., 1997; Schwarze et al., 1999; Shoichet and Winn, 2000; Siegel et al., 2000; Zhang et al., 2002; Kunwar, 2003; Westphal et al., 2006). Nevertheless, the delivery approaches available clinically or in clinical trials for delivery of drugs to brain include, intracerebral implantation of controlled release implants, intracerebroventricular infusion, convention enhanced delivery, BBB disruption by osmotic agents (Fishman and Christy, 1965; Siegel et al., 2000; Kunwar, 2003; Westphal et al., 2006). These strategies are either highly invasive and compromise BBB properties or have low efficacy due to limited diffusion of drug to the surrounding tissue. Hence, there is a continuing need to design suitable therapeutic strategies that deliver drugs to CNS in a safe and more effective manner. In theory, the best treatment would be the one which does not alter BBB, but delivers high payloads of drugs to the target site in the brain.

Various studies have shown that immunoliposomes with transferrin vectors (Cerletti et al., 2000; Shi et al., 2001; Huwyler et al., 2002; Pardridge, 2002a) and nanoparticles coated with poloxamer can deliver drugs to brain (Alyautdin et al., 1997; Gulyaev et al., 1999). It was also reported that nanoparticles made with PEGylated amphiphilic copolymers can penetrate into brain without significant alteration of BBB (Calvo et al., 2001). Thus, designing carriers with nanometric size, PEGylated surface and vector for BBB receptors is an attractive strategy. However, the poor stability of liposome has limited their applicability and market potential. On the other hand, binding ligands to the surface of nanoparticles remains problematic due to the small number of available surface groups and steric hindrance. Dendrimers can provide an interesting alternative to these delivery systems because of their highly branched and complex architecture, which endows them with properties such as monodisperse size, presence of internal cavities and high surface functionality (Klajnert and Bryszewska, 2001). The presence of large number of surface groups provides opportunity to conjugate ligands not only for transport across BBB but also for targeting to specific cells e.g. tumors. At the same time the presence of internal cavities allows encapsulation of guest molecules (Esfand and Tomalia, 2001; Cloninger, 2002; Patri et al., 2002; Aulenta et al., 2003). Another most lucrative property of the dendrimers is the easy tunability of their architecture, size, solubility etc. (Lee et al., 2005; Svenson and Tomalia, 2005). It is well known that permeability of drug molecules across BBB is dependent on the lipophilicity as well as the size of molecules. Keeping these factors in consideration, unlike any other drug carrier, dendrimers can be easily modified to obtain desired lipophilicity and size to enhance their permeability across BBB. Thus, dendrimers can serve as a tailorable delivery system for improved delivery of drugs to the brain.

There is abundant evidence in the literature that a range of endocytotic processes occur at BBB, which provide entry to variety of nutrients, amino acids and hormones (Smith and Gumbleton, 2006). In fact, endocytosis is the major port for entry into the

brain. Hence, attempts have been made to utilize it for the delivery of therapeutics across BBB and indeed, some impressive results have been documented in the literature using nanoparticles and liposomes as drug carriers (Shi et al., 2001; Huwlyer et al., 2002; Pardridge, 2002a). Application of dendrimers in brain delivery is relatively a new area of research. The mechanism of uptake and transfer of dendrimers across BBB has not been extensively evaluated so far. A detailed characterization of internalization and uptake pathways of dendrimers, specifically understanding whether entry of dendrimers takes place randomly or at preferential sites in plasma membrane will be important for the design and use of dendrimers in brain delivery. With this premise, this study was designed to explore the mechanism of uptake and transfer of polyether-co-polyester (PEPE) dendrimers across BBB. A series of PEPE dendrimers were synthesized largely from polyethylene glycol (PEG) (Dhanikula and Hildgen, 2006) so that they would have lower toxicity, higher permeability across BBB (Calvo et al., 2001) and also longer circulation half-life (Klibanov et al., 1990; Gabizon et al., 1993). In order to select a candidate with desirable characteristics, cytotoxicity and hemolytic potential of a series of dendrimers was determined. Plasma protein adsorption on dendrimers was also evaluated to detect opsonization of their surface. The dynamics and mechanism of internalization of dendrimers into brain endothelial cells (bEnd.3) was assessed to understand the endocytotic process. Finally, ability of these dendrimers to cross BBB was evaluated by using an *in vitro* BBB model.

4.3. Experimental

4.3.1. Materials

1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), 4-(dimethyl amino) pyridine (DMAP), sodium thiosulphate, silver nitrate, sodium carbonate, sodium chloride, calcium chloride, glucose, HEPES, sucrose, Triton-X 100,

chlorpromazine, ammonium per sulfate, sodium dodecyl sulfate (SDS), atenolol, theophylline, dithioerythritol, silver stain SDS molecular weight standard mixtures, cytochalasin B, methyl β -cyclodextrin, poly-d-lysine (MW 196,400), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), MTT solubilisation solution, Hoechst 33258 and sodium fluoride were purchased from Sigma-Aldrich Canada, (Oakville, ON). N,N-dimethyl formamide (DMF), paraformaldehyde and acetic acid were supplied by Sigma-Aldrich Canada, (Oakville, ON). Dialysis tubing (MWCO 3500 and 6000-8000 Da) were obtained from Fisher Scientific Co. (Ottawa, ON). The bicinchonic acid (BCA) protein assay kit used to characterize protein levels was obtained from Pierce Biotechnology (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM), Hank's Balanced Salt Solution (HBSS), non-essential amino acids, fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen Canada, (Burlington, ON). 30% acrylamide/Bis solution (37.5:1) (2.6% C) for polyacrylamide gel was purchased from Bio-Rad laboratories (Hercules, CA). All other chemicals and solvents were of reagent grade and were used without purification unless specified otherwise.

4.3.2. Methods

4.3.2.1. Dendrimers evaluated

A series of PEPE dendrimers (Figure 4.1) consisting of 1,2,3,4 butane tetracarboxylic acid as the core molecule, PEO in the interior cavity, gallic acid or dihydroxy benzoic acid (DHBA) or bis(hydroxyl methyl) butyric acid (BHBA) at the branching points and PEG methacrylate in the terminal branches were synthesized as reported previously (Dhanikula and Hildgen, 2006; Dhanikula and Hildgen, 2007). Dendrimers are referred as den-1-(Gn)-M, den-2-(Gn)-M or den-3-(Gn)-M in the manuscript, where 1, 2 and 3 represent dendrimers containing DHBA, gallic acid and BHBA, respectively; while, Gn represents the generation of the dendrimer and M denotes

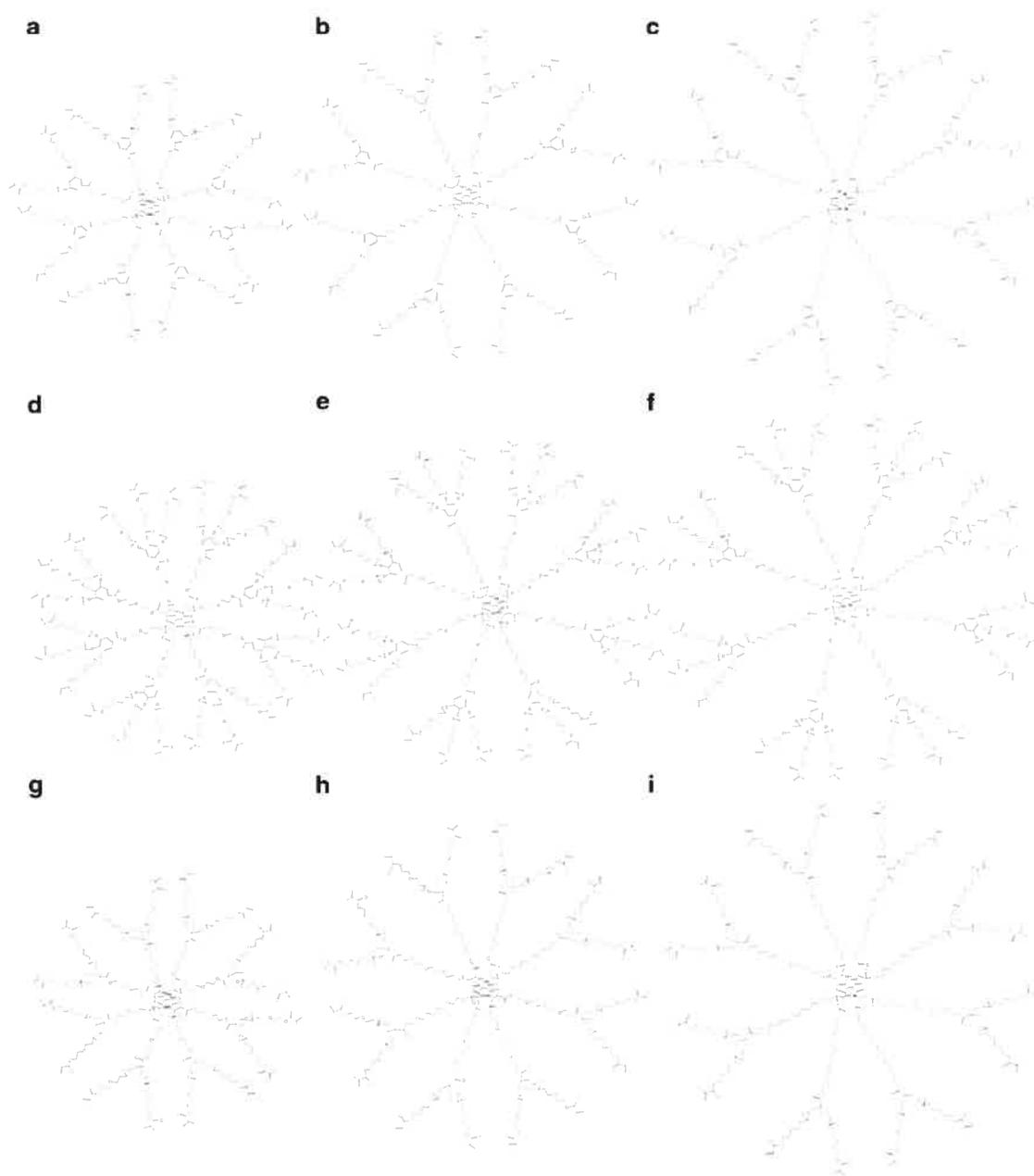


Figure 4.1. Chemical structures of various dendrimers showing their architecture (a) den-1-(G2)-200, (b) den-1-(G2)-300, (c) den-1-(G2)-400, (d) den-2-(G2)-200, (e) den-2-(G2)-300, (f) den-2-(G2)-400, (g) den-3-(G2)-200, (h) den-3-(G2)-300, (i) den-3-(G2)-400.

Table 4.1. Characteristics of PEPE dendrimers

<i>Dendrimer</i>	<i>Generation</i>	<i>Branching molecule</i>	<i>Terminal functional group</i>	<i>Hydrodynamic size (nm)^a</i>
Den-1-(G2)-200	2	DHBA	-COCH(CH ₃)CH=CH ₂	3.58 (0.11)
Den-1-(G2)-300	2	DHBA	-COCH(CH ₃)CH=CH ₂	4.74 (0.13)
Den-1-(G2)-400	2	DHBA	-COCH(CH ₃)CH=CH ₂	5.41 (0.27)
Den-2-(G2)-200	2	Gallic acid	-COCH(CH ₃)CH=CH ₂	4.19 (0.11)
Den-2-(G2)-300	2	Gallic acid	-COCH(CH ₃)CH=CH ₂	4.83 (0.19)
Den-2-(G2)-400	2	Gallic acid	-COCH(CH ₃)CH=CH ₂	7.41 (0.14)
Den-3-(G2)-200	2	BHBA	-COCH(CH ₃)CH=CH ₂	2.45 (0.12)
Den-3-(G2)-300	2	BHBA	-COCH(CH ₃)CH=CH ₂	3.34 (0.06)
Den-3-(G2)-400	2	BHBA	-COCH(CH ₃)CH=CH ₂	4.94 (0.22)
Den-1-(G2)-OH	2	DHBA	-CO CH(CH ₃)CH ₂ CH ₂ OH	5.11 (0.17)

^aData are mean (PI), n= 5

the molecular weight of PEO used in the interior cavity of the dendrimers. Characteristics of these dendrimers are shown in table 4.1.

4.3.2.2. Fluorescent labeling of dendrimers

Dendrimers (Den-1-(G2)-400, Den-2-(G2)-400 and Den-3-(G2)-400) were labeled with rhodamine B as a fluorescent probe. For this purpose, pendant allyl surface functional groups were oxidized to hydroxyl groups utilizing the conditions optimized previously (Dhanikula and Hildgen, 2006). Later, appropriate amount of freeze dried dendrimer (25 mg) was dissolved in DMF. EDC (0.1 mM), DMAP (0.08 mM) and rhodamine B (0.08 mM) were added to the flask and reaction was allowed to occur for 48 h. Precipitate of EDC-urea was removed by filtration, product was purified by dialysis (MWCO 6000-8000 Da) against deionized water for 96 h, until no more free rhodamine was detected in the medium. Later, dialysate was freeze dried to obtain the product (Yield 75%). Conjugation of rhodamine to dendrimers was verified by ¹HNMR (Bruker ARX400 NMR spectrometer, Bruker Biospin, Billerica, MA) (Figure 4.1s, supporting information) and spectrofluorimetry (AMINCO Bowman™, Thermo Spectronic Instruments, Rochester, NY). *In vitro* stability of rhodamine-dendrimer conjugates at 37°C in PBS (pH 7.4) revealed no dissociation of rhodamine from dendrimers for 3 days, indicating a stable linkage.

4.3.2.3. Cytotoxicity of dendrimers

Brain Endothelial cell lines (bEnd.3) (American Type Culture Collection, Rockville, MA) were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids and penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. Cellular growth inhibition was evaluated by MTT assay. bEnd.3 cells (passage 20-28) were seeded at a concentration of 5×10^5 cells/mL (100 μ L/well) in 96 well cell culture plates and were allowed to adhere to the wells for 24 h. Dendrimers (0.1 to 5 mg/mL in HBSS) were added

to the cells and incubated for 72 h. Later, 10 μ L of 5 mg/mL MTT solution was added to each well, followed by incubation at 37°C for 4 h. Formazan crystals produced by the reduction of MTT by viable cells were dissolved in 100 μ L of MTT solubilization solution. Absorbance was measured at 570 nm using Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria).

4.3.2.4. Hemolytic potential of dendrimers

The lysis of erythrocytes by PEPE dendrimers was determined by incubating them with freshly isolated rat RBCs. Blood was collected from Wistar rats in heparinated collection vials as per Canadian guidelines for laboratory animals and animal ethical committee of University of Montreal. It was centrifuged at 500 g for 10 min, washed with 0.1 M phosphate buffered saline (PBS, pH 7.4) three times to obtain RBCs. RBCs were diluted to 10% with PBS (Ohtani et al., 1989; Li et al., 2004). 0.9 mL of dendrimer samples (0.16 to 5 mg/mL in PBS) were incubated with 0.1 mL of RBC suspension at 37°C for 2 h. After incubation, tubes were centrifuged at 500 g to remove non-lysed RBCs. Control experiments to determine basal lysis of RBCs by PBS and 100% lysis by distilled water were also carried out. The supernatant was collected and analyzed by UV spectrophotometer at 540 nm to estimate the amount of hemoglobin released.

4.3.2.5. Adsorption of plasma proteins on dendrimers

Plasma protein adsorption on dendrimers was determined by incubating them with rat serum (200 μ L) in an incubator shaker (200 rpm) at 37°C for 1 h. After incubation, serum was diluted to 1 mL with PBS (pH 7.4, 50 mM) and samples were centrifuged at 50,000 g for 1 h. The pelleting efficiency of dendrimers by centrifugation was found to be between 25-30%. Pellets obtained were resuspended in PBS by vortexing for 15 min and centrifuged again under similar conditions. This washing cycle was repeated 3 times, followed by resuspension of the pellet in 50 μ L of protein solubilizing solution (10% w/v

SDS and 2.32% w/v dithioerythritol) and incubation at 95°C for 5 min. Samples were cooled and centrifuged to remove the dendrimers. Supernatants were loaded, resolved on 7.5% polyacrylamide gel (Laemmli, 1970) and developed with silver staining as mentioned by Scevchenko et al. (Scevchenko et al., 1996). Developed gels were scanned using MultiImage™ plate reader and analyzed by ChemImager™ 5500 software (Alpha Innotech Corp., San Leandro, CA).

For the determination of total amount of protein adsorbed on dendrimers, dendrimer pellets were incubated with micro BCA™ protein assay reagent for 1 h and centrifuged at 50,000 g for 30 min. Absorbance of the supernatant was measured at 562 nm using Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria) and protein content was estimated using albumin standard curve.

4.3.2.6. Endocytosis of dendrimers

bEnd.3 (passage 20-28) were seeded at a concentration of 1×10^6 cells/mL (300 μ L/well) into 24 well cell culture plates and were allowed to adhere to the wells for 24 h. Rhodamine labeled dendrimers (200-700 μ g/mL in HBSS) were added to each well and incubated for 4 h at 37°C. Subsequently, cells were washed four times with HBSS to remove dendrimer adhering to the cellular surface. Later cells were lysed with 200 μ L of 0.5% Triton-X containing 0.1 N NaOH. Dendrimer concentrations in cell lysate were determined by measuring rhodamine fluorescence (λ_{ex} 550 nm and λ_{em} 625 nm) using Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria). Standard curve generated using lysed cells and rhodamine labeled dendrimers was used for the quantification of dendrimer concentration in the experiments. Protein content of each well was estimated using BCA™ protein assay reagent.

4.3.2.7. Mechanism of endocytosis

To understand the mechanism of uptake of dendrimers, bEnd.3 cells (passage 20-28) were incubated with medium containing various inhibitors namely, sucrose (0.45 M), chlorpromazine (10 $\mu\text{g}/\text{mL}$), methyl β -cyclodextrin (5 mM), cytochalasin B (10 $\mu\text{g}/\text{mL}$), sodium fluoride (0.02 M), acetic acid (20 μM) for 1 h. Effect of K^+ depletion was observed by incubating cells with K^+ free buffer (140 mM NaCl, 20 mM HEPES, 1 mM CaCl_2 , 1 mg/mL glucose; pH 7.4) for 3 h. After these treatments, cells were washed with HBSS three times and complete growth medium was replaced in the wells. This was followed by incubation of rhodamine labeled dendrimers with cells in the presence of complete growth medium for 4 h, washing with HBSS and lysis with 0.5% Triton-X in 0.1 N NaOH. Dendrimer levels in cell lysate were measured by fluorescence plate reader (λ_{ex} 550 nm and λ_{em} 625 nm). Toxicity of these inhibitors on bEnd.3 cell viability was also evaluated by MTT assay, to confirm that the observed effects were not due to their influence on cellular proliferation.

Oxidative burst produced during endocytosis was evaluated by using luminol (1 mM, PBS) (Rudt and Muller, 1992; Rudt and Muller, 1993). bEnd.3 cells (1×10^6 cells/mL, in PBS) and luminol solution were transferred to the cuvette and incubated in luminometer (AMINCO BowmanTM, Thermo Spectronic Instruments, Rochester, NY) at 37°C for 15 min to quantify the background luminescence. Later, dendrimers (100 $\mu\text{g}/\text{mL}$ in PBS) were added to the cuvette and chemiluminescence intensity was measured for 180 min at 37°C. In order to understand the influence of plasma protein adsorption on the oxidative burst, dendrimers were pre-incubated with FBS (10%) for 1 h prior to the experiment.

4.3.2.8. Microscopic examination of endocytosis

bEnd.3 cells (passage 20-28) were plated at the density of 0.5×10^6 cells/mL on glass cover slips treated with 0.05% w/v of poly-d-lysine solution. Cover slips were placed in 24 well cell culture plates and cells were allowed to grow to 80% confluence. Cells were incubated with rhodamine labeled dendrimers (200 $\mu\text{g/mL}$) for 4 h. Later, cover slips were washed four times with HBSS and fixed with paraformaldehyde (1% w/v in PBS). Nuclei were stained with HOE 33258 (10 $\mu\text{g/mL}$) and the cells were washed again four times with HBSS. Cover slips were then mounted on slides using GelTol mounting medium (Thermo Electron Corporation, PA, USA). Fluorescence images were acquired at 60 X (inverted oil immersion objective) using an Axiovert epifluorescence microscope (Zeiss, Oberkochen, Germany), a Magnafire CCD camera (Optronics, Goleta, CA) and analyzed using Image-Pro Plus software (version 6).

4.3.2.9. BBB model

bEnd.3 cells (passage 25-28) were plated onto polycarbonate Transwell[®] inserts at the seeding density of 5×10^5 cells/mL maintained in DMEM supplemented with 10% FBS, 1% non-essential amino acids and penicillin/streptomycin. Cells achieved a growth to confluent monolayer in 4-5 days of seeding. U373 MG, astrocytoma cells (kind gift from Dr. J. F. Bouchard, University of Montreal) were maintained in the same medium as bEnd.3 cells and were seeded on Transwell[®] inserts at the density of $0.5 - 5 \times 10^5$ cells/mL. Influence of co-culturing U373 MG cells on transendothelial electrical resistance (TEER) and permeability of markers was also evaluated by seeding them (a) on different days after seeding of bEnd.3 cells (i.e. 2, 3 days), (b) at different densities on upper surface of inserts or (c) on the floor of cell culture wells 3 days prior to the seeding of bEnd.3 cells.

A). P-gp expression assay

Rhodamine-123 accumulation assay was used to determine the expression of P-gp in bEnd.3 cells (Omidi et al., 2003). Confluent mono layers of bEnd.3 cells (9 days post-seeding) were incubated with rhodamine-123 (0.2 $\mu\text{g}/\text{mL}$) in the presence or absence of P-gp inhibitor verapamil (40 μM) for 1 h at 37°C (this phase representing cellular accumulation of rhodamine-123). Efflux of rhodamine-123 from cells was conducted over a period of 2 h at 37°C using the same protocol. At the end of incubation, cells were washed four times with HBSS and lysed with 200 μL of ethanol. Quantification of rhodamine-123 was done with fluorescence plate reader (λ_{ex} 510 nm and λ_{em} 532 nm) using rhodamine-123 standard curve generated in the presence of cell lysates. Protein content of each well was estimated using BCA™ protein assay reagents. For evaluating the transport of rhodamine-123 across BBB model, it was added to the donor compartment (0.2 $\mu\text{g}/\text{mL}$) in the presence or absence of P-gp inhibitor verapamil (40 μM). The samples (300 μL) were collected from the receptor compartment at specified time points and analyzed by fluorescence plate reader.

B). Bioelectric properties and permeability assessment

For the assessment of bioelectric properties i.e. TEER and transendothelial permeability of solutes, either bEnd.3 cells alone or bEnd.3 and U373 MG cells were cultured on Transwell® inserts. TEER was measured using Millicell®-ERS voltohmmeter (Millipore, Billerica, MA) to assess the growth of cells on inserts. Theophylline and atenolol were used as transcellular and paracellular permeability probes, respectively (Hilgendorf et al., 2000; Augustijns and Mols, 2004; Miret et al., 2004). Transport studies were conducted 9-10 days post-seeding (the time at which TEER was found to be highest). Study was initiated by adding theophylline (4 mM) and atenolol (4 mM) into the donor compartment and PBS in the receiver compartment. Samples (100 μL) were taken from the receiver compartment at predetermined time points with replacement. They were analyzed

by HPLC using C-18 column, mobile phase consisting of methanol: 10 mM phosphoric acid: 10 mM KH_2PO_4 (26:16:58 v/v, pH 4.5) at a flow rate of 0.9 mL/min and wavelength of 290 nm using a WatersTM 717 system (Waters Corporation, Milford, MA) equipped with tunable absorbance detector (WatersTM 486).

4.3.2.10. Transport of dendrimers across BBB

bEnd.3 and U373 MG cells were seeded onto polycarbonate Transwell[®] inserts at the density of 5×10^5 cells/mL and 2×10^5 cells/mL, and confluent monolayers (9-10 days) were used for transport studies. Permeability was determined in apical to basolateral direction using HBSS as a transport medium. Dendrimers (500 $\mu\text{g}/\text{mL}$ in HBSS) were placed in the donor compartment and plates were incubated at 37°C. Samples (300 μL) were collected from the receiver compartment every 60 min for 6 h, later at 18 and 24 h and were analyzed for rhodamine labeled dendrimer using fluorescence plate reader (λ_{ex} 550 nm and λ_{em} 625 nm). Basal to apical permeability was determined by adding dendrimers (500 $\mu\text{g}/\text{mL}$ in HBSS) in the receiver compartment and sampling (100 μL) at specified time points from the donor compartment. The effect of dendrimers on BBB integrity was investigated by monitoring TEER through out the experiment. In order to ascertain that rhodamine labeled dendrimers are crossing the BBB model and not rhodamine, ¹HNMR spectrum of samples collected at 24 h of permeation study was acquired (Figure 4.2s, supporting information).

4.4. Results and Discussion

4.4.1. Cytotoxicity of dendrimers

Cationic dendrimers like polyamido amine (PAMAM), diamino butane (DAB), diamino ethane (DAE) dendrimers are cytotoxic and display IC_{50} values between 50–300 $\mu\text{g}/\text{mL}$

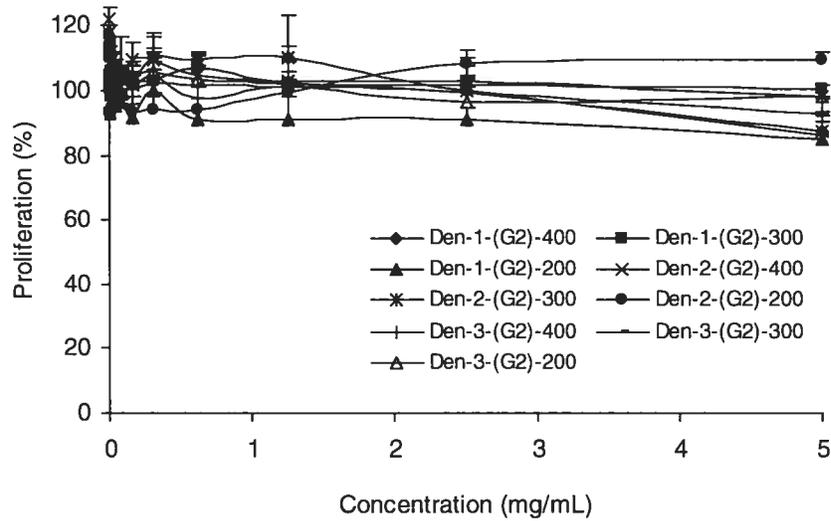


Figure 4.2. Viability of bEnd.3 cells assessed by MTT assay after 72 h of incubation with PEPE dendrimers. Data are mean + SD (n = 6).

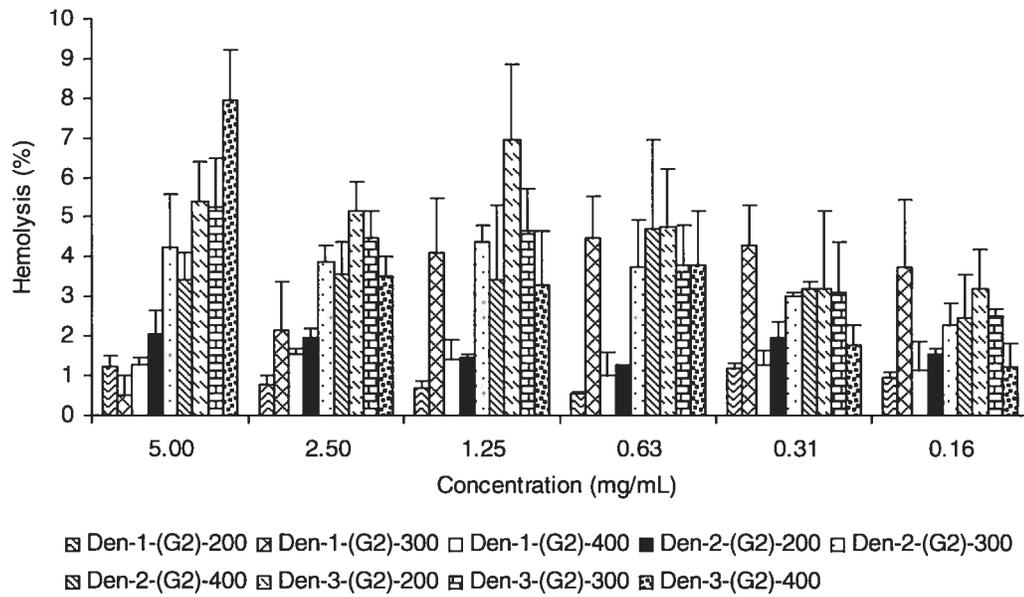


Figure 4.3. Extent of hemolysis of rat RBCs produced by PEPE dendrimers after 2 h of incubation at 37°C. Data are mean + SD (n = 4).

depending on dendrimer-type, cell-type and generation (Malik et al., 2000; Fischer et al., 2003). However, for successful application in drug delivery it is essential to design dendrimers which have minimal toxicity. Surface modification of dendrimers by PEG or lauryl chains has been reported to reduce the cytotoxicity of PAMAM dendrimers (Jevapresphant et al., 2003). Therefore, these dendrimers were synthesized largely from PEG. As seen in figure 4.2, none of the dendrimers showed significant reduction in the viability of bEnd.3 cells even after exposure to concentrations as high as 5 mg/mL. Previous study has also shown that PEPE dendrimers did not cause significant death of murine macrophage cells at the concentrations evaluated (Dhanikula and Hildgen, 2007). Based on the results of these studies and the chemical composition of PEPE dendrimers, it is contemplated that they would be safe for drug delivery applications.

4.4.2. Hemolytic potential of dendrimers

Extensive RBC lysis limits the i.v. delivery of a delivery system or an agent and often enhances the toxicity when administered by i.v. route. Duncan and coworkers have shown that dendrimers with carboxylate and malonate surfaces produced 15 to 40% hemolysis in 1 h (Malik et al., 2000). In another study, second generation PAMAM dendrimers were reported to cause 50% hemolysis at 1 mM, while third and fourth generation dendrimers induced 70 and 95% hemolysis (Domanski et al., 2004). PEPE dendrimers evaluated in this study produced less than 10% hemolysis at concentration as high as 5 mg/mL (Figure 4.3). Further, no changes in the shape of RBCs were observed during the study (data not shown), suggesting their safety for i.v. administration.

4.4.3. Adsorption of plasma proteins on dendrimers

After i.v. injection plasma proteins are immediately adsorbed onto the particles resulting in recognition and phagocytosis by mononuclear phagocytic systems (MPS) (Luck et al., 1998). Uptake of drug carriers by MPS is one of the major limitations for drug

targeting to the brain. Therefore, plasma protein adsorption studies were performed to get a preliminary idea about their *in vivo* fate.

Protein adsorption on dendrimers was found to be low. Total amount of protein adsorbed ranged between 4.65 and 7.56 $\mu\text{g}/\text{mg}$ of dendrimer (Table 4.2). There was no significant difference in the extent of protein binding on dendrimers of different series ($p > 0.1$, One way ANOVA). The molecular weight of PEG within the same series also did not have any considerable effect. Even the pattern of specific protein adsorption was also not influenced by the architecture of dendrimer and molecular weight of PEG (Figure 4.4). All dendrimers showed bands around 66,000, 50-60,000 and 14,000 Da corresponding to albumin, α -globulins (like Zn- α_2 -Glycoprotein (41,000 Da), α_2 HS-Glycoprotein (49,000 Da), GC globulin (52,000 Da), and low molecular weight proteins (like lysozyme (14,000 Da), 2 S γ_2 -globulin (14,000 Da), pos γ -globulin (13,260 Da), respectively. Only den-2-(G2)-200 showed a band around 48,500 corresponding to β -globulins like β_2 -glycoprotein I (48,000Da). High molecular weight proteins like immunoglobulins ($\geq 150,000$ Da) and complement factors ($\geq 80,000$ Da) were not found to be adsorbed on dendrimers. Immunoglobulins and complement factors are typical opsonins that adsorb on particulate system and enhance their uptake by MPS. The lack of adsorption of these proteins on dendrimers shows that they might have low clearance by MPS after i.v. administration. Further, these dendrimers are largely composed of PEG which has been shown to obtain long circulating nanoparticles (Klibanov et al., 1990; Gabizon et al., 1993). Consequently, these PEPE dendrimers might have long *in vivo* circulation half-life, which would allow sufficient time for delivery to the CNS (Calvo et al., 2001).

4.4.4. Endocytosis of dendrimers in brain endothelial cells

Dendrimers with higher molecular weight in each series i.e. den-1-(G2)-400, den-2-(G2)-400 and den-3-(G2)-400 were selected for endocytosis studies because, molecular

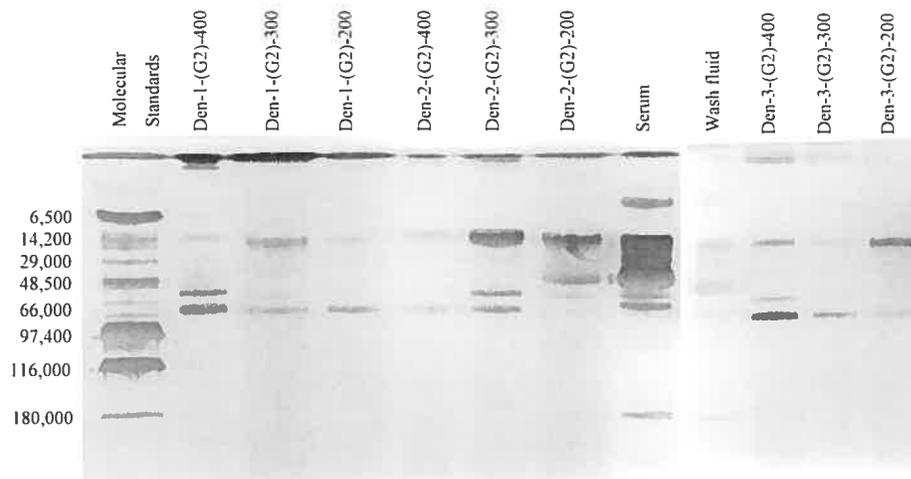


Figure 4.4. SDS-PAGE images of plasma proteins adsorbed on various dendrimers after 1 h of incubation with rat serum at 37°C. Images show pattern and extent of adsorption of different plasma proteins on dendrimers.

Table 4.2. Total amount of plasma proteins adsorbed on the surface of dendrimers

<i>Dendrimer</i>	<i>Total protein adsorbed ($\mu\text{g}/\text{mg}$ of dendrimer)</i>
Den-1-(G2)-200	5.19 \pm 0.37
Den-1-(G2)-300	5.97 \pm 0.90
Den-1-(G2)-400	7.04 \pm 0.39
Den-2-(G2)-200	7.56 \pm 0.27
Den-2-(G2)-300	7.07 \pm 1.01
Den-2-(G2)-400	6.23 \pm 0.70
Den-3-(G2)-200	4.83 \pm 0.66
Den-3-(G2)-300	4.65 \pm 1.75
Den-3-(G2)-400	6.28 \pm 0.92

Data are mean \pm SD (n=3)

weight of dendrimers did not have significant influence on their toxicity, hemolysis and plasma protein adsorption. PEPE dendrimers were efficiently (70-80%) internalized by bEnd.3 cells in time and concentration dependent manner (Figure 4.5). Uptake of rhodamine labeled dendrimers increased 2-2.3 fold with increase in concentration from 200 to 700 $\mu\text{g/mL}$; however, saturation was seen to occur at 500 $\mu\text{g/mL}$ demonstrating the presence of saturable endocytosis (Figure 4.5b). It has been reported that linear uptake is indicative of fluid phase endocytosis while, saturable uptake is suggestive of receptor mediated endocytosis (Rupper and Cardelli, 2001). Given the fact that these dendrimers do not have cationic or anionic surface groups, their uptake by cells due to non-specific interaction would be minimal and hence, adsorptive endocytosis would be low. The extent of internalization of dendrimers was significantly reduced at 4°C ($p < 0.001$, Paired t-test), with uptake being reduced to 5 to 15% of the control experiment at 37°C (Figure 4.6). The temperature dependence of dendrimer uptake signifies the presence of energy dependent internalization of approximately 85 to 95% of dendrimers. Sodium Fluoride (Silverstein et al., 1977), also inhibited 60, 68 and 79% uptake of den-3-(G2)-400, den-2-(G2)-400, and den-1-(G2)-400, respectively (Figure 4.6). Cytochalasin B, an inhibitor of hexose transport and various cell movements (Axline and Reaven, 1974), produced 68, 47 and 44% reduction in the uptake of den-3-(G2)-400, den-2-(G2)-400, and den-1-(G2)-400, respectively. The partial inhibition of uptake process by energy depletion and inhibition of cell motility suggests endocytotic uptake of dendrimers.

Phagocytosis is known to produce oxidative burst in the cells, therefore peroxide formation during the uptake of dendrimers in bEnd.3 cells was determined. Interestingly, no oxidative burst was observed during the uptake of dendrimers both with or without serum protein adsorption. Since, cytochalasin B, an inhibitor of cell motility produced partial inhibition of dendrimer internalization, presence of phagocytosis by bEnd.3 cells can not be excluded. But at the same time, it is speculated that phagocytosis would be low due to the small size (4.9-7.4 nm, Table 4.1) of these dendrimers (Catizone et al., 1993).

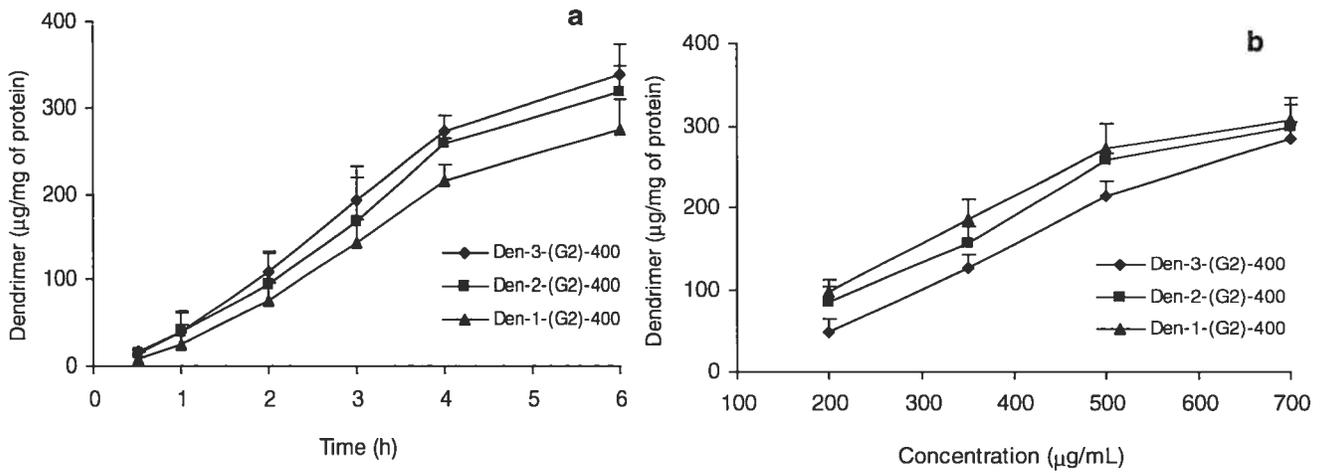


Figure 4.5. Time (a) and concentration (b) dependent uptake of rhodamine labeled dendrimers by bEnd.3 cells. Cells were incubated with 500 µg/mL of dendrimers for different time or with different concentration of dendrimers for 4 h. Data are mean + SD (n = 6).

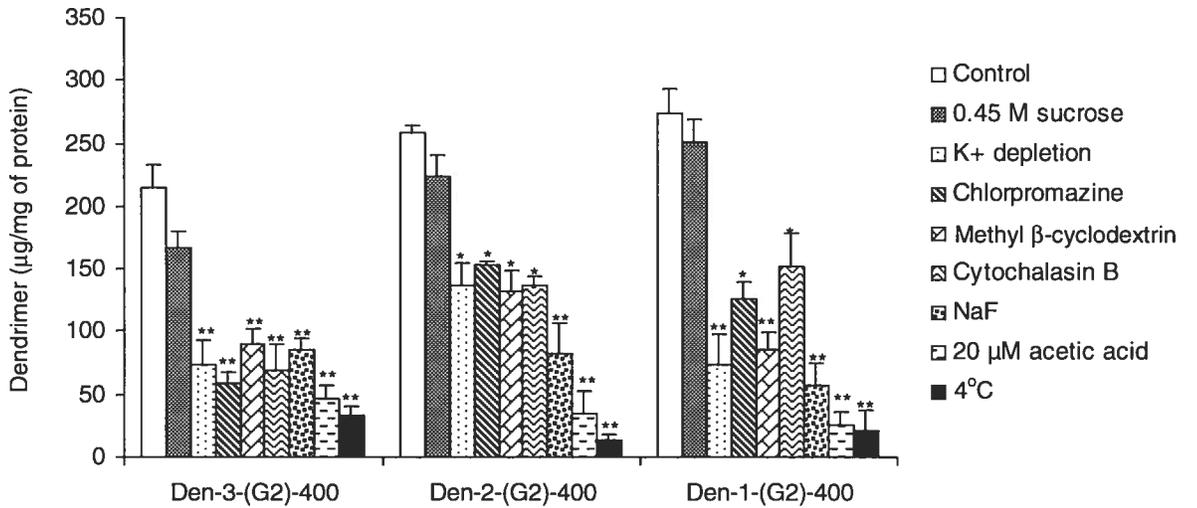


Figure 4.6. Influence of various endocytotic inhibitors on the uptake of rhodamine labeled dendrimers by bEnd.3 cells. Cells were incubated with of 500 µg/mL dendrimers for 4 h at 37°C. Data are mean + SD (n = 6). (* Statistically significant difference at p < 0.05 and ** p < 0.01, Repeated measures one way ANOVA)

Hypertonic, 0.45 M sucrose induced 8 to 22% inhibition in the dendrimer internalization, with uptake being 78%, 87%, and 92% of control experiment in den-3-(G2)-400, den-2-(G2)-400, and den-1-(G2)-400, respectively (Figure 4.6). This implies that internalization in den-1-(G2)-400 does not occur by fluid phase endocytosis (Heuser and Anderson, 1989), while in den-2-(G2)-400 and den-3-(G2)-400 it plays a minor role. Cytoplasmic acidification by acetic acid produced 78 to 91% reduction in endocytosis of three dendrimers (Figure 4.6). Reduction in uptake of dendrimers by 20 μ M acetic acid was statistically different from that of controls ($p < 0.05$, Paired t-test). Cytoplasmic acidification is reported to induce a general arrest in the formation and pinching off of coated vesicles from the cell membrane and thus inhibiting receptor mediated endocytosis (Sandvig et al., 1987; Davoust et al., 1988). Therefore, receptor mediated endocytosis is a major contributor in the internalization of dendrimers by bEnd.3 cells.

To evaluate the role played by clathrin dependent endocytosis in the internalization of dendrimers, potassium depletion was employed (Larkin et al., 1983; Larkin et al., 1985). It produced 47 to 73% inhibition in the internalization of dendrimers by bEnd.3 cells. Chlorpromazine, another inhibitor of clathrin-mediated endocytosis (Wang et al., 1993; Minana et al., 2001), produced 40%, 73% and 54% inhibition in the uptake of den-2-(G2)-400, den-3-(G2)-400 and den-1-(G2)-400, respectively (Figure 4.6). Both K^+ depletion and chlorpromazine did not completely abolish the internalization of dendrimers in bEnd.3 cells, suggesting probable involvement of clathrin independent pathways. Therefore, the effect of methyl β -cyclodextrin (Furuchi and Anderson, 1998) on the uptake of dendrimers was also evaluated. It produced 58, 49 and 69% inhibition in uptake of den-3-(G2)-400, den-2-(G2)-400, and den-1-(G2)-400, respectively (Figure 4.6). These results indicate that both caveolin-mediated and clathrin-mediated endocytosis are responsible for the uptake of dendrimers. However, greater inhibition of internalization of den-2-(G2)-400 by methyl β -cyclodextrin as compared to chlorpromazine and K^+ depletion suggests that caveolin-mediated uptake plays a major role in the uptake of den-2-(G2)-400. On the other hand,

clathrin-mediated endocytosis occurs to a greater extent in den-1-(G2)-400 and den-3-(G2)-400. Thus, uptake of these dendrimers into brain endothelial cells is the combination of several pathways, which could be advantageous because even during saturation of one mechanism other alternative mechanism would operate and hence permeation of dendrimers across BBB would occur. The cellular entry of dendrimers was also visualized by using epi-fluorescence microscopy (Figure 4.7). High fluorescence intensity in epi-fluorescence image of bEnd.3 cells, obtained after 4 h of incubation with rhodamine labeled den-1-(G2)-400 corroborate significant internalization of dendrimer by the cells (Figure 4.7). It is evident that dendrimers entered the cells and localized mostly in the cytoplasm at this time scale. Further, the presence of punctuated fluorescence in the cells suggests localization of dendrimers in the endocytotic vesicles, which is a characteristic feature of endocytotic uptake.

4.4.5. Characterization of BBB model

bEnd.3 cell line has been previously reported to express various transporters like GLUT-1, OAT1, amino acid carriers, P-gp and tight junction elements like ZO-1, occludin, claudin-1 etc. (Omidi et al., 2003; Song and Pachter, 2003). Therefore, in the present study bEnd.3 cells were utilized for evaluating transport of dendrimers across BBB. These endothelial cells were particularly characterized for expression of P-gp transporter (Figure 4.8) by studying the efflux of rhodamine-123, a fluorescent substrate of P-gp (Batrakova et al., 1998; Omidi et al., 2003). Rhodamine-123 efflux from the cells was seen to occur within 2 h of incubation and verapamil could reduce the extent of efflux suggesting expression of P-gp in bEnd.3 cells within 9 days of culture (Figure 4.8a). Verapamil also significantly increased the permeability of rhodamine-123 across the BBB (Figure 4.8b), substantiating the expression of p-gp in the BBB model.

TEER of the bEnd.3 cells and co-culture of bEnd.3 and U373 MG cells was maximum around 9-10 days of culture. Co-culture of bEnd.3 cells with U373 MG cells was

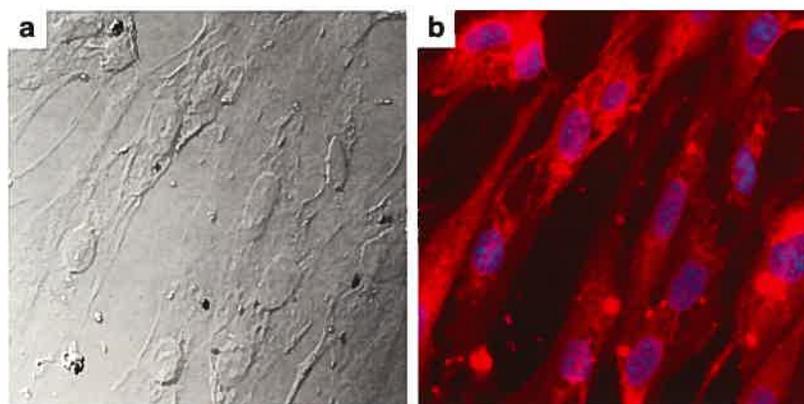


Figure 4.7. Epi-fluorescence images of bEnd.3 cells incubated with rhodamine labeled den-1-(G2)-400 (200 $\mu\text{g}/\text{mL}$) for 4 h. (a) DIC image of the cells (b) Overlay of red and blue fluorescent images. Image shows localization of dendrimer in the cells. Red fluorescence corresponds to rhodamine labeled dendrimers and blue to nuclear stain HOE 33258.

Table 4.3. TEER and permeability coefficients of theophylline and atenolol across bEnd.3 cells alone and co-cultures of bEnd.3 and U373 MG cells.

<i>BBB model</i>	<i>TEER^a</i> ($\Omega.\text{cm}^2$)	<i>P_{app}</i> ($\times 10^{-6} \text{cm}/\text{sec}$)	
		<i>Theophylline</i>	<i>Atenolol</i>
bEnd.3 cells	75 \pm 15	8.8 \pm 2.0	1.8 \pm 0.5
bEnd.3 cells + U373 MG cells on inserts	165 \pm 16	8.9 \pm 1.6	0.2 \pm 0.05
bEnd.3 cells + U373 MG cells on well floor	81 \pm 13	8.9 \pm 1.7	1.8 \pm 0.4

Data are mean \pm SD (n=6)

^aTEER was measured after 10 days of culture

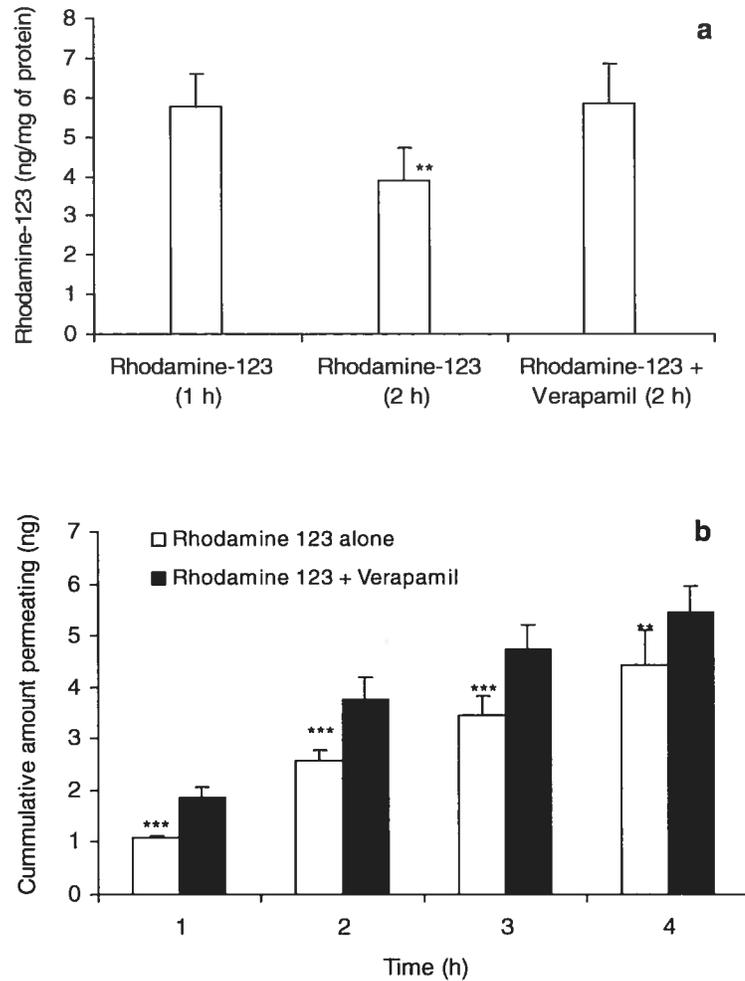


Figure 4.8. (a) Retention of rhodamine-123 inside bEnd.3 cells after 1 and 2 h of incubation in the presence and absence of verapamil (P-gp efflux inhibitor). Presence of verapamil resulted in increased retention of rhodamine-123 inside cells after 2 h, suggesting expression of P-gp in the cells. (b) Permeation of rhodamine-123 across BBB model in the presence and absence of verapamil. Data are mean + SD (n = 4 or 12). (** Statistically significant difference at $p < 0.01$ and *** $p < 0.005$, t-test)

found to increase the TEER from 60-90 Ωcm^2 to 150-180 Ωcm^2 (Table 4.3). The day of seeding U373 MG cells on Transwell[®] insert caused no significant difference in the TEER of the co-culture. Further, plating U373 MG cells on the floor of cell culture plates produced TEER similar to that of bEnd.3 cells alone. However, density of U373 MG cells was found to influence TEER, with cell density of $1.5\text{-}2 \times 10^5$ cells/mL generating maximum resistance. Therefore, U373 MG cells were seeded on Transwell[®] inserts at 2×10^5 cells/mL on the same days as bEnd.3 cells and were allowed to grow for 9-10 days. P_{app} of theophylline and atenolol across bEnd.3 cells alone were $8.8 \pm 2.0 \times 10^{-6}$ and $1.8 \pm 0.54 \times 10^{-6}$ cm/sec, respectively (Table 4.3). On co-culturing bEnd.3 cells with U373 MG cells, P_{app} of atenolol was $0.23 \pm 0.05 \times 10^{-6}$ cm/sec, signifying significant decrease in its permeability and hence, paracellular transport. However, permeability of theophylline was not significantly reduced (Table 4.3). All these results indicated that co-culture of bEnd.3 and U373 MG cells had augmented tight junction properties as compared to culture of bEnd.3 cells alone and thus, it was used for further studies.

4.4.6. Transport of dendrimers across BBB

In the view of higher extent of internalization (Figure 4.6) and minimal uptake by fluid phase endocytosis (which is reported to be restricted in BBB (Smith and Gumbleton, 2006)) of den-1-(G2)-400 as compared to den-2-(G2)-400 and den-3-(G2)-400, it was selected for transport studies. Den-1-(G2)-400 was able to cross BBB model in significant amounts with 102 μg of rhodamine labeled dendrimer being transported across the barrier in 24 h (Figure 4.9). ¹HNMR spectrum of 24 h permeation sample (Figure 4.2s, Supporting information) proved that rhodamine labeled dendrimers are able to cross BBB intact and also that it is not merely rhodamine that is crossing the BBB. Transport of den-1-(G2)-400 across BBB model was rapid and approximately 5 μg of dendrimer permeated in first 1 h. P_{app} of den-1-(G2)-400 was also significantly high ($19.7 \pm 1.9 \times 10^{-6}$ cm/sec). Interesting observation was that, TEER decreased only up to 86% in first 4 h of the transport and

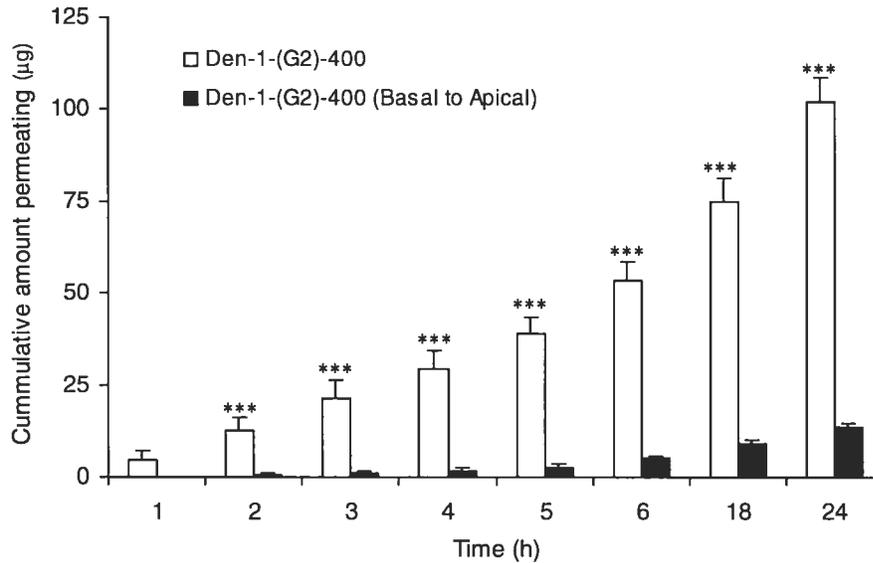


Figure 4.9. Permeation of den-1-(G2)-400 across BBB model (bEnd.3 and U373 MG cells co-culture). Cells were grown on polycarbonate Transwell[®] inserts for 9-10 days and incubated with 500 µg/mL of rhodamine labeled den-1-(G2)-400. Data are mean + SD (n = 4). (***) Statistically significant difference at $p < 0.005$, t-test)

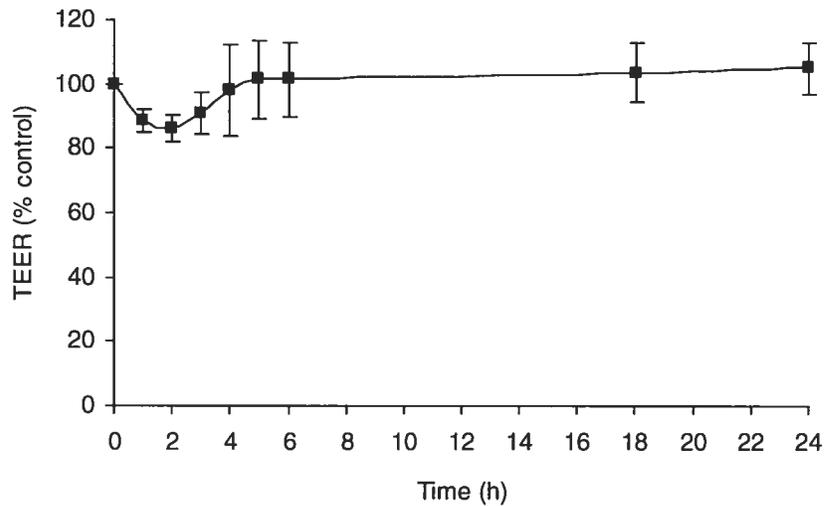


Figure 4.10. Percentage decrease in the TEER of BBB model after incubation with 500 µg/mL of rhodamine labeled den-1-(G2)-400 in the donor compartment at 37°C. Data are mean ± SD (n = 4).

restored back to normal in 6 h (Figure 4.10). This indicates that dendrimers did not cause significant disruption of the tight junctions during permeation. It also implies that dendrimers are able to cross BBB in high amounts without compromising its barrier properties. The basal to apical efflux of dendrimers was very low (1-12%) (Figure 4.9). Because of the low basal to apical efflux of dendrimers, they would be able to transport the drugs to the brain without being effluxed out. Altogether, the results of the present study are very promising and indicate the potential of these dendrimers for enhanced delivery across BBB.

4.5. Conclusion

In the present study, suitability of PEPE dendrimers for delivery across blood brain barrier was established. Dendrimers were found to be non-toxic to the brain endothelial cells and safe for i.v. administration. Due to low plasma protein adsorption, it is expected that these dendrimers would exhibit longer circulation half-life; which would in turn result in improved delivery to the brain. Dendrimers internalized efficiently in the brain endothelial cells by both clathrin and caveolin mediated endocytosis. They were also able to cross *in vitro* BBB model in high amount without significant disruption of tight junction properties. Further *in vivo* specificity for brain delivery can be easily achieved by conjugating the ligands for brain targeting to the surface functional groups of these dendrimers. Thus, these PEPE dendrimers can be potentially utilized as biocompatible, long-circulating delivery systems for the enhanced delivery of therapeutics across BBB.

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4.8. Supporting Information

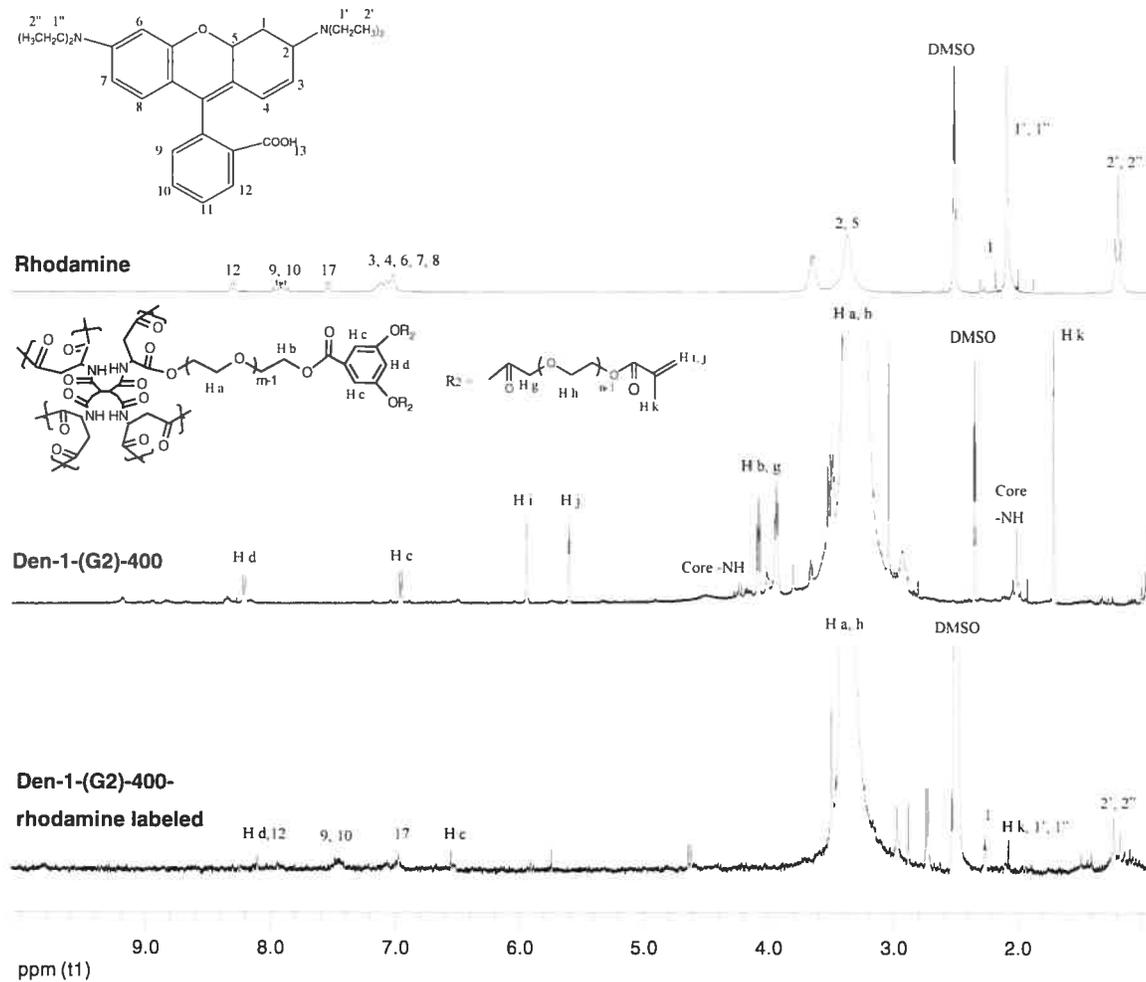


Figure 4.1s. ¹H NMR spectra of den-1-(G2)-400, rhodamine and rhodamine labeled den-1-(G2)-400 in DMSO at 300 MHz.

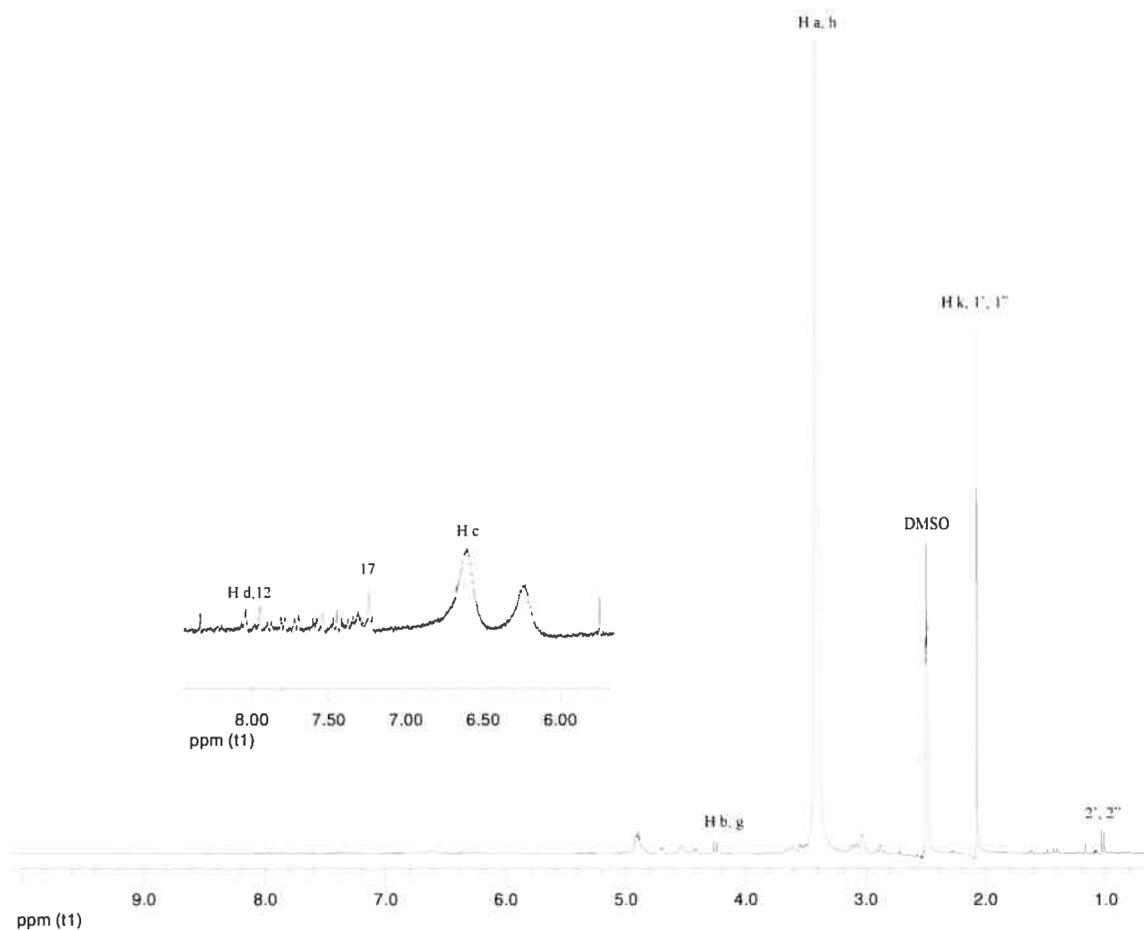


Figure 14.2s. ^1H NMR spectrum of rhodamine labeled den-1-(G2)-400 obtained at 24 h from permeation experiments. Permeation samples collected at 24 h were lyophilized and reconstituted in DMSO prior to recording NMR spectrum at 300 MHz.

CHAPTER FOUR

RESEARCH PAPER

Methotrexate loaded polyether-co-polyester dendrimers for the treatment of gliomas: enhanced efficacy and intratumoral transport capability

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5.1. Abstract

Therapeutic benefit in glial tumors is often limited due to low permeability of delivery systems across blood brain barrier (BBB), drug resistance and poor penetration into the tumor tissue. In an attempt to overcome these hurdles, polyether-co-polyester (PEPE) dendrimers were evaluated as the drug carriers for the treatment of gliomas. Dendrimers were conjugated to D-glucosamine as the ligand for enhancing BBB permeability and tumor targeting. The efficacy of MTX loaded dendrimers was established against U87 MG and U343 MG-A cells. Permeability of rhodamine labeled dendrimers and MTX loaded dendrimers across *in vitro* BBB model and their distribution into avascular human glioma tumor spheroids was also studied. Glucosylated dendrimers were found to be endocytosed in significantly higher amounts than non-glucosylated dendrimers by both the cell lines. IC₅₀ of MTX after loading in dendrimers was lower than that of free MTX, suggesting that loading MTX in PEPE dendrimers increases its potency. Similar higher activity of MTX loaded glucosylated and non-glucosylated dendrimers was found in the reduction of size of tumor spheroids. These MTX loaded dendrimers were able to kill even MTX resistant cells highlighting their ability to overcome MTX resistance. In addition, amount of MTX transported across BBB dendrimers was 3-5 times more after loading in dendrimers. Glucosylation further increased the cumulative permeation of dendrimers across BBB and hence increased the amount of MTX available across it. Glucosylated dendrimers distributed through out the avascular tumor spheroids within 6 h, while, non-glucosylated dendrimers had taken 12 h. The results show that glucosamine can be used as an effective ligand not only for targeting glial tumors but also for enhanced permeability across BBB. Thus, glucosylated PEPE dendrimers can serve as potential delivery system for the treatment of gliomas.

Keywords: dendrimer, gliomas, tumor spheroids, methotrexate, D-glucosamine, BBB model, permeability

5.2. Introduction

Though not highly prevalent, brain tumors are one of the most lethal forms of cancer. They are the leading cause of solid tumor death in children under age of 20 and are the third leading cause of cancer death in young adults ages 20-39 years (Landis et al., 1999). In the year 2000, approximately 176,000 new cases of brain and other central nervous system (CNS) tumors were diagnosed worldwide, with an estimated mortality of 128,000 (Parkin et al., 2001). It is reported that only 5% of patients survive from brain tumors after 5 years of diagnosis. In fact, the patients with brain tumors have poorer survival rates than breast cancer patients. Most of the systemically administered chemotherapeutic agents do not enter brain in adequate amounts. Consequently, high doses of drugs are administered systemically to obtain required brain tumor concentration causing systemic toxicity, and there by compromising the quality of patient life. Due to these limitations of the conventional delivery methods, brain tumors remain as unsolved clinical problem in spite of decades of research. Thus, there is a need for multi-functional carrier that can be engineered into a single nano-platform such that it can carry drug, cross blood brain barrier (BBB) and target the tumors. In this direction, dendrimers can serve as a versatile targeting platform due to their unique structural and functional advantages instigating from the multiple surface groups that can be used for conjugating multi-functional ligands (Aulenta et al., 2003; Klajnert and Bryszewska, 2001), and from the presence of internal voids in which drugs can be easily encapsulated or complexed (Esfand and Tomalia, 2001; Patri et al., 2002). These interior voids provide a nano-compartment in the dendrimers where the loaded drug is protected from the external environment. Further, dendrimers have small nanometric size which can provide additional advantage of increased permeability across the barriers.

Receptor-mediated endocytosis is one of the major ports of entry into the brain. The receptors for insulin, transferrin, endothelial growth factors, amino acids and various metabolic nutrients are expressed on BBB (Smith and Gumbleton, 2006). Glucose

transporter GLUT-1 is one such transporter found in high density on BBB (Pardridge et al., 1990; McAllister et al., 2001). Glucose transporters such as GLUT-1 are also known to be over-expressed on the tumors of brain, colon, liver, lung, pancreas, stomach and retina (Airley et al., 2001; Pedersen et al., 2002; Luciani et al., 2004). Targeting to various tumors by the glucose transporter has been successfully done for positron emission tomography (Haberkorn et al., 1994; Maublant et al., 1998), magnetic resonance contrast imaging (Luciani et al., 2004) and gene targeting (Park et al., 2005). Glucose conjugation to the delivery system confers tumor-targeting property through facilitative glucose metabolism by the glucose transporters in the tumors (Noguchi et al., 2000). Thus, glucose can be used not only for enhanced delivery across BBB but also for targeting to the brain tumors. Considering the advantage of dual targeting using the same ligand and synthetic simplicity of conjugating a single ligand, glucose was used as a targeting moiety in the present work.

Most of the brain tumors are solid tumors. Recently, it has been suggested that limited penetration of drugs in solid tumors is one of the causes of poor therapeutics indices of many chemotherapeutic agents (Minchinton and Tannock, 2006). In particular the avascular regions of solid tumors represent a major obstacle in achieving the effective control of the tumor growth (Kostarelos et al., 2005). In many solid tumors the cellular population could be more than 100 μm apart from the vasculature as a consequence the drug and nutrient distribution to the distant cells is limited. In addition to the limited vasculature, high cell density, elevated interstitial pressure, hypoxia and acidic pH (Brown and Giaccia, 1998) impede the penetration, distribution and cellular accumulation of chemotherapeutic agents in these distant tumor cells (Kostarelos et al., 2004). For a treatment to be effective it should access the entire tumor, since survival of even a few cells could result in cancer reoccurrence. Indeed, the poor therapeutic indices of delivery systems like liposomes due to poor diffusion/penetration within the interstitial space of tumors have been repeatedly documented in the literature (Yuan et al., 1994; Ishida et al., 1999). Thus, distribution of drug in avascular regions of the tumor is one of the most challenging tasks. For these reasons, preliminary evaluation of interaction and diffusion of

delivery systems within an avascular tumor model can serve as an extremely valuable tool for optimizing delivery systems for anticancer therapeutics (Kostarelos et al., 2005). Tumor spheroids display a three-dimensional representation of avascular regions found in many solid tumor tissues (Mellor et al., 2005; Mellor et al., 2006). They have extensive cell-cell contact, elevated interstitial pressure, hypoxia, presence of quiescent cells and gradient of nutrient concentration and cellular proliferation from the exterior to the centre (Desoize and Jardillier, 2000; Kostarelos et al., 2004). Therefore, they can serve as invaluable tool for this purpose.

The objective of this study was to determine the prospective of polyether-copolyester (PEPE) dendrimers loaded with methotrexate (MTX) in the treatment of gliomas. PEPE dendrimers were conjugated to D-glucosamine for enhanced delivery across the BBB as well as for targeting the tumors. The potency of these MTX loaded dendrimers was evaluated against glioma cells and the avascular human glioma tumor spheroids. The ability of fluorescently labeled dendrimers to penetrate within the tumor spheroids was investigated using confocal laser scanning microscopy.

5.3. Experimental

5.3.1. Materials

D-glucosamine, di-succinimidyl carbonate, rhodamine-B, fluorescein isothiocyanate (FITC), 4-(dimethyl amino) pyridine (DMAP), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), Triton-X 100, poly-d-lysine (MW 196,400), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), MTT solubilisation solution, ethidium bromide and dihydrofolate reductase assay kit were purchased from Sigma-Aldrich Canada Chemical Co. (Oakville, ON). N,N-dimethyl formamide (DMF), and paraformaldehyde were supplied by Sigma-Aldrich Canada

(Oakville, ON). Dialysis tubing (MWCO 3500 and 6000-8000 Da) were obtained from Fisher Scientific Co. (Ottawa, ON). The bicinchonic acid (BCA) protein assay kit used to characterize protein levels was obtained from Pierce Biotechnology (Rockford, IL). MTX was purchased from Toronto Research Chemicals Inc. (North York, ON). Dulbecco's modified Eagle's medium (DMEM), Hank's Balanced Salt Solution (HBSS), non-essential amino acids, fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen Canada (Burlington, ON). All other chemicals and solvents were of reagent grade and were used without purification unless specified otherwise.

5.3.2. Methods

5.3.2.1. Dendrimers evaluated

Two series of PEPE dendrimers, namely den-1-series and den-2-series were evaluated in this study (Table 5.1). The dendrimers are referred as den-1-(Gn)-M or den-2-(Gn)-M in the manuscript, where 1 and 2 represent dendrimers containing dihydroxy benzoic acid (DHBA) and gallic acid respectively; Gn represents the generation and M denotes the molecular weight of polyethylene oxide (PEO) used in the interior cavity of the dendrimers. The synthesis and chemical characterization of these dendrimers has been reported elsewhere (Dhanikula and Hildgen, 2006; Dhanikula and Hildgen, 2007).

5.3.2.2. Conjugation of glucosamine to dendrimers

D-glucosamine (4.5 mM) was dissolved in dry acetone, later di-succinimidyl carbonate (0.54 mM) and dimethyl aminopyridine (2.3 mM) were added and the reaction was allowed to occur for 48 h. Acetone was evaporated on rotatory evaporator, product was dissolved in methanol and purified by extraction with ether (3 times) and chloroform (3 times). Subsequently, methanol was evaporated and product was dried under vacuum. The allyl pendant terminal groups of dendrimers were oxidized to hydroxyl groups as reported

previously (Dhanikula and Hildgen, 2006). Succinimidyl carbonate derivative of glucosamine (2.7 μM) was then conjugated to these hydroxyl dendrimers (2.7 μM) by incubating them in dry acetone and DMF (10:1) at room temperature for 96 h. Product was purified by dialysis against water for 96 h using dialysis membrane of MWCO 6000-8000 Da. Conjugation of glucosamine to dendrimers was determined by ^1H NMR.

5.3.2.3. Fluorescent labeling of the dendrimers

The conjugation of rhodamine B to the dendrimers was carried out as reported previously (Dhanikula and Hildgen, (Submitted)). In the case of den-1-(G2)-400, pendant allyl terminal functional groups were oxidized to hydroxyl groups (Dhanikula and Hildgen, 2006) to obtain hydroxyl derivative of the dendrimers. Later, hydroxyl derivatives of den-1-(G2)-400 or glucosamine conjugates of den-1-(G2)-400 (den-1-(G2)-400-Glu) were dissolved in DMF. EDC (0.1 mM), DMAP (0.08 mM) and rhodamine B (0.08 mM) were added to the flask and the reaction was allowed to occur for 48 h. Precipitate of EDC-urea was removed by filtration and product was purified by dialysis (MWCO 6000-8000 Da) against deionized water for 96 h. *In vitro* stability of rhodamine-dendrimer conjugates was assessed in PBS (pH 7.4) at 37°C. No dissociation of rhodamine from dendrimers was observed for 3 days, indicating a stable linkage.

5.3.2.4. MTX encapsulation

Dendrimers were dissolved in DMF in screw capped vials, MTX was added to these vials and samples were stirred at room temperature for 48 h. Unencapsulated drug was removed by dialysis (MWCO 3500 Da) for 4 h against 4 L deionized water. The dialysate was lyophilized to obtain freeze dried dendrimer loaded with MTX. Drug loading was determined by adding freeze dried dendrimers to DMF, sonicating them (550 Sonic dismembrator, Fisher Scientific, Ottawa, ON) for 1 min and later agitating for 24 h at 200

rpm. MTX was analyzed using UV spectrophotometer U-2001 (Hitachi high technologies, Orlando, FL) at 376 nm with appropriate blank corrections.

5.3.2.5. Internalization of dendrimers by glioma cells

U87 MG and U343 MG-A cells (kind gift from J.-F. Bouchard, University of Montreal, ATCC (Rockville, MD)) (passage 15-20) were seeded at a concentration of 1×10^5 cells/mL (300 μ L/well) into 24 well cell culture plates and were allowed to adhere to the wells for 24 h. Rhodamine labeled dendrimers (100 μ g/mL, in HBSS) were added to each well and incubated for 4 h at 37°C. Subsequently, cells were washed four times with HBSS to remove dendrimer adhering to the cellular surface. Cells were then lysed with 200 μ L of 0.5% Triton-X containing 0.1 N NaOH. Dendrimer concentrations in cell lysate was determined by measuring rhodamine fluorescence (λ_{ex} 550 nm and λ_{em} 625 nm) using Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria). Standard curve generated using lysed cells and rhodamine labeled dendrimers was used for the quantification of fluorescence. Protein content of each well was estimated by using micro BCA™ protein assay reagents.

5.3.2.6. Intracellular localization of dendrimers

U343 MG-A cells (passage 15-20) were plated at the density of 0.5×10^6 cells/mL on glass cover slips treated with 0.05% w/v of poly-d-lysine solution. Cells were allowed to grow to 80% confluence. Later, rhodamine labeled dendrimers (200 μ g/mL) were added to each well and incubated for 4 h. After incubation, slides were washed four times with HBSS, fixed with formaldehyde (1% w/v in PBS) for 30 min and again washed with HBSS two times. Cover slips were then mounted on slides using GelTol mounting medium (Thermo Electron Corporation, PA, USA). Confocal laser scanning microscope images were acquired at 100 X using DMRXE microscope (Zeiss, Oberkochen, Germany)

equipped with Leica TCS SP 2 confocal system (Leica Microsystems, Heidelberg, Germany).

5.3.2.7. Development of MTX resistant U87 MG cells

U87 MG cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, penicillin/streptomycin and 500 nM of MTX at 37°C in a 5% carbon dioxide atmosphere. The production of MTX resistance in cells was followed by observing the size of the cells and nuclei, MTX-FITC accumulation in the cells and dihydrofolate reductase activity.

A). Enzyme assay

Activity of dihydrofolate in cells was determined by preparing cell extracts as described previously by Alt et al., 1976 (Alt et al., 1976). Cells were harvested from monolayer by trypsinization, washed three times with HBSS and suspended in ice-cold 50 mM potassium phosphate (pH 7.0). This suspension was disrupted with a probe sonicator for 5 min. Later, it was centrifuged at 100,000 g for 60 min at 4°C to yield supernatant called cell extract. Folate reductase activity in the cell extract was determined using dihydrofolate reductase (DHFR) assay kit. Protein content of the cell extract was determined by BCATM protein assay kit.

B). MTX-FITC accumulation

MTX was conjugated with FITC according to the procedure mentioned by Gapski et al., 1975 (Gapski et al., 1975) and Kaufman et al., 1978 (Kaufman et al., 1978). U87 MG cells (MTX resistant and sensitive) were plated into 24 well plates and were allowed to adhere for 24 h. Later they were incubated with medium containing 30 µM MTX-FITC for 10 h at 37°C. After incubation cells were washed four times with HBSS and lysed with 200 µL of 0.5% Triton-X containing 0.1 N NaOH. MTX-FITC concentrations in cell lysates

were determined by measuring fluorescence (λ_{ex} 504 nm and λ_{em} 538 nm) using Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria). Standard curve generated using lysed cells and MTX-FITC was used for the quantification of fluorescence in the experiments. Protein content of each well was estimated using micro BCA™ protein assay reagents.

5.3.2.8. Antiproliferative activity of dendrimers against glioma cells

Human glioma cell lines, U87 MG and U343 MG-A were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids and penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. MTX resistant U87 MG cells were cultured as mentioned in the previous section. Cells (passage 15-20) were seeded at a concentration of 1×10^4 cells/mL (100 μ L/well) in 96 well cell culture plates and were allowed to adhere to the wells for 24 h. Cellular growth inhibition was evaluated by MTT assay. Dendrimers loaded with MTX (2 to 1000 μ M, in HBSS) were added to the cells and incubated for 72 h. Later, 10 μ L of 5 mg/mL MTT solution was added to each well, followed by incubation at 37°C for 4 h. Formazan crystals produced by the cells were dissolved in 100 μ L of MTT solubilization solution. Absorbance was measured at 570 nm using Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria).

5.3.2.9. Transport of dendrimers and MTX across BBB

Co-culture of bEnd.3 and U373 MG cells was used as the model for BBB (Omidi et al., 2003; Song and Pachter, 2003; Dhanikula and Hildgen, (Submitted)). bEnd.3 and U373 MG cells were seeded onto polycarbonate Transwell® inserts (Corning, Lowell, MA) at the density of 5×10^5 cells/mL and 2×10^5 cells/mL, and confluent monolayers (9-10 days) were used for transport studies (Dhanikula and Hildgen, (Submitted)). Permeability was determined in apical to basolateral direction using HBSS as a transport medium. Rhodamine labeled dendrimers or MTX loaded dendrimers were placed in the donor

compartment and plates were incubated in a humidified and 5% CO₂ atmosphere at 37°C. Samples were collected from the receiver compartment at predetermined time points. They were analyzed for rhodamine labeled dendrimer using fluorescence plate reader (λ_{ex} 550 nm and λ_{em} 625 nm). MTX was analyzed by HPLC using C-18 column, mobile phase consisting of methanol, 10mM phosphoric acid and 10 mM KH₂PO₄ (26:16:58 v/v, pH 4.5) at a flow rate of 0.9 mL/min and wavelength of 290 nm using a WatersTM 717 system equipped with tunable WatersTM 486 absorbance detector (Waters Corporation, Milford, MA). The effect of dendrimers on BBB integrity was investigated by monitoring transendothelial electrical resistance (TEER) through out the experiment.

5.3.2.2.10. Avascular human glioma tumor spheroids

Tumor spheroids of U87 MG and U343 MG-A cells were grown *in vitro* using liquid overlay system (Rofstad et al., 1986; Kobayashi et al., 1993). Agarose solution was prepared in serum free DMEM (2 % w/v) by heating it at 80°C for 30 min. Each well of cell culture plates was coated with a thin layer (0.3 mL) of this sterilized solution. Tumor cells at the density of 1×10^5 cells/mL (in complete medium) were seeded into each well. Subsequently, plates were gently agitated for 5 min on the first day of seeding and tumor spheroids were allowed to grow for 7 days at 37°C in a humidified, 5% CO₂ atmosphere. Medium of the wells was changed every 2-3 days.

A). Growth inhibition of tumor spheroids

For evaluating the inhibition of tumor growth, tumor spheroids were incubated with PBS, MTX or MTX loaded dendrimers. Samples were prepared in PBS (pH 7.4) and 100 μ L sample was added per well to obtain a concentration of 200-400 μ M. Growth inhibition was monitored by measuring the size of tumor spheroids using an inverted phase microscope fitted with an ocular micrometer. The major (d_{max}) and minor (d_{min}) diameters

of each spheroid were determined and spheroid volume was calculated as mentioned previously (Ballangrud et al., 1999) by using the following formula:

$$V = \frac{\pi \times d_{\max} \times d_{\min}^2}{6} \dots\dots\dots \text{Equation 3}$$

B). Determination of cell viability in tumor spheroids

Ethidium bromide was used as a fluorescent probe for determining dead cells in the tumor spheroids after treatment with MTX or MTX loaded dendrimers. For this purpose, tumor spheroids were incubated with PBS, MTX or MTX loaded dendrimers for 7 days. Subsequently, they were incubated with ethidium bromide (50 µg/mL) for 20 min at 4°C. After incubation, they were washed four times with PBS and disintegrated with 0.5% Triton-X containing 0.1 N NaOH. Fluorescence in the cell lysate was measured by using Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria). Protein content of tumor spheroids was estimated using micro BCA™ protein assay reagents.

C). Diffusion of dendrimers into tumor spheroids

Tumor spheroids were incubated with rhodamine labeled dendrimers (200 µg/mL) for 1, 2, 4, 6, 12, and 24 h. At specified time points spheroids were washed four times with HBSS, fixed with formaldehyde (10% w/v in PBS) for 30 min and placed in cavity microscope slides. Confocal laser scanning microscope images were acquired at 10 X using confocal microscope system described above. Eight µm thick optical sections were acquired from the top towards the center of the spheroids until approximately 136 µm deep into the spheroid.

5.4. Results

5.4.1. Conjugation of glucosamine to the dendrimers

Conjugation of glucosamine to the dendrimers was carried out by using di-succinimidyl carbonate so that the amino group of the glucosamine is selectively activated to form succinimidyl derivative. This would in turn result in glucosamine conjugated to the dendrimers by a carbamate linkage. Characterization of products by FTIR and ^1H NMR (Figure 5.1s, Supporting information) proved the successful synthesis. The number of glucosamine molecules attached to each dendrimer was also determined by ^1H NMR spectroscopy. Three glucosamine molecules per dendrimer were found to be conjugated in den-1-(G2)-400-Glu; while, den-2-(G2)-400-Glu had 5 glucosamine molecules conjugated per dendrimer.

5.4.2. MTX loading in dendrimers

MTX loading in the dendrimers of den-1-series ranged between 17.2 and 21.9% w/w, while in that of den-2-series between 20.3 and 24.5% w/w (Table 5.1). Dendrimers with PEO 400 Da in the interior cavity encapsulated higher amount of MTX as compared to dendrimers with PEO 300 and 200 Da in both den-1-series and den-2-series; with MTX loading of 21.9% w/w in den-1-(G2)-400 and 24.5% w/w in den-2-(G2)-400. MTX loading in glucosylated dendrimers was lower than corresponding non-glucosylated dendrimers, with den-1-(G2)-400-Glu and den-2-(G2)-400-Glu encapsulating 19.4% w/w and 20.8% w/w of MTX, respectively.

5.4.3. Internalization of dendrimers by glioma cells

U87 MG and U343 MG-A cells endocytosed approximately 76 and 347 $\mu\text{g}/\text{mg}$ of protein of rhodamine labeled den-1-(G2)-400 in 6 h (Figure 5.1a). For both the cell lines,

Table 5.1. Hydrodynamic size and MTX loading in dendrimers

<i>Dendrimer</i>	<i>Hydrodynamic size (nm)^a</i>	<i>Drug loading (w/w)^b</i>
Den-1-(G2)-200	3.58 (0.11)	17.2 ± 1.8
Den-1-(G2)-300	4.74 (0.13)	19.1 ± 1.1
Den-1-(G2)-400	5.41 (0.27)	21.9 ± 1.7
Den-1-(G2)-400-Glu	7.52 (0.34)	19.4 ± 1.3
Den-2-(G2)-200	4.19 (0.11)	20.3 ± 1.0
Den-2-(G2)-300	4.83 (0.19)	22.9 ± 1.2
Den-2-(G2)-400	7.41 (0.14)	24.5 ± 1.1
Den-2-(G2)-400-Glu	11.24 (0.41)	20.8 ± 1.5

^a Data are mean (PI) (n= 5)

^b Data are mean ± SD (n= 4-6)

amount of dendrimers internalized by the cells increased with time till 6 h, but decreased at 12 h (Figure 1). Extent of internalization of dendrimers was 3-5 times greater in U343 MG-A cells than in U87 MG cells. The reason for the higher endocytosis of dendrimers in U343 MG-A cells is not understood as yet. Conjugation of glucosamine significantly increased the endocytosis of the dendrimer to 603 $\mu\text{g}/\text{mg}$ of protein i.e. by 8 folds in U87 MG and to 672 $\mu\text{g}/\text{mg}$ of protein i.e. by approximately 2 folds in U343 MG-A cells (Figure 5.1b). Confocal laser scanning microscopy images of the human glioma cells incubated with rhodamine labeled dendrimers also showed that dendrimers internalized in high amount within 4 h of incubation and localized mainly in the cytoplasm (Figure 5.2).

5.4.4. Characterization of MTX resistant U87 MG cells

Microscopic examination of MTX resistant cells showed marked differences in the morphological features; primarily MTX resistant U87 MG cells were larger, with larger nuclei and had cellular granules in the cytoplasm (Figure 5.2s, Supporting information). The DHFR activity in the cell extracts of MTX resistant U87 MG cells was found to be 0.89 ± 0.22 $\mu\text{M}/\text{min}/\text{mg}$ of protein, while in MTX sensitive cells it was 0.39 ± 0.19 $\mu\text{M}/\text{min}/\text{mg}$ of protein. The difference in the activity of DHFR proved that MTX resistant cells were successfully developed (Alt et al., 1976). It also indicated that MTX sensitive U87 MG cells had a higher ability to convert folic acid to dihydrofolic acid and thus, lower activity of DHFR enzyme in the cells was one of the source of MTX resistance. MTX resistant cells accumulated MTX-FITC approximately two times higher than MTX.

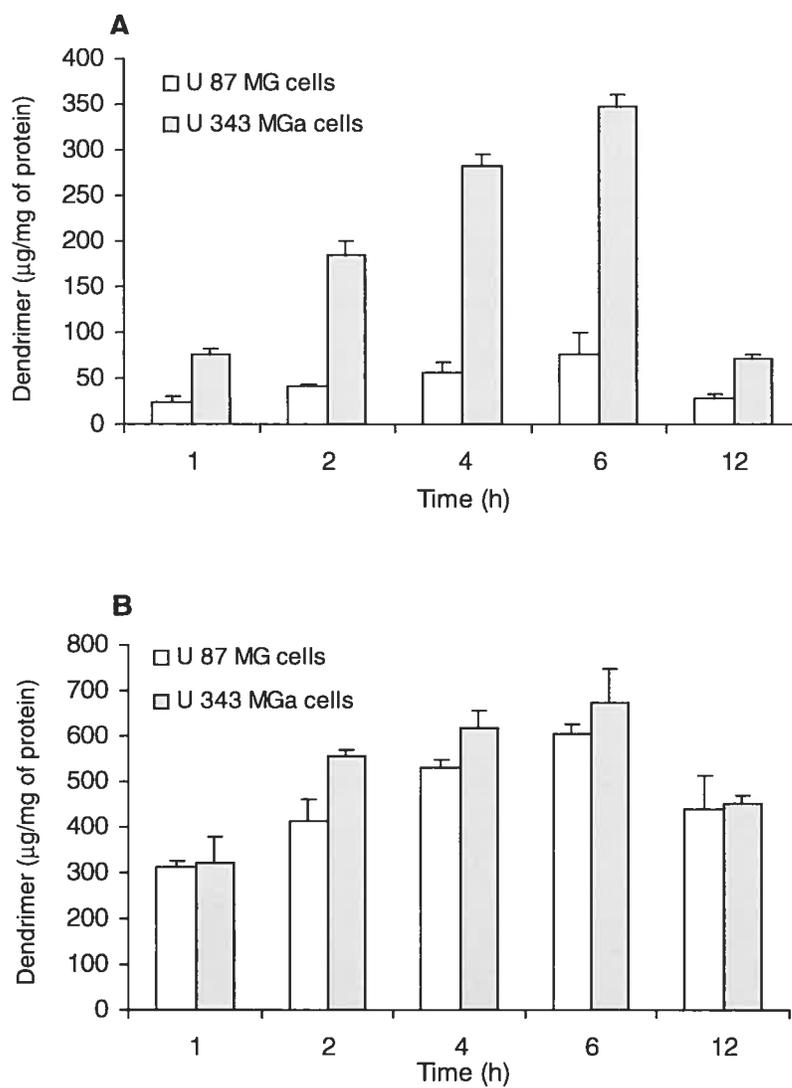


Figure 5.1. Extent of internalization of rhodamine labeled (A) den-1-(G2)-400 (B) den-1-(G2)-400-Glu in the human glioma cells. Data are mean values ($n = 6 + SD$)

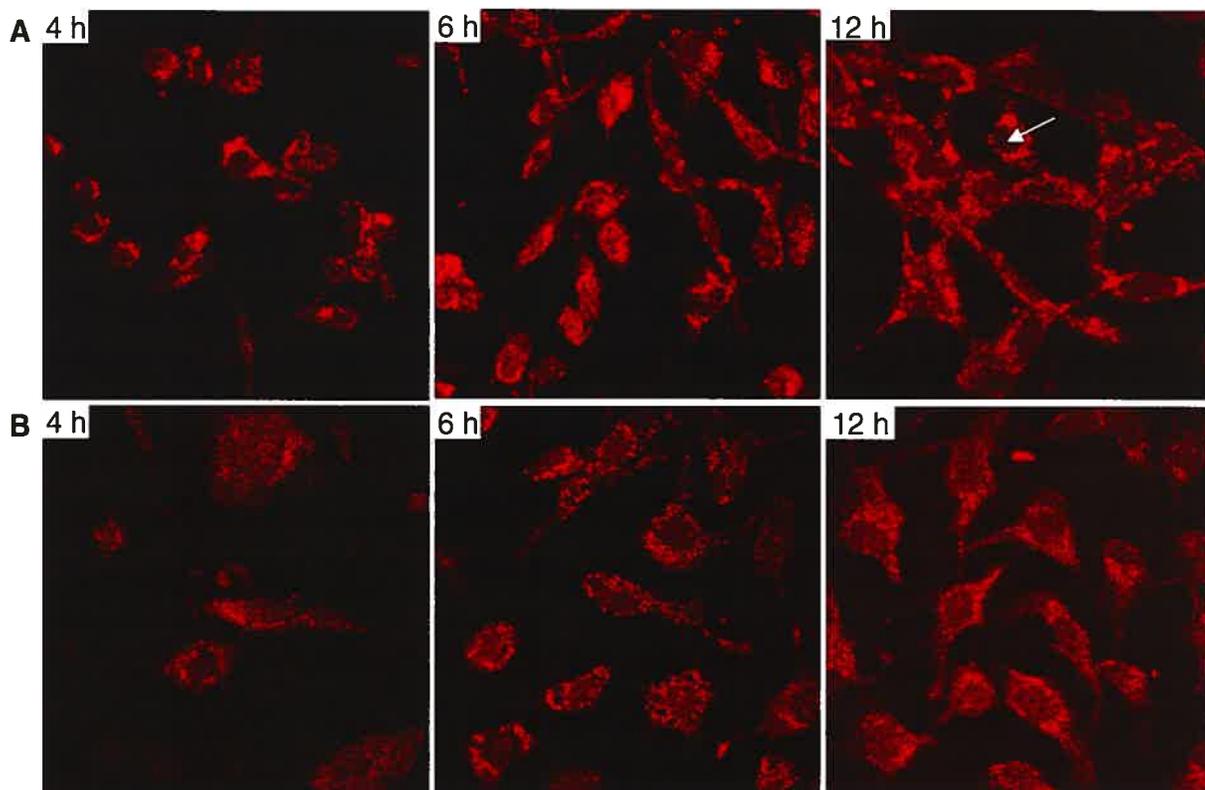


Figure 5.2. Confocal microscope images of U343 MG-A cells incubated with (A) rhodamine labeled den-1-(G2)-400 (B) rhodamine labeled den-1-(G2)-400-Glu. Images were acquired at 100 X. White arrow indicates nuclei of the cell.

5.4.5. Antiproliferative activity of dendrimers against glioma cells

The cytotoxicity of the dendrimers alone on the brain endothelial cells (Dhanikula and Hildgen, (Submitted)) and U87 MG cells were evaluated to ascertain their safety. None of the dendrimers showed significant cytotoxicity against bEnd.3 and U87 MG cells in the concentration of 0.01 mg/mL to 5 mg/mL (data not shown). Inhibition in the growth of two glioma cell lines was tested in the present study to evaluate their potential in different types of gliomas. IC₅₀ values of MTX towards U87 MG and U343 MG-A cells were 2.14 and 2.79 μ M, respectively (Table 5.2). In both the cell lines, MTX loaded in dendrimers had 1.5-5 times lower IC₅₀ value than free MTX. In MTX resistant U87 MG cells, MTX encapsulated in dendrimers had IC₅₀ values 9 to 15 times lower (1.17 to 5.22 μ M) than that of free MTX (85.95 μ M). The conjugation of glucosamine to dendrimers further reduced the IC₅₀ of MTX, but the extent of reduction was dependent on the cell line. MTX loaded dendrimer-glucosamine conjugates were 3.5 to 4.5 times more potent than dendrimers alone towards U87 MG cells with IC₅₀ values of 0.35 ± 0.08 and 0.43 ± 0.05 μ M and 2 times more toxic towards U343 MG-A cells with IC₅₀ values of 0.39 ± 0.13 and 0.42 ± 0.15 μ M (Table 5.2). This indicates that there is variation in the extent of uptake of glucosylated dendrimers and hence, expression of glucose transporters in these cell lines. Even in MTX resistant cell lines glucosylated dendrimers were 2-5 times more effective in inhibiting the cell growth (Table 5.2). The influence of increasing the number of glucosamine units from 3 (den-1-(G2)-400-Glu) to 5 (den-2-(G2)-400-Glu) on potency was not apparent in the IC₅₀ values.

5.4.6. Transport of dendrimers and MTX across BBB

The cumulative permeation of rhodamine labeled dendrimers across BBB model was highest for den-1-(G2)-400-Glu (345 μ g) followed by den-2-(G2)-400-Glu (242 μ g), den-2-(G2)-400 (204 μ g) and den-1-(G2)-400 (102 μ g) (Figure 5.3). No significant

Table 5.2. IC₅₀ values of MTX and MTX loaded dendrimers against different human glioma cell lines

<i>Sample</i>	<i>U87 MG cells (μM)</i>	<i>U343 MG-A cells (μM)</i>	<i>MTX resistant U87 MG cells (μM)</i>
MTX	2.14 ± 0.34	2.79 ± 0.64	85.95 ± 3.52
Den-1-(G2)-200	1.28 ± 0.08 ^{**}	0.62 ± 0.16 ^{***}	-
Den-1-(G2)-300	1.91 ± 0.25 ^{**}	0.59 ± 0.07 ^{***}	-
Den-1-(G2)-400	1.93 ± 0.44 ^{**}	0.70 ± 0.11 ^{***}	5.22 ± 0.86 ^{***}
Den-1-(G2)-400-Glu	0.43 ± 0.05 ^{***}	0.39 ± 0.13 ^{***}	1.17 ± 0.41 ^{***}
Den-2-(G2)-200	1.42 ± 0.81 ^{**}	1.30 ± 0.18 ^{**}	-
Den-2-(G2)-300	1.41 ± 0.26 ^{**}	1.35 ± 0.19 ^{**}	-
Den-2-(G2)-400	1.22 ± 0.36 ^{**}	0.90 ± 0.22 ^{***}	3.88 ± 0.61 ^{***}
Den-2-(G2)-400-Glu	0.35 ± 0.08 ^{***}	0.42 ± 0.15 ^{***}	2.02 ± 0.52 ^{***}

Data are mean ± SD (n=5-6)

Statistical analysis was performed using t-test and *post-hoc* Man-Whitney rank sum test (*** = p < 0.001, ** = p < 0.005, * = p < 0.05)

reduction in TEER was observed throughout the study (data not shown), indicating that dendrimers were able to cross BBB without disruption of its barrier properties. Glucosylated dendrimers were able to permeate across BBB more than dendrimers alone. In the case of den-1-(G2)-400-Glu, the extent of permeation was 3.5 times higher than that of den-1-(G2)-400, while in den-2-(G2)-400-Glu it was 1.2 times den-2-(G2)-400 (Figure 5.3). The permeability of dendrimer with five glucosamine units (den-2-(G2)-400-Glu) was marginally higher than one with three units (den-1-(G2)-400-Glu), suggesting that three glucosamine units could be ideal for enhanced delivery across BBB.

Encapsulation of MTX in dendrimers increased the amount of MTX transported across BBB by three-four folds. Approximately 0.11 mM MTX was transported across BBB over a period of 12 h (Figure 5.4). This was increased to 0.37 mM by den-1-(G2)-400, to 0.73 mM by den-1-(G2)-400-Glu, to 0.58 mM by den-2-(G2)-400, and to 0.69 mM by den-2-(G2)-400-Glu (Figure 5.4). It was also seen that conjugation of three glucosamine molecules to the dendrimers (den-1-(G2)-400-Glu) elevated the amount of MTX permeating across BBB by approximately two folds. However, the presence of 5 glucosamine units per dendrimer in den-2-(G2)-400-Glu only marginally increased the amount of MTX available across BBB, primarily due to greater hydrodynamic size of the corresponding dendrimer (Table 5.1).

5.4.7. Interaction with avascular human glioma tumor spheroids

Confocal laser scanning microscopic imaging of the tumor spheroids showed that in the first 4 h of incubation dendrimers are present only in the periphery; however the extent of diffusion was greater for the dendrimer with glucosamine ligand (Figure 5.5). Within 6 h of incubation, glucosylated dendrimer were able to distribute throughout the tumor spheroids; while dendrimers without ligand had taken 12 h for the complete distribution (Figure 5.5). More importantly it was also seen that the extent of distribution was much higher in glucosamine conjugated dendrimers. These results show that dendrimers *per se*

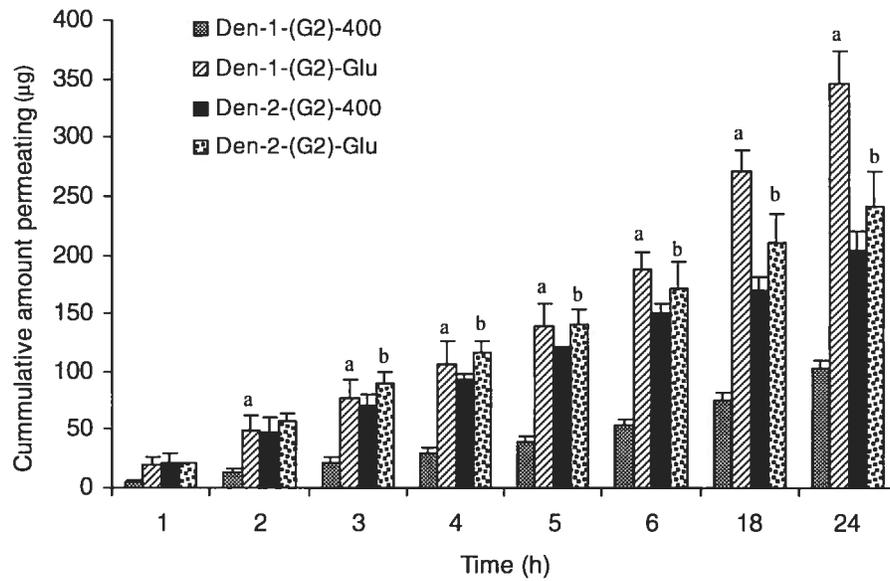


Figure 5.3. Transport of rhodamine labeled dendrimers across the BBB model (co-culture of bEnd.3 and U373 MG cells) at 37°C. Data are mean values (n = 4 + SD)

^aStatistically significant difference between den-1-(G2)-400 and den-1-(G2)-400-Glu, den-2-(G2)-400 and den-2-(G2)-400-Glu ($p < 0.001$, t-test and *post-hoc* Man-Whitney rank sum test) and ^bbetween den-2-(G2)-400 and den-2-(G2)-400-Glu after 2 h ($p < 0.05$, t-test and *post-hoc* Man-Whitney rank sum test).

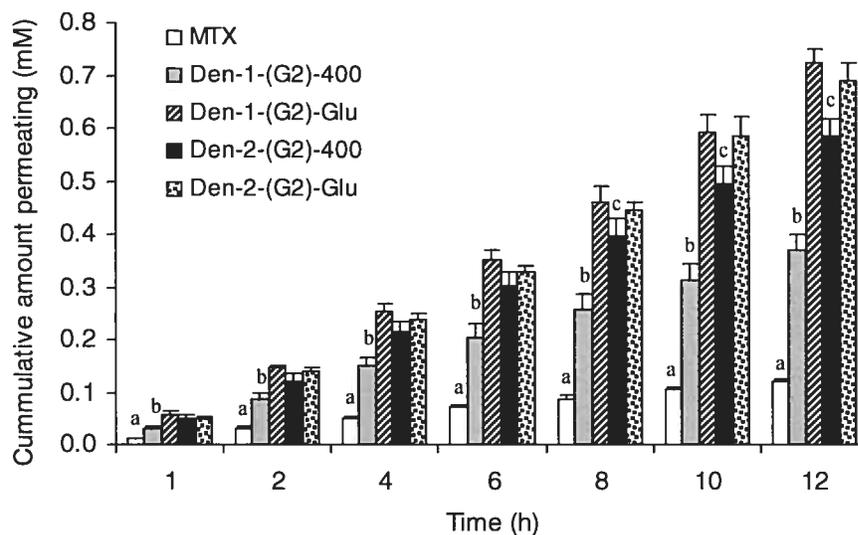


Figure 5.4. Cumulative amount of MTX permeating across the BBB model at 37°C. MTX or MTX loaded dendrimers were placed in the donor compartment of Transwell® inserts and the amount of MTX permeating into the receiver compartment was analyzed by HPLC. Data are mean values (n = 4 + SD).

^aStatistically significant difference between free MTX and all dendrimers ($p < 0.0001$, t-test and *post-hoc* Man-Whitney Rank Sum test), ^bbetween den-1-(G2)-400 and den-1-(G2)-400-Glu, den-2-(G2)-400, den-2-(G2)-400-Glu ($p < 0.001$, t-test and *post-hoc* Man-Whitney rank sum test) and ^cbetween den-2-(G2)-400 and den-2-(G2)-400-Glu after 8 h ($p < 0.05$, t-test and *post-hoc* Man-Whitney rank sum test).

are able to diffuse through out the avascular tumor spheroids. Conjugating glucosamine ligands increased the rate and extent of distribution of dendrimers; thus, making it faster and more efficient. The influence of various treatments on the growth of tumor spheroids was also studied. It was observed that tumor spheroids continued to grow in size and volume in the absence of any drug (Figure 5.6b). Free MTX was able to inhibit the growth for few days but produced only marginal reduction in the tumor spheroid volume (Figure 5.6c); on the other hand, MTX loaded dendrimers produced considerable reduction in size (Figure 5.6d and 5.6e). The extent of reduction in the tumor volume was different for U87 MG and U343 MG-A tumor spheroids. In U87 MG tumor spheroids, tumor spheroid volume was 79%, 75%, 66% and 65% of the control after 7 days at 0.2 mM MTX and 70%, 69%, 63% and 71% at 0.4 mM MTX for den-1-(G2)-400, den-1-(G2)-400-Glu, den-2-(G2)-400 and den-2-(G2)-400-Glu, respectively (Figure 5.7). Reduction in the size of tumor spheroids was higher in case of U343 MG-A tumors spheroids, at 0.2 mM MTX, tumor spheroid volume was 46%, 49% and 43% of the control after 7 days of incubation; while, at 0.4 mM it was 31%, 35% and 33% for den-1-(G2)-400-Glu, den-2-(G2)-400 and den-2-(G2)-400-Glu, respectively (Figure 5.7). The higher reduction in U343 MG-A tumor spheroids indicates that U87 MG tumor spheroids are more resistant to MTX than U343 MG-A tumor spheroids. In both tumor spheroids increase in concentration increased the activity for all dendrimers except in den-2-(G2)-400-Glu, perhaps due to aggregation at high concentration which reduced uptake by the tumor spheroids. Ethidium bromide is known to stain the DNA of dead cells; hence, its concentration is related to the population of dead cells in the sample. Concentration of ethidium bromide (stains DNA of dead cells) in the tumor spheroids treated with MTX loaded dendrimer was 13.54 $\mu\text{g}/\text{mg}$ of protein, while, in control and in those treated with free MTX it was 3.77 and 5.45 $\mu\text{g}/\text{mg}$ of protein, respectively (Figure 5.8). In tumor spheroids treated with glucosylated dendrimer loaded with MTX, ethidium bromide concentration was 18.49 $\mu\text{g}/\text{mg}$ of protein. This clearly indicates that PEPE dendrimers induce statistically significant increase in cell death as compared to free MTX ($p < 0.01$, One way ANOVA, *post hoc* Student-Newman-Keuls

test) and also that glucosylation further enhanced this effect ($p < 0.01$, One way ANOVA, *post hoc* Student-Newman-Keuls test).

5.5. Discussion

The basic GLUT is reported to play a major role in glucose uptake by the tumor cells. Though glucose transporters are expressed in all tissues, the major difference between normal and cancer cells is the presence of facilitative GLUT genes in the tumors cells (Noguchi et al., 2000). Glucose conjugated niosomes have been reported to have higher tumor-to-muscle accumulation in glioma xenografts (Luciani et al., 2004); Kim et al., (Kim et al., 2004) have also demonstrated that glucose conjugated PEI enhance gene delivery to the lung cancer. Additionally, the presence of GLUT-1 can be potentially employed for enhanced delivery of the carriers across BBB (Pardridge et al., 1990). Dendrimer conjugated to glucose are small and mimic glucose, in fact that they have been reported to compete with glucose in glucose assay (Beier et al., 2007). Thus, they can be taken up by the GLUT on BBB as well as tumor cells. Due to these advantages offered by glucose ligand, glucosamine conjugates of dendrimers were synthesized in this study. D-glucosamine (2-amino-2-deoxy-D-glucose) was chosen as a ligand in this study because, it is reported that modification at 2 position of the glucose ring do not alter its interaction with glucose transporters, additionally presence of amino group would allow easy and reproducible conjugation at 2-amino position. The influence of glucosamine ligand on the targetability of the dendrimers was determined by studying the extent of internalization of dendrimers by the glioma cells. It was found that glucosylated dendrimers had 2 to 8 folds higher levels in all the glioma cells (U87 MG and U343 MG-A) in 6 h (Figure 5.1). This puts forward the fact that presence of glucosamine ligand significantly enhanced the targetability of the dendrimers to glioma cells. Interestingly, compared to non-glucosylated

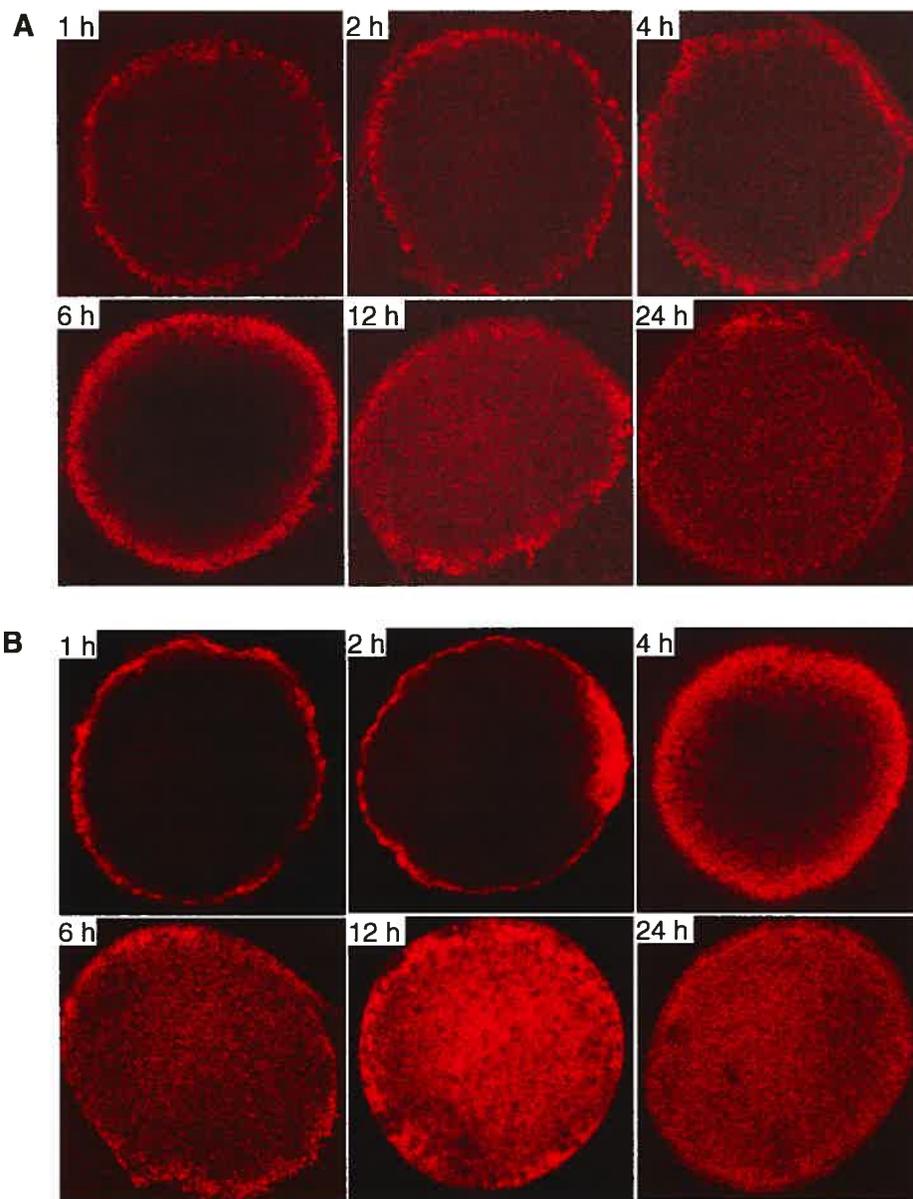


Figure 5.5. Confocal microscope images of U87 MG tumor spheroids incubated with (A) rhodamine labeled den-1-(G2)-400 (B) rhodamine labeled den-1-(G2)-400-Glu as a function of time. Images were acquired at 10 X.

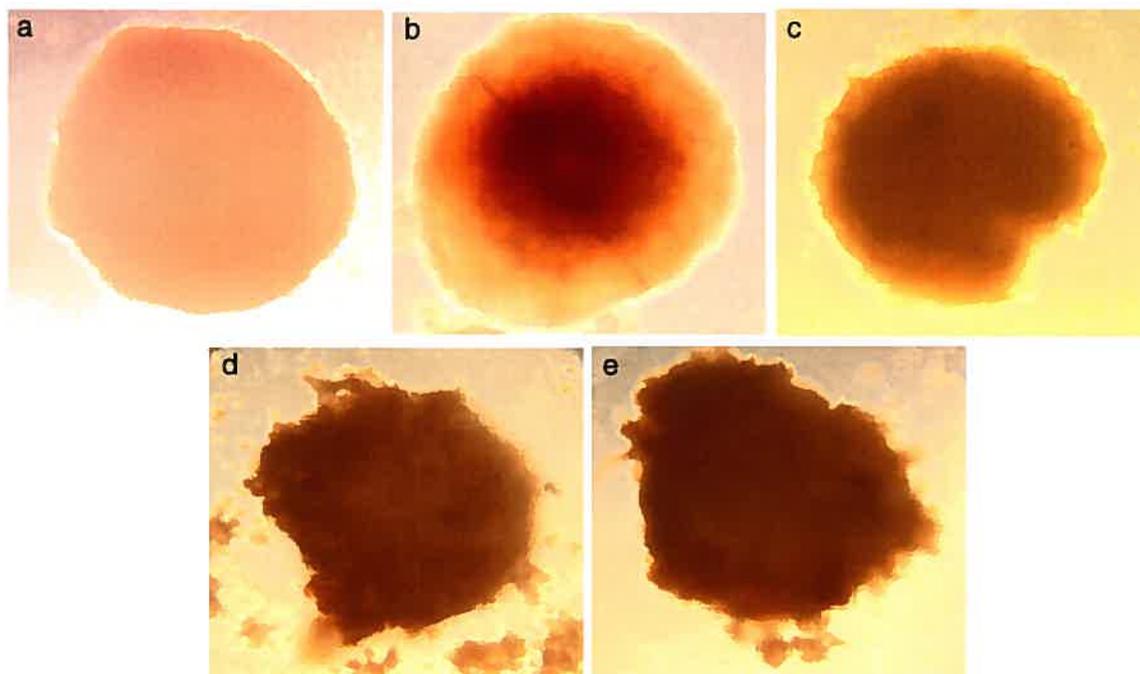


Figure 5.6. Representative micrographs of U87 MG tumor spheroids (a) before treatment, 4 days after treatment with (b) PBS (c) MTX (0.4 mM) (d) den-2-(G2)-400 loaded with MTX (0.4 mM) (e) den-1-(G2)-400-Glu loaded with MTX (0.4 mM). Images were acquired using 10 X objective.

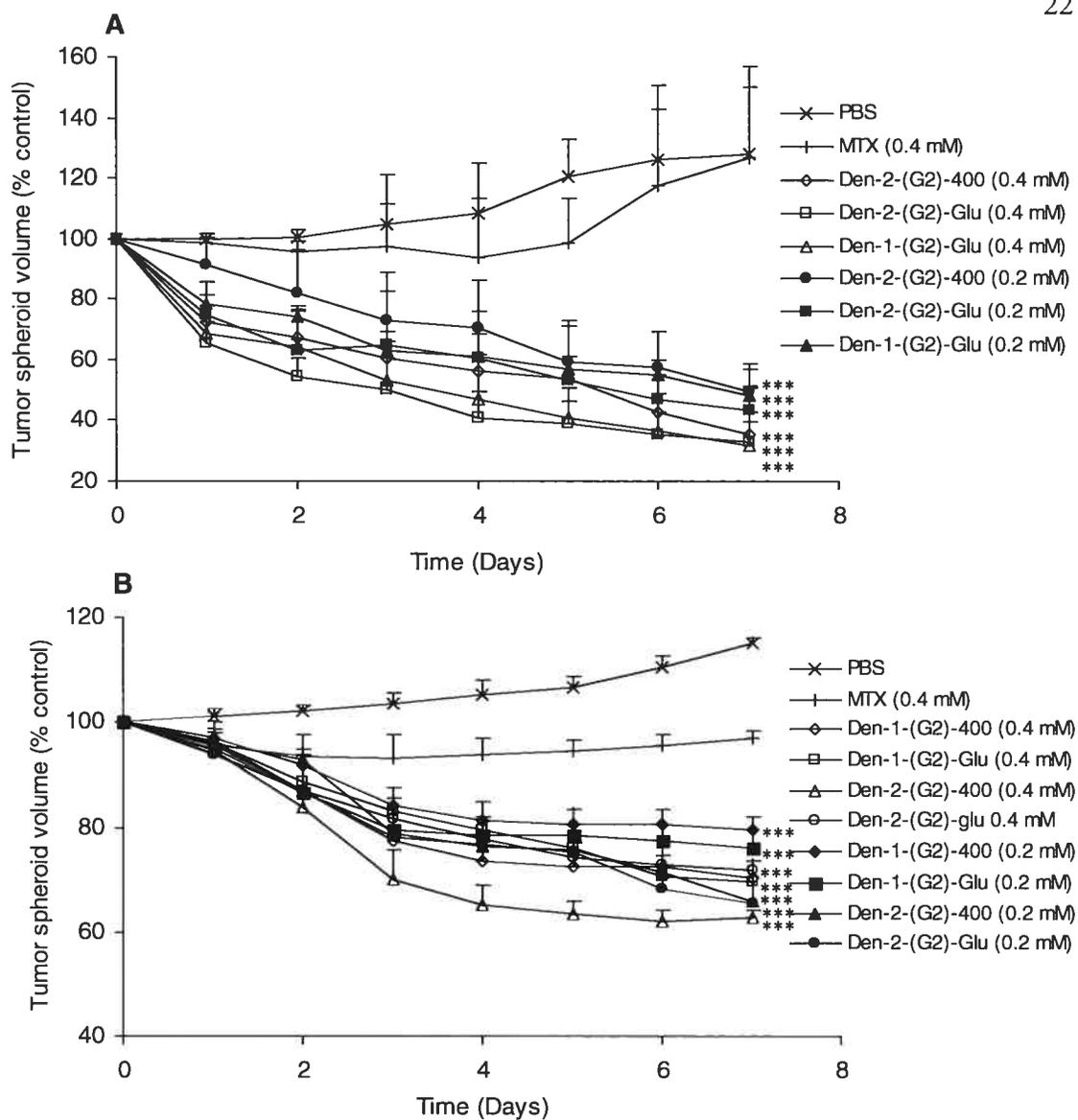


Figure 5.7. Inhibition in the growth of (A) U343 MG-A (B) U87 MG tumor spheroids on treatment with MTX or MTX loaded dendrimers. The diameter of tumor spheroids were measured using a microscope fitted with an ocular micrometer and the volume of the spheroids was calculated. Data are mean values ($n = 4 + SD$). *** Statistically significant difference with respect to MTX ($p < 0.005$, One way RM ANOVA, *post hoc* Student-Newman-Keuls test)

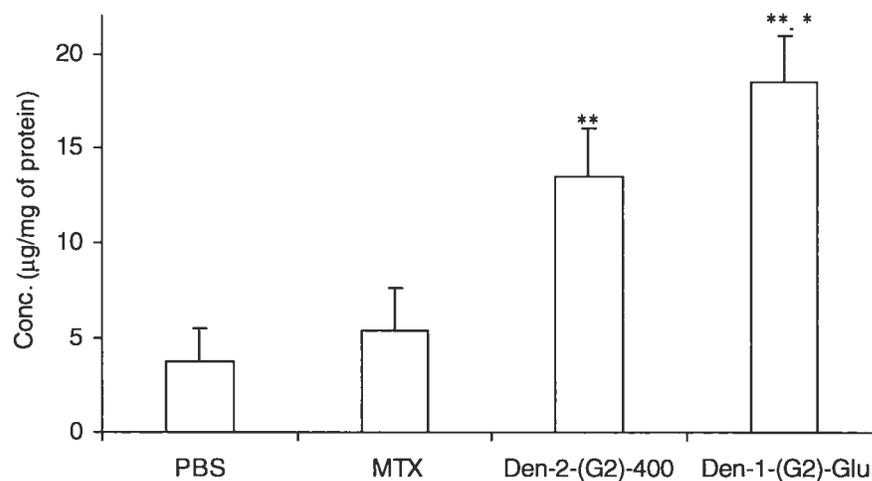


Figure 5.8. Accumulation of ethidium bromide in U87 MG tumor spheroids subjected to different treatments. Ethidium bromide stains the DNA of dead cells and thus its concentration relates to dead cell population. Data are mean values ($n = 4 + SD$). ** Statistically significant difference with respect to PBS and MTX ($p < 0.05$, One way ANOVA, *post hoc* Student-Newman-Keuls test); * Statistically significant difference with respect to den-2-(G2)-400-Glu ($p < 0.01$, One way ANOVA, *post hoc* Student-Newman-Keuls test)

Note: Den-2-(G2)-400 and den-2-(G2)-400 were selected because they produced greater reduction in U87 MG tumor spheroid size at 0.4 mM.

dendrimers, fluorescence of glucosylated dendrimers was retained at higher levels in the cells even at 12 h (Figure 5.1), probably due to more stable rhodamine dendrimer linkage or could be due to lower efflux of glucosylated dendrimers out of the cells. Dendrimers internalized very fast in the cells and were present throughout the cytoplasm within 4 h (Figure 5.2). The pattern of distribution of dendrimers in the cells was not found to be influenced by the conjugation of glucosamine to the dendrimers. However, greater fluorescence was seen in the nucleus of cells treated with den-1-(G2)-400-Glu suggested their entry into the nucleus.

Considering the limitation of drug-dendrimer conjugates, MTX was loaded in PEPE dendrimers in the present study. PEPE dendrimers were able to encapsulate high amount of MTX (20.3-24.5% w/w, Table 5.1) as compared to polyethylene glycol (PEG) grafted polyamido amine (PAMAM) dendrimers, which could encapsulate 10 to 13 mol of MTX/mol of dendrimer (Kojima et al., 2000). We have previously reported that this is due to augmentation in the size of interior voids of PEPE dendrimers with increase in the molecular weight of PEO (Dhanikula and Hildgen, 2007). Further, dendrimers of den-2-series have higher number of terminal PEG branches as compared to den-1-series and thus, have greater encapsulation ability (Dhanikula and Hildgen, 2007). The amount of MTX loaded in dendrimers increased with the molecular weight of PEG in the interior in the both series. Conjugation of glucosamine to the dendrimers was seen to decrease the loading for both the den-1-series and den-2-series (Table 5.1). This could happen due to the hindered diffusion of the MTX molecules into the interior of the dendrimers because of steric hindrance offered by the glucosamine units at the surface or due to the bending of glucosamine units into the dendritic structure. Nevertheless, even in the presence of glucosamine units the drug loading was significantly high.

Recently, dendrimers have been utilized for the delivery of various chemotherapeutic agents including MTX. However, in most of these studies MTX was conjugated to the terminal groups of the dendrimers. These conjugates have failed to

provide increase in the therapeutic benefit; for instance, Wu et al., 2006 (Wu et al., 2006) reported that MTX conjugated to PAMAM dendrimers and EGFR antibody did not show any improvement in the potency either due to reduced binding of the conjugate to DHFR or due to low cleavage of MTX from the conjugate. In another study Patri et al., 2005 (Patri et al., 2005) reported that the PAMAM, folic acid and MTX conjugate had lower inhibitory potential than free MTX. They also reported that MTX loaded dendrimers were equally toxic to free MTX and did not demonstrate any increase in the potency after encapsulation of drug in the dendrimers (Patri et al., 2005). This was attributed to fast release of MTX from PAMAM dendrimers. However, in the present study MTX loaded dendrimers were more efficacious than free MTX (Table 5.2). In figure 5.1 and 5.2 it is evident that these dendrimers were endocytosed effectively by the cells. Glucosylation of the dendrimers further improved the potency of these MTX loaded dendrimers against glioma cells by approximately 2 to 4.5 folds (Table 5.2). The higher potency of glucosylated dendrimers can be correlated to their higher cellular uptake as compared to non-glucosylated dendrimers by the glioma cells (Figure 5.1). Further, dendrimers were able to notably overcome the MTX-resistance and reduce the IC_{50} by approximately 9-15 times (Table 5.2). Ability of these dendrimers to improve the potency of MTX towards resistant glioma cell lines suggests that they contain the drug before entering the cells i.e. drug is released from the dendrimers after entering the cells. And that dendrimers enter the cells by mechanism different from that of the uptake of MTX. MTX is reported to be taken up into the mammalian cells by the reduced folate receptors (Matherly and Goldman, 2003). Loss of reduced folate receptor functions is a common mode of MTX resistance in the cells (Zhao and Goldman, 2003). Thus, lower uptake of MTX by U87 MG cells is also one of the factors in the resistance of these cells to MTX, in addition to lower DHFR activity. From these results it is evident that PEPE dendrimers deliver high payload of MTX intracellularly.

Various attempts have been made in the past to improve the therapeutic index of MTX for treating brain tumors by using anti-EGFR mAbs (Brady et al., 1992; Faillot et al.,

1996; Loscher and Potschka, 2005) or by using MTX loaded immuno-liposomes (Saito et al., 2004). However, major limitation of these systems is low permeability across BBB. Consequently, intrathecal delivery or convection enhanced delivery have been used to increase the therapeutic benefits. These delivery strategies are highly invasive in nature. Thus, in order to understand the influence of glucosamine conjugation on the permeability of dendrimers across BBB.

Recently, various *in vitro* BBB model have emerged as a valuable method to investigate permeability of drug and delivery systems across BBB in early development stages. These models allow rapid screening of large number of drugs or delivery systems which would otherwise a time consuming process. They retain many features of BBB like presence of tight junction elements like ZO-1, occludin, claudin-1 and various transporters like GLUT-1, OAT1, amino acid carriers, P-gp (Omidi et al., 2003; Song and Pachter, 2003). A strong correlation between permeability of various drug across BBB model consisting of co-culture of brain endothelial cells and astrocytes and human brain concentration is reported (Dehouck et al., 1992; Lundquist et al., 2002). Thus, permeability of dendrimers and glucosylated dendrimers across *in vitro* BBB model were determined in the present study. Dendrimers were able to permeate the BBB and reach the receiver compartment in high amounts (Figure 5.3). Notably, the extent of permeation of den-2-(G2)-400 was 2 times higher than of den-1-(G2)-400. It is speculated that this higher permeation of den-2-(G2)-400 across BBB was due to higher number of PEG chains present on the surface of the latter (Dhanikula and Hildgen, 2007). Den-1-(G2)-400-Glu, with 3 glucosamine molecules per dendrimer had 3.5 times greater permeability in comparison with den-1-(G2)-400; while Den-2-(G2)-400-Glu, with 5 glucosamine molecules per dendrimer had moderately higher permeation (1.2 folds) than den-2-(G2)-400 across BBB model (Figure 5.3). It is hypothesized that moderate augmentation in permeability of glucosylated dendrimer, den-2-(G2)-400-Glu as compared to den-2-(G2)-400 is because of the larger size of the dendrimer in the presence of 5 glucosamine moiety which reduces their endocytosis (Table 5.1). Nonetheless, higher permeability of

glucosylated dendrimers suggests that glucosamine serves to increase the permeability of dendrimers across BBB. The effect of higher permeability of glucosylated dendrimers were reflected in the amount MTX delivered by these dendrimers across BBB (Figure 5.4). Both glucosylated and non-glucosylated dendrimer were able to transport more amount of MTX across the barrier than free MTX. Encapsulation of MTX in den-1-(G2)-400 and den-2-(G2)-400 increased the amount of MTX available across BBB 4 and 6 times, respectively (Figure 5.4). Elevated amount of MTX available in the receiver compartment of BBB model in the latter dendrimer is due to its higher permeation across BBB resulting from higher number of terminal PEG branches in its chemical structure (Dhanikula and Hildgen, 2007). Glucosylation of den-1-(G2)-400 and den-2-(G2)-400 increased the availability of MTX across BBB by two folds in den-1-(G2)-400-Glu, but only by approximately one fold in den-2-(G2)-400-Glu (Figure 5.4). Lower increment in MTX availability by den-2-(G2)-400-Glu can be explained by its lower permeability across BBB (Figure 5.3) due to larger size in the presence of 5 glucosamine moiety (Table 5.1). Interestingly, den-1-(G2)-400-Glu transported 7 times higher amount of MTX across BBB as compared to free MTX. Thus, conjugation of 3 glucosamine molecules per dendrimer as in the case of den-1-(G2)-400-Glu seems to be ideal for enhancing the permeability of PEPE dendrimers across BBB. In the previous study (Dhanikula and Hildgen, (Submitted)) we have established that these PEPE dendrimers cross BBB intact. Thus, these PEPE dendrimers can be used for the delivery of higher amount of MTX across BBB and the conjugation to glucosamine can further augment it.

Due to the stagnation of delivery systems at the periphery of the tumor vasculature and poor permeation into the hypoxic and necrotic regions of the tumor that are distant from the vascular bed, the amount of drug accessing inside the solid tumors is low (Yuan et al., 1994; Kostarelos et al., 2004; Minchinton and Tannock, 2006). As a consequence the overall therapeutic effect of chemotherapeutic agents is restricted, leading to the relapse of cancer. These limitations of delivery systems are particularly dangerous in malignant gliomas which are one of the most refractory tumors (Graham and Cloughesy, 2004;

Desjardins et al., 2005). It is postulated that enhancement in the ability of the delivery system to penetrate deeper into the tumor tissues can significantly reduce the tumor regrowth and augment the therapeutic benefit of the treatment. Thus, in this study targeting, interstitial penetration and diffusion of dendrimers into avascular regions of solid tumors was studied using tumor spheroids as a model. Rhodamine labeled dendrimers were able to reach the central necrotic region of the dendrimers in 12 h of incubation (Figure 5.5) suggesting that they can diffuse across the avascular region of tumor tissue and can deliver the drug load to these regions. Glucosylation was found to enhance the rate and extent of diffusion of the dendrimers in the tumor spheroids. This is because the central regions of the solid tumors are hypoxic and they are reported to demonstrate a hypoxia related increase in glucose transport (Clavo et al., 1995; Airley et al., 2001). Thus, greater demand of glucose in the hypoxic regions of the tumors drives the faster and enhanced distribution of glucosylated dendrimers in the tumors spheroids. Hence, these PEPE dendrimers would be able reach the avascular regions of the tumors within 150-200 μm and deliver their payload and thus can provide more effective control of the tumor growth.

Tumor spheroids of U87 MG and U343 MG-A cells were prepared to evaluate the efficacy of these dendrimers in treating solid tumors. MTX loaded dendrimers were able to considerably reduce the size of tumor spheroids during 7 days of the study (Figure 5.6 and 5.7). In U87 MG tumor spheroids, tumor volume was reduced to 63-79% of the control while, in U343 MG-A tumor spheroids it was decreased to 31-49% of the control (Figure 5.7), indicating that U87 MG tumor spheroids are more resistant to MTX than U343 MG-A tumor spheroids. The potency of the MTX loaded dendrimers was always higher than that of the free MTX (significant difference, $p < 0.005$, one way RM ANOVA). However, the therapeutic gain obtained from glucosylated dendrimers on tumor spheroids was different depending on the type of glioma cells. In both tumor spheroids, den-1-(G2)-400-Glu was more effective in reducing the volume as compared to den-1-(G2)-400, while den-2-(G2)-400-Glu was either more effective than or as effective as non-glucosylated den-2-(G2)-400 dendrimers alone at both concentrations tested (Figure 5.7). These results could also be

due to the fact that den-2-(G2)-400-Glu has greater size than den-1-(G2)-400-Glu and hence lower penetration in the tumors (Table 5.1). The concentration of ethidium bromide in the lysate of tumor spheroids treated with MTX loaded dendrimer was significantly higher than that of free MTX treated tumor spheroids and control ($p < 0.01$, One way ANOVA, *post hoc* Student-Newman-Keuls test) (Figure 5.8). Treatment of tumor spheroids with MTX loaded glucosylated dendrimers also produced augmentation in the accumulation of ethidium bromide ($p < 0.01$, One way ANOVA, *post hoc* Student-Newman-Keuls test). Ethidium bromide is known to stain the DNA of dead cells; hence, its concentration is related to the population of dead cells in the sample. In view of the above fact, MTX loading in the dendrimers significantly enhanced the percentage kill of human glioma cells in the tumor spheroids. This is attributed to enhanced entry of dendrimers into tumor spheroids as well as slow release of MTX after loading in these dendrimers (Dhanikula and Hildgen, 2007). Since, the tumor spheroids mimic the microenvironment of solid tumors, the higher efficacy of MTX loaded dendrimers suggests that they would provide significantly higher therapeutic benefit than MTX.

In a nutshell, this study shows that MTX loaded PEPE dendrimers are more potent than free MTX as well as effective against MTX resistant glioma cells. They can deliver MTX across BBB in high amounts and can penetrate into the central necrotic regions of avascular tumor spheroids. Additionally, glucosylation not only increases their potency but also enhances their permeability across BBB and diffusion into the avascular regions of the tumor tissue. And hence, glucosylated PEPE dendrimers can serve as effective delivery systems for the treatment of gliomas. Further *in vivo* studies to test the proof-of-the-concept would be carried out in future. It is speculated that these glucosylated dendrimers with ability to counteract drug resistance and enhance drug delivery to the central avascular regions of the tumor can serve as a step forward to improving the efficacy of chemotherapy.

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5.8. Supporting information

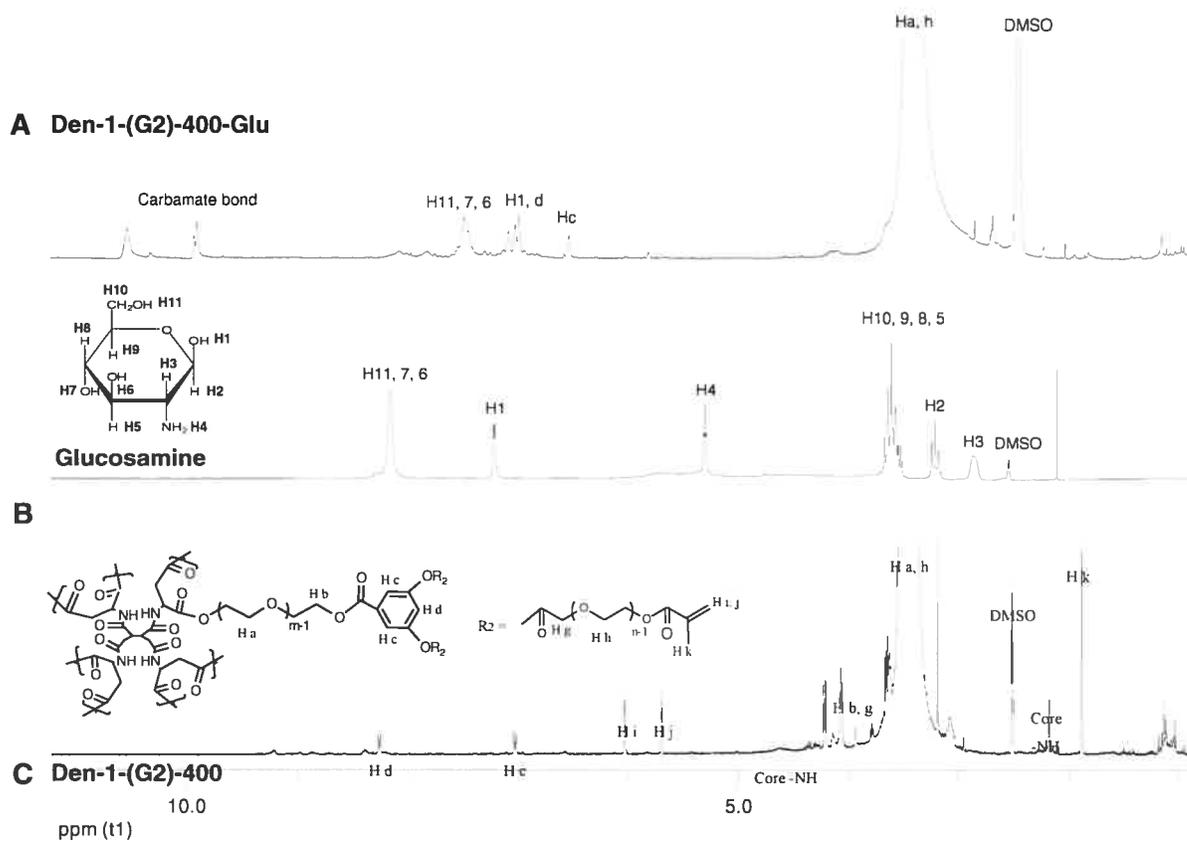


Figure 5.1s. ^1H NMR spectra of (A) Den-1-(G2)-400-Glu (glucosamine conjugated den-1-(G2)-400), (B) glucosamine and (C) Den-1-(G2)-400 in DMSO at 400 MHz.

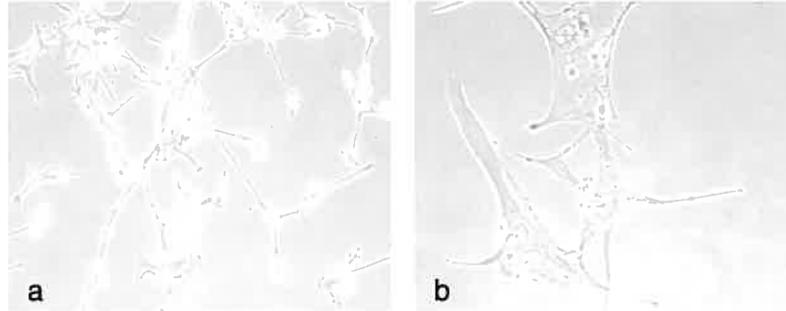


Figure 5.2s. Micrographs of U87 MG and MTX resistant U87 MG cells.

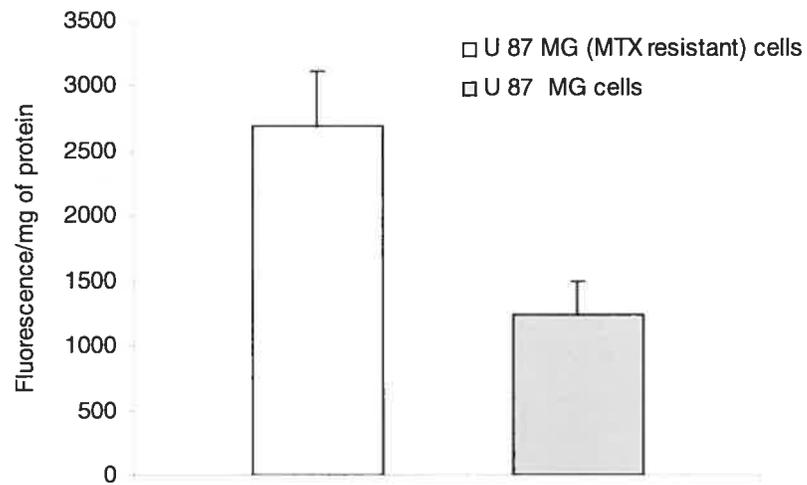


Figure 5.3s. Accumulation of MTX-FITC in the U87 MG and MTX resistant U87 MG cells in 2 h.

DISCUSSION

The effective delivery of drug for the treatment of diseases has always remained a formidable task in pharmaceutical industry. Extremely potent molecules can be rendered less effective due to inefficient delivery of the drug to the desired site of action; conversely, molecules with lower potency can provide a better therapeutic benefit if they reach the tissue/region for desired action (Charman et al., 1999). The clinical success of a formulation is defined not only by therapeutic benefit it provides but also by the toxicity to the patient after its administration. Thus, a balance of efficacy and toxicity has to be achieved. This could be accomplished by either site specific delivery and/or by minimizing exposure to undesirable tissues of the body. Nano-delivery systems like nanoparticles, liposomes, micelles etc. help to realize these objectives by encapsulating the drug, camouflaging it from the bodies' environment, releasing it slowly and also targeting it to the site of action (Braun et al., 2005; Yokoyama, 2005). The need for these delivery systems is felt more in diseases like cancer where drugs have significant toxicity on the body if administered alone. The promising advantages of these delivery systems in terms of improving patient compliance without compromising therapeutic benefit and also giving a second life to existing drug molecules has resulted in their appreciation by the pharmaceutical industry. In fact, the advancement in application of these pharmaceutical carrier technologies has progressed well beyond experimental/conceptual stages with number of successful products already in the market, and many more in the clinical trials or in development pipeline (Limited, 2005).

Though nanoparticles and liposomes have been widely utilized as deliver systems for site specific delivery, yet relatively newer polymeric scaffolds called dendrimers hold a promising future. Their unique, controlled and branched architecture of dendrimers has opened a new avenue for therapeutics primarily due to their monodispersity and high surface functionality (Tomalia et al., 1990; Aulenta et al., 2003; Gillies and Fréchet, 2005). Features of dendrimers that could result in superior performance relative to other carrier systems include, their size and shape (Bosman et al., 1999; Klajnert and Bryszewska,

2001c). At approximately 5 to 15 nm in diameter, dendrimers are approximately the size and shape of a hemoglobin molecule. They have globular shape with internal cavities and large number of surface functional groups. And most importantly they are monodisperse which would avoid variable and unpredictable pharmacokinetics of drug (Patri et al., 2002; Lee et al., 2005). By virtue of these exhilarating advantages dendrimers are envisaged to be the carriers of future nanotechnology based drug delivery. However, in spite of extensive research, design of dendrimers for drug delivery applications is still in its formative years. Much remains to be done to optimize their properties for more efficient drug delivery, mainly the issues of toxicity, drug loading, and drug release need to be addressed. In the present project these issues pertaining to dendrimers have been addressed and an attempt has been made to device delivery system which could provide effective delivery of model chemotherapeutic agent, MTX to brain tumors.

6.1. Synthesis and characterization of dendrimers

The dendritic macromolecules have greater degree of complexity and sophistication as compared to other polymeric systems, but the degree of control over macromolecular structure is significantly greater. And this ability to regulate the size, shape, molecular weight and properties by varying the monomers, core molecule etc. (Boas and Heegaard, 2004) is the most lucrative attribute of the dendrimers. Most of the dendrimers used for drug delivery like PAMAM dendrimers, DAB dendrimers, polyarylether dendrimers are synthesized by repeated reaction of small monomer units. As a consequence large number of synthetic steps are required to obtain desirable molecular weight resulting in a tedious process, increased structural defects and also impurity. Synthesis of dendrimers by successive grafting of polymeric building blocks, like PEG, poly(ϵ caprolactone) etc., a high molecular weight dendritic polymer can be obtained in only a few steps (Trollsås and Hedrick, 1998; Trollsås et al., 2000). This approach is also reported to simplify purification of dendrimers which is otherwise a tedious process (Trollsås and Hedrick, 1998). In fact, it

has been recently demonstrated by Gilles et al., (Gillies and Fréchet, 2002) that use of PEG can facilitate tunability of dendritic properties and architecture due its higher molecular weight. Thus, in this project PEO was used for the synthesis of the dendrimers. Dendrimers were synthesized using simple synthetic scheme by which second generation dendrimers could be obtained in seven reaction steps (Scheme 2.2). In addition, a combination of convergent and divergent method was used during synthesis so that dendrimers would have least structural defects and higher purity. To ensure the biodegradability of these dendrimers ester groups were incorporated in the structure.

The architecture of biomaterial not only originates from the chemistry of the smallest molecular units, but also from the way they are connected to each other and from the way they are arranged in the three dimensional network (Grest et al., 1987). Trollsås et al. (Trollsås et al., 2000) have shown that by changing the number of branching point, generation as well as the molecular weight (i.e. degree of polymerization) of poly(caprolactone), different shape and sizes of dendrimer can be obtained. Similar variation in these parameters was shown to influence the architecture of PEO based dendrimer like bow type hybrids by Gillies and Fréchet (Gillies and Fréchet, 2002). Thus, by playing with these parameters in the construction of dendrimers, different architectures can be obtained. Hence, in this project dendrimers of varying architecture were produced by using PEO of varying molecular weight as well as by changing the number of branching point and generation. The objective of designing dendrimers of different architecture was to determine the influence of factors like variation in molecular weight of PEO, density of branching etc. on the encapsulation of drug, release properties, toxicity, internalization in cells etc.

In brief, following variations were carried out to obtain dendrimers of different architecture:

- Three different series of dendrimers were synthesized; namely, den-1-series consisting of dihydroxy benzoic acid (DHBA), den-2-series, having gallic acid and den-3-series utilizing bis(hydroxyl methyl) butyric acid (BHBA) as the branching molecule.
- The variation in molecular weight in each series of dendrimer was produced by using PEO of different molecular weights i.e. 200, 300 and 400 Da in the synthesis of the interior cavity.
- Dendrimers were also synthesized for two different generations i.e. second generation and third generation.

All dendrimers consist of 1,2,3,4 butane tetracarboxylic acid and aspartic acid as the core molecule, PEO in the interior cavity, DHBA, gallic acid or BHBA at the branching points and PEG methacrylate in terminal branches (Figure 2.1). The three series of dendrimers mentioned above differ with respect to branching molecule in the dendron, with den-1-series, den-2-series and den-3-series consisting of DHBA, gallic acid and BHBA, respectively (Figure 3.1). These branching molecules were used to generate dendrimers with hydrophobic region in the structure (den-1-series and den-2-series), or dendrimers devoid of hydrophobic region (den-3-series). Because of the greater functionality of gallic acid, dendrimers of den-2-series also had greater number of terminal branches (24), as compared to dendrimers of den-1-series and den-3-series which had 16 terminal branches. Since, the presence of non-ionizable group would generate dendrimers with reduced toxicity; thus, allyl group was selected as the terminal functional group. Because, allyl group is non-ionizable and can be easily modified to hydroxyl, or carboxyl group (Nadeau et al., 2005) when required, for conjugating ligands and other bioactive molecules. PEO was incorporated in the interior cavity of the dendrimers to provide a hydrotropic interior as

well as to increase the size of the interior cavity for higher encapsulation of the guest molecules. In addition, chain length of PEO (200, 300 and 400 Da) in the interior cavity of dendrimers was varied to achieve variation in the size of dendritic voids. All these modifications in the chemical structure produced dendrimers with different architecture as shown in figure 3.1. The series of PEPE dendrimers were synthesized using the synthetic procedure shown in scheme 2.1-2.3, 3.1s and 3.2s. The characterization of synthesized dendrimers by ^1H NMR and FTIR proved the successful synthesis (Figures 2.2, 2.3b and 3.2). Molecular weight of dendrimers determined by MALDI-TOF and ^1H NMR indicated low polydispersity index ($M_n/M_w \leq 1.02$) and a good correlation with the expected values (Table 2.2, 3.1). Differences in the structures of the dendrimers of three different series i.e. den-1-(G2)-400, den-2-(G2)-400 and den-3-(G2)-400 were demonstrated by ^1H NMR spectra (Figure 3.2).

Differential scanning calorimetry (DSC) studies showed that dendrimers had very low T_g values, ranging between -12 and -21.37°C (Table 2.3), suggesting that they would be stable at room temperature. Additionally, presence of minor melting peak in the thermograms of all dendrimers indicated the existence of crystalline form (Gowariker et al., 1987). Thus, these dendrimers have zones of crystallinity in their structure along with amorphous nature.

The hydrodynamic size of dendrimers ranged between 3 and 8 nm. In den-1-series, size ranged from 3.58 to 5.41 nm, while in den-2-series from 4.19 to 7.41 nm and in den-3-series from 2.45 to 4.94 nm. Hydrodynamic size of dendrimers increased with the molecular weight of PEO incorporated in the interior cavity in a given series (Table 3.2). This substantiates augmentation in the size of dendritic voids with molecular weight of PEO incorporated in the interior cavity. Visualization of these dendrimers with AFM showed that they are oval or spherical in shape and also that their shape changes with change in the chemical structure (Figure 2.6). Interestingly, all dendrimers showed aggregation at concentration above 0.1 mg/mL. It is speculated that this aggregation occurs

due to the presence of large amount of PEG in dendrimers which result in chain entanglement during lyophilization. However, this problem could be easily overcome by lyophilization of dendrimers in the presence of lyoprotectant like lactose, sorbitol, glucose etc. (Hirsjärvi et al., 2006).

6.2. Biological characterization

Safety of carrier is of primary importance for application in drug delivery. It is desirable to establish the compatibility of drug delivery systems prior to demonstrating their efficacy and effectiveness to deliver a drug molecule. Considering the fact that components of delivery system can play vital role in determining the toxicity of the product (Fischer et al., 2003); these dendrimers were synthesized largely from PEG, amino acid aspartic acid, gallic acid and benzoic acid (which are used as preservatives and have antioxidant properties) and butane tetracarboxylic acid (which was found to be non-toxic to murine macrophages (RAW 264.7) cells in the concentration of 0.1-5 mg/mL). The toxicity of the dendrimers was evaluated against murine macrophages, bEnd.3 cells as well as U87 MG cells by lactate dehydrogenase (LDH) and MTT assay which are based on the assay of LDH enzyme released from the cells with damaged cell membrane and oxidation of MTT to formazan by mitochondria of the cells. Dendrimers produced no toxicity to RAW 264.7 cells in the tested concentration range of 0.1 to 250 $\mu\text{g/mL}$ (Figure 3.3) and also to bEnd.3 and U87 MG cells upto the concentration of 5 mg/mL (Figure 4.2). These results show that PEPE dendrimers lack toxicity and hence are biocompatible. It is speculated that, this is due to presence of PEG in the dendrimers which is known to reduce the toxicity (Bhadra et al., 2002; Jevapresphant et al., 2003). This lack of toxicity is very promising attribute of PEPE dendrimers, because most of the dendrimers studied in literature for drug delivery like PAMAM, DAB, DAE and polyaryl ether dendrimer are toxic and have IC_{50} values between 50-300 $\mu\text{g/mL}$ (Malik et al., 2000; Fischer et al., 2003). So far only bow-tie

dendrimers reported by Gilles et al., (Gillies et al., 2005) have shown non-toxicity at such high concentrations.

In addition to cellular toxicity, toxicity of dendrimers towards red blood cells (RBCs) was also evaluated. These dendrimers produced less than 10 % hemolysis and also produced no significant change in shape of rat RBCs after 2 h of incubation (Figure 4.3), suggesting that they are safe for i.v. administration unlike PAMAM dendrimers and dendrimers with malonate and carboxylate surface groups (Malik et al., 2000; Domanski et al., 2004). Both toxicity towards cells and RBCs was not influenced by the molecular weight of the dendrimer and chemical structure (Figure 4.3). Although these studies are preliminary and extensive toxicological studies in animal models are needed to ascertain safety of PEPE dendrimers for drug delivery, yet the results are quite promising.

It has been reported that after i.v. administration plasma proteins are adsorbed on the colloidal systems resulting in rapid uptake of by mononuclear phagocytic system (MPS) (Vonarbourg et al., 2006). Indeed, it is a major hurdle in delivering drug to the target site. Therefore, in order to gain preliminary information about the interaction between plasma proteins and dendrimers, they were incubated with serum for specified time. Amount of protein and type of protein adsorbed on them was determined. It was found that dendrimers adsorbed very low amount of protein (Table 4.2), additionally, opsonic proteins like immunoglobulins and complement factors were also not adsorbed on their surface (Figure 4.4). PEG is known to increase circulation half-life of liposomes, nanoparticles etc. (Klibanov et al., 1990; Gabizon et al., 1993). Based on this fact and also absence of opsonic proteins on the surface of dendrimers it is expected that they would have low clearance by MPS and hence long circulation half-life, which would allow sufficient time for delivery to the brain (Calvo et al., 2001). Since, no influence of molecular weight or architecture on toxicity and plasma protein adsorption on dendrimer surface was observed; thus, all PEPE dendrimers were evaluated further for drug loading and release.

6.3. Drug loading in PEPE dendrimers

Dendrimers exhibited high ability to encapsulate both model fluorescent compounds, rhodamine B and β -carotene (Table 2.4), suggesting that they offer microenvironment suitable for encapsulating both hydrophilic and hydrophobic guest molecules. Advantage of this characteristic is that PEPE dendrimers can be used to deliver both water soluble and water insoluble compounds; thus, eliminating the need to synthesize dendrimers with ability to encapsulate either hydrophilic or hydrophobic molecules.

After ascertaining that these dendrimers have good ability to encapsulate guest molecules, MTX was encapsulated in series of PEPE dendrimers to select the dendrimer with good encapsulation efficiency. MTX encapsulation in these dendrimers was found to vary with the chemical structure and architecture of the dendrimer (Table 3.2). Loading was low in dendrimers of den-3-series, which had no hydrophobic region in their structure. However, in dendrimer of den-2-series and den-1-series it was significantly high (17.2 to 24.5% w/w) (Table 3.2). Drug loading in these two series of dendrimers was higher than reported in literature for PEGylated PAMAM dendrimers (Kojima et al., 2000; Pan et al., 2005). This difference in encapsulation ability of dendrimer with and without hydrophobic region demonstrated that aromatic rings interact by hydrophobic interactions with MTX. Thus, incorporation of hydrophobic region in the interior of dendrimer is substantial for obtaining high drug loading in dendrimers. This principle is well known and has been extensively utilized in designing micelles but its application in dendrimer technology is rare; generally, dendrimers are synthesized either from only aromatic rings like polyaryl ether dendrimers or from small hydrophilic monomers like PAMAM, DAB, DAE or polyethylene dendrimers. Loading in dendrimers of den-2-series was higher than in dendrimers of den-1-series. The former dendrimer have higher number of terminal PEG branches, suggesting that increasing PEG grafting density also augments drug loading. Thus, it is other structural feature desirable for improving drug loading. We have

hypothesized that incorporation of PEO in the interior cavity of dendrimers would increase the size of the dendritic voids and would improve the encapsulation of guest molecules. This was validated by the fact that increasing the molecular weight of PEO from 200 to 400 Da improved MTX loading in dendrimers of all three series. There are number of reports in literature on the use of PEG grafts of various dendrimers to increase the encapsulation of drugs (Kojima et al., 2000; Yang et al., 2004; Pan et al., 2005). However, in this study molecular weight of PEO incorporated in the cavity of the dendrimer was varied. This is the first report in the literature where an attempt has been made to increase the size of dendritic voids and observe its influence on drug loading. In this series of dendrimer, increase in generation was not found to improve MTX loading (Table 3.2); in fact, loading in third generation dendrimer was lower than second generation dendrimer. This was anticipated to be due to hindered diffusion of MTX into the interior of dendrimer (Pan et al., 2005). The mechanism of encapsulation of MTX in PEPE dendrimers was studied by FTIR, ¹HNMR and UV spectroscopy (Figures 3.5-3.7). Variation in the mechanism of interaction of MTX with PEPE dendrimers was found to change with modifications in the chemical structure. However, weak hydrogen bonding, hydrophobic interactions and physical entrapment were established to be main mechanisms of encapsulation. DSC studies showed that MTX was present either in molecularly dispersed or amorphous form in these dendrimers (Figure 3.4). These studies also proved that MTX was encapsulated inside the dendrimers and was not surface bound.

6.4. Release of encapsulated drug

Release of model fluorescent compounds rhodamine B and β -carotene from dendrimer den-1-(G2)-400 was sustained for 170 h, with no burst release (Figure 2.10). However, release of MTX was biphasic with initial burst release during 6 h followed by a slower release over 168 h (Figure 3.8). This was due to high solubility of MTX in PBS at

pH 7.4 (Modi et al., 2006). In fact, burst release of MTX has also been reported from microspheres and nanoparticles (Liang et al., 2004; Reddy and Murthy, 2004; Modi et al., 2006). Interesting observation of this study was that, release of MTX was significantly altered by the architecture of the dendrimer. In dendrimers without aromatic ring (den-3-series) MTX was released within 50 h, while in dendrimers with aromatic it occurred over a period of 168 h (Figure 3.8). Additionally, burst released was decreased by 20% in dendrimers of den-2-series. Alterations in the molecular weight of PEO in the interior cavity of dendrimers did not have any significant effect on MTX release, indicating that altering size of interior voids has no influence on release of drug but enhances its encapsulation. Thus, increasing the size of dendritic voids could be harnessed an attribute for increasing the drug loading in dendrimers without influence on drug release; thereby providing an opportunity to deliver higher amount of drug in sustained manner. It is noteworthy to mention that release of MTX from den-1-series and den-2-series PEPE dendrimers was much slower than has been reported by Patri et al. (Patri et al., 2002) and Kojima et al., (Kojima et al., 2000) for its release from PEGylated PAMAM dendrimers. This demonstrates the potential of these PEPE dendrimers to serve as sustained delivery carriers. Release of MTX from higher generation dendrimer was not significantly different from second generation (Figure 3.8). Considering lower loading and similar release profile as second generation dendrimer, third generation dendrimer (den-1-(G3)-400) was not evaluated for delivery across BBB.

6.5. Understanding mechanism of uptake by brain endothelial cells

BBB is highly impermeable barrier, it prevents the entry of not only large (MW > 500 Da) but also hydrophilic molecules (Brightman, 1977). Brain endothelial cells lining the brain blood capillaries lack fenestrations thus, fluid phase endocytosis is relatively negligible. Nonetheless, receptor mediated, carrier and adsorptive endocytotic process occur in abundance at this barrier (Pardridge, 1998; Tsuji and Tamai, 1999; Smith and

Gumbleton). Consequently, molecules or delivery systems exploiting these pathways get an easy access to otherwise formidable region of the body. Considering the limited avenues by which delivery systems can enter brain, it is always attractive to conduct preliminary studies for understanding the mechanism by which they can enter endothelial cells. These studies provide insight into feasibility of developing a delivery system into a carrier suitable for brain delivery. It is noteworthy to mention that these endocytotic processes occur in endothelial cells, thus in this study we have evaluated mechanism of uptake of PEPE dendrimers into brain endothelial cells.

PEPE dendrimers of different architecture were evaluated in this study to observe the influence of alterations in chemical structure in the mechanism of uptake into bEnd.3 cells. By and large uptake of dendrimers into bEnd.3 cells was rapid and dendrimers were present in good amount within 1 h of incubation (Figure 4.5) and within 4 h they were found to present through out the cytoplasm (Figure 4.7). Only incubation at 4°C and acetic acid, inhibitors of energy dependent endocytosis (Silverstein et al., 1977) and coated vesicles formation (Sandvig et al., 1987; Davoust et al., 1988), could inhibit internalization of all dendrimers by more than 85% (Figure 4.6), suggesting that these dendrimers are largely taken up by energy dependent endocytosis into the endothelial cells. The influence of molecular architecture of dendrimers on the extent of contribution of each pathway in the uptake of particular dendrimer into endothelial cells was evident only after incubation with specific inhibitors like chlorpromazine, β -cyclodextrin etc. For instance, dendrimer den-3-(G2)-400 showed approximately 20 % internalization by fluid phase endocytosis while, den-1-(G2)-400 and den-2-(G2)-400, 8 and 13%, respectively (Figure 4.6). In addition, it was found that caveolin-mediated endocytosis had major contribution in the uptake of den-2-(G2)-400, on the other hand, den-3-(G2)-400 and den-1-(G2)-400 were mainly taken up by clathrin-mediated endocytosis. Nevertheless, neither chlorpromazine, β -cyclodextrin, cytochalasin B or K^+ depletion produced complete inhibition in internalization of any dendrimer, suggesting that all these dendrimers were taken up into endothelial cells by multiple endocytotic pathway but the extent of each pathway differed

with the structure of the dendrimer. This could be advantageous because, even during saturation of one mechanism other alternative pathway would operate and hence uptake of dendrimers into endothelial cells would occur. The extent of uptake of dendrimers into bEnd.3 cells was very high, which showed that they are efficiently taken up by these cells. To further look if these dendrimers are just retained by endothelial cells or are able to permeate and cross BBB, their permeability across BBB model was studied. Since, den-3-(G2)-400 had shown low loading, fast release of MTX and greater uptake by fluid phase endocytosis, which occurs to limited extent at BBB it was not evaluated further for permeability across BBB model. To the best of our knowledge, this is the first study in which the mechanism of uptake of delivery system into brain endothelial cells has been studied.

6.6. Permeability of PEPE dendrimers across BBB model

Permeability of delivery system across BBB is a major determinant of the amount of drug reaching the brain tumors. Recently, various *in vitro* BBB models have emerged as a valuable method to investigate permeability of drug and delivery systems across BBB in early development stages (Lundquist and Renftel, 2002). These models allow rapid screening of large number of drugs or delivery systems which is otherwise a time consuming process. They also offer advantage of understanding the mechanism of uptake and transfer of drug molecules and their carriers across BBB (Smith and Gumbleton). These models retain many features of BBB like presence of tight junction elements like ZO-1, occludin, claudin-1 and various transporters like GLUT-1, OAT1, amino acid carriers, P-gp (Omidi et al., 2003; Song and Pachter, 2003). A strong correlation between permeability of various drug across BBB model consisting of co-culture of brain endothelial cells and astrocytes and human brain concentration has been reported (Dehouck et al., 1992; Lundquist et al., 2002). Thus, *in vitro* BBB model was employed in this study to understand permeability of these dendrimers into the brain. BBB model based on bEnd.3

cells was used in this study, because it has been extensively characterized for expression of various proteins and transporters (Omidi et al., 2003; Song and Pachter, 2003). However, we have also evaluated for the tightness of junctions by measuring TEER and permeability of transcellular and paracellular markers across the model. The expression of P-gp is reported to be one of the major efflux transporter at BBB was also studied. Rhodamine-123, a fluorescent substrate of P-gp was used for examining the expression of P-gp (Batrakova et al., 1998). P-gp expression was evaluated both in monolayer bEnd.3 cells as well as in the co-culture of bEnd.3 and U373 MG cells grown on polycarbonate Transwell[®] inserts. Rhodamine-123 was found to be effluxed out of the bEnd.3 cells within 2 h and co-incubation with verapamil (P-gp inhibitor) increased its cellular concentration (Figure 4.8a). In addition, verapamil also significantly increased the permeability of rhodamine-123 across the BBB (Figure 4.8b), substantiating the expression of P-gp in the BBB model. P_{app} of theophylline and atenolol across co-culture of bEnd.3 and U373 MG cells was found to be 8.8 ± 2.0 and $0.23 \pm 0.05 \times 10^{-6}$ cm/sec (Table 4.3). The significantly low permeability of atenolol across this model suggested low paracellular transport signifying development of appropriate tight junction properties. Further, TEER of this co-culture BBB model ($150\text{--}180 \Omega\text{cm}^2$) was found to be maximum after 9-10 days of culture (Table 4.3). Though this TEER is much lower than would under in vivo conditions but it is similar and in some cases higher than that has been reported in literature for models used for studying permeability across BBB (Lundquist et al., 2002; Song and Pachter, 2003). Further, considering low permeability of paracellular marker atenolol, which is also indicator of tight junction properties this model was used for evaluating permeability of dendrimers across BBB.

Dendrimers were labeled with rhodamine to follow their transport into the receptor compartment and amount of dendrimer available was analyzed by fluorescence plate reader. It was found that dendrimers were transported rapidly across BBB model with approximately 5 μg of den-1-(G2)-400 available in first 1 h; while, 22 μg of den-2-(G2)-400 was transported in 1 h. P_{app} of den-1-(G2)-400 was also high, $19.7 \pm 1.9 \times 10^{-6}$ cm/sec.

It is evident that permeability of den-2-(G2)-400 was higher than that of den-1-(G2)-400 (Figure 4.9 and 5.3), perhaps due to the presence of higher number of PEG chains at the surface. Conjugation of PEG has been reported to increase permeability of nanoparticles and liposomes into the brain (Calvo et al., 2001). Interestingly, TEER was reduced only by 14% by den-1-(G2)-400 (Figure 4.10) and 5% by den-1-(G2)-400 (data not shown). This clearly suggest that these dendrimers do not compromise tight junctions of BBB during their permeation and also that greater the number of PEG chains in the structure of dendrimer, higher is the permeability and lower is the effect on tight junction properties of BBB. As seen in Figure 4.9, basal to apical transfer of these dendrimers was very low, suggesting that they are not effluxed out of BBB and also that there is preferential apical to basal transfer of these dendrimers. In order to establish that it is dendrimers that are crossing BBB and not rhodamine, ¹HNMR spectrum of 24 h permeation sample was acquired (Figure 4.2s, Supporting information). It proved that rhodamine labeled dendrimers were able to cross BBB intact and also that it is not merely rhodamine that is crossing the BBB. These results demonstrate that PEPE dendrimers *per se* have good permeability across BBB.

Colloidal delivery systems like liposomes and nanoparticles are reported to act as molecular Trojan horses for delivery of chemotherapeutic agents into the brain (Olivier et al., 2002; Pardridge, 2006). They protect the drug from external biological environment and ferry it across BBB without being recognized by the efflux transporters. PEPE dendrimers had shown commendable permeability across BBB model (Figure 5.3). Subsequently, we have ascertained their ability to improve the availability MTX across BBB; for this purpose, MTX loaded dendrimers were placed in the donor compartment of the Transwell[®] inserts and the amount of MTX available in the receptor compartment was determined as a function of time. Amount of MTX permeating across BBB was increased approximately 4 times by den-1-(G2)-400 and 6 times by den-2-(G2)-400 as compared to free MTX (Figure 5.4). The increased amount of MTX delivered by den-2-(G2)-400 correlates well with the

higher permeability of this dendrimer across BBB as compared to den-1-(G2)-400. These results showed that PEPE dendrimers can serve to enhance delivery of MTX into the brain.

6.7. Efficacy against glioma cell lines and avascular tumor spheroids: Enhanced delivery by D-glucosamine ligand

Conjugation of the drug molecules to the terminal groups of dendrimers has been utilized as a preferred method for their delivery as compared to physical encapsulation, because, it is reported that drugs loaded non-covalently into dendrimers are released rapidly (Kojima et al., 2000; Liu et al., 2000; Kolhe et al., 2003; Patri et al., 2005), rendering them ineffective for targeted delivery. However, largely dendrimer-drug conjugates have failed to provide better therapeutic benefit than free drug. For instance, MTX conjugated to PAMAM dendrimers and EGFR antibody did not show any improvement (Wu et al., 2006) and in another study PAMAM, folic acid and MTX conjugate had lower inhibitory potential than free MTX (Patri et al., 2005). However, we have shown that by manipulating the structure of the dendrimer drug release can be manipulated. Further, considering limitations of drug conjugation, MTX was encapsulated in these PEPE dendrimers and its efficacy was evaluated against U87 MG and U343 MG-A cells. MTX loaded PEPE dendrimers were found to be more efficacious than free MTX (Table 5.2). The higher activity of the dendrimers compared to free MTX could be due to cellular uptake of dendrimers and intracellular release of the drug. In figure 5.1 and 5.2 it is evident that dendrimers were endocytosed effectively by the cells. Further, dendrimers were able to notably overcome the MTX-resistance and reduce the IC_{50} by approximately 9-15 times (Table 5.2). Ability of PEPE dendrimers to improve the potency of MTX towards resistant glioma cell lines suggests that they contain the drug before entering the cells i.e. drug is released from the dendrimers after entering the cells. And that dendrimers enter the cells by mechanism different from that of the uptake of MTX. MTX is reported to be taken up into

the mammalian cells by the reduced folate receptors (Matherly and Goldman, 2003; Zhao and Goldman, 2003).

D-glucosamine was used a ligand for targeting highly expressed glucose transporters on BBB (GLUT-1) and also on the tumor cells (Pardridge et al., 1990; McAllister et al., 2001; Luciani et al., 2004). D-glucosamine was used because, the amino group present at 2 position of the glucose ring can be easily linked to carboxylic group of dendrimers obtained after oxidation; in addition, it avoids loss in recognition of this sugar by glucose transporters on random conjugation to any hydroxyl group. On an average 3 glucosamine units were conjugated to den-1-(G2)-400 and 5 to den-2-(G2)-400. Conjugation to glucosamine served to significantly increase the internalization of dendrimers by glioma cells, U87 MG and U343 MG-A. In U343 MG-A cells it was increased by 2 folds and in U87 MG cells by 8 folds (Figure 5.1), suggesting ability of glucosylated dendrimers to be recognized by the glucose transporters on these glioma cells. Glucosylation of the dendrimers further improved the potency of these MTX loaded dendrimers against glioma cells by approximately 3 to 4 folds (Table 5.2). The higher potency of glucosylated dendrimers can be correlated to their higher cellular uptake as compared to non-glucosylated dendrimers by the glioma cells (Figure 5.1). The effect of glucosylation was also observed on permeability of dendrimers alone across BBB. Glucosylated den-1-(G2)-400 could permeate 3.5 times higher than non-glucosylated dendrimers, but in the case of den-2-(G2)-400, glucosylation increased the permeability only by 1.2 folds. Similar trend was observed for MTX loaded glucosylated dendrimers which could increase the availability of MTX across BBB by approximately 3 and 1.5 times for den-1-(G2)-400 and den-2-(G2)-400 (Figure 5.4). It hypothesized that lower permeability of den-2-(G2)-400 after glucosylation is due to higher number of glucosamine units (five per dendrimer) in this dendrimer which increase its size (Table 5.1) and reduce its transport by GLUT. Based on this observation it was concluded that 3 glucosamine ligand per dendrimer are suitable for enhanced delivery across BBB. Most interesting observation was that, glucosylation increased the rate and extent of diffusion of dendrimers

into avascular tumor spheroids. Glucosylated den-1-(G2)-400 could diffuse through out the tumor spheroid within 6 h, while, non-glucosylated dendrimer had taken 12 h for the same (Figure 5.5). The tumor microenvironment is characterized by high cell density, elevated interstitial pressure, poor vascularization, hypoxia and acidic pH (Brown and Giaccia, 1998). These multicellular factors impede the distribution, penetration and cellular accumulation of chemotherapeutic agents (Kostarelos et al., 2004). Drug delivery to the central regions of tumors and the regions far away from vascular bed has always remained a difficult task. The avascular regions of the solid tumors in particular represent a major obstacle in achieving the effective control of the tumor growth (Yuan et al., 1994; Minchinton and Tannock, 2006). It has been reported that delivery systems stagnate at the periphery of the tumor vasculature and have poor permeation into the hypoxic and necrotic regions of the tumor that are distant from the vascular bed. This results in very low amount of drug reaching inside the solid tumors, resulting in low therapeutic benefit and often regrowth of the tumor. Enhancement in the penetration of delivery systems into the central necrotic regions is believed to significantly improve the efficiency of solid tumor treatment. Tumor spheroids have been continuously utilized as the models for the study of early tumor development (Sarfaraz and Wessels, 1999; Gilead et al., 2004), avascular micrometastatic tumors (Walker et al., 1988; Waleh et al., 1995) and models for tumor interstitial space (Paulus et al., 1994). Tumor spheroids mimic growing tumors (Sutherland and Durand, 1984; Sutherland, 1988) more closely compared to the mono layer cell culture, they help in understanding tumor development and also acting as a bridge between *in vitro* and *in vivo* models (Helmlinger et al., 1997). Thus, in this project they were used to evaluate the intratumoral transport capabilities of these dendrimers. As seen in figure 5.5, glucosylation of PEPE dendrimers enhanced the rate and extent of diffusion of the dendrimers in the tumor spheroids. This improved diffusion of glucosylated dendrimers to the central regions of tumor spheroids is probably because, central regions of the solid tumors are hypoxic and they demonstrate a hypoxia related increase in glucose transport (Haberkorn et al., 1994; Clavo et al., 1995). Thus, greater demand of glucose in the hypoxic regions of the tumors

drives the faster and enhanced distribution of glucosylated dendrimers in the tumors spheroids. Hence, these glucosylated PEPE dendrimers would be able reach the avascular regions of the tumors within 150-200 μm and deliver their payload and thus can provide more effective control of the tumor growth.

To further investigate if MTX loaded PEPE dendrimers would be able to provide therapeutic benefit as compared to free MTX, reduction in the volume of U87 MG tumor spheroids as well as accumulation of ethidium bromide (stains DNA of dead cells) after treatment with PEPE dendrimers was examined. In the absence of any drug tumor spheroids continued to increase in size (Figure 5.6 and 5.7), MTX was able to only marginally reduce their size for 2-3 days (Figure 5.7). However, MTX loaded dendrimers produced 30-80% reduction in the size of both U343 MG-A and U87 MG tumor spheroids. Glucosylated den-1-(G2)-400 was more potent in reducing the size of both U343 MG-A and U87 MG tumors as compared to non-glucosylated dendrimer (Figure 5.7) at both the evaluated concentrations. However, glucosylated den-2-(G2)-400 was more effective or as effective as non-glucosylated den-2-(G2)-400. It is hypothesized that this is due to the greater number of glucosamine ligands per dendrimer (five) in den-2-(G2)-400-Glu as compared to den-1-(G2)-400-Glu (three) which result in increase in size (Table 5.1) and hence reduced uptake by glucose transporters. Thus, three glucosamine units per dendrimer are ideal for enhanced delivery of these dendrimers to tumor cells as well as permeability across BBB. Treatment of U87 MG tumor spheroids with PEPE dendrimers also increased the ethidium bromide concentration per mg of protein from 3.77 $\mu\text{g}/\text{mg}$ of protein (for control) and 5.45 $\mu\text{g}/\text{mg}$ of protein (for MTX treated) to 13.54 and 18.49 $\mu\text{g}/\text{mg}$ of protein for den-2-(G2)-400 and den-1-(G2)-400-Glu, respectively (Figure 5.8). All these results indicate that loading of MTX in PEPE dendrimers enhances death of glial tumor cells. Enhanced tumor cell kill is believed to happen because PEPE dendrimers enter the tumor spheroids in high amount and release MTX slowly for longer duration of time. Since, the tumor spheroids mimic the microenvironment of solid tumors, the higher efficacy of MTX

loaded dendrimers suggests that they would provide significantly higher therapeutic benefit than MTX.

CONCLUSION

In this work, novel polyether-co-polyester (PEPE) dendrimers based on PEG were synthesized and proposed for the enhanced delivery of chemotherapeutic agents across BBB. We have succeeded in achieving high loading capacity in these dendrimers by increasing the size of the interior cavity and also by incorporating hydrophobic regions in the structure of dendrimers. The latter feature was also found to be valuable in retarding the release of MTX from these dendrimers. MTX loaded dendrimers have shown to significantly increase the efficacy of MTX in killing human glioma cells. They were also found to be effective in overcoming MTX resistance. By virtue of the small size, lack of toxicity, plasma protein binding and high permeability across BBB, PEPE dendrimers offer a unique opportunity for delivery of therapeutics to brain. High loading capacity and slow release of loaded guest molecule further potentiates their application by enhancing the drug payload that could be delivered to the brain. Glucosamine was found to be a valuable ligand not only for enhancing permeability across BBB, targeting to glioma cells but also for delivery to the hypoxic regions of the solid tumors. Collectively, the results of this research aid in understanding the relationship between the architecture of PEPE dendrimers in encapsulating drug, its release and permeability across BBB with prospect of designing novel carriers for delivery to the brain. It is believed that the present project has offered a new and very promising option for delivery of anticancer drugs to brain, warranting their further evaluation in animal models.

6.8. References

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