Antibiotic delivery across the blood-brain barrier by nanoparticle system

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ANTIBIOTIC DELIVERY ACROSS THE BLOOD-BRAIN BARRIER BY NANOPARTICLE SYSTEM

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SUMMARY

Brain infection, especially bacterial and fungal meningitis, remains to be life-threatening disease despite the existence of numerous clinically available antibiotics. Important challenges include the antimicrobial agent transportation restricted by the blood-brain barrier (BBB) and multi-drug resistance presented by pathogenic microorganisms. The aim of this study is to design multifunctional micelles for brain delivery of antimicrobial agents.

Biologically active polymer core/shell nanoparticles (i.e. micelles) self-assembled from TAT-poly(ethylene glycol) (PEG)-b-cholesterol (TAT-PEG-b-Chol) were fabricated and used as a carrier for delivery of antibiotics across the BBB. Ciprofloxacin as a model antibiotic was efficiently loaded into the nanoparticles by a membrane dialysis method. The actual loading level of ciprofloxacin was dependent on initial loading weight of ciprofloxacin and dialysis temperature. The blank and ciprofloxacin-loaded nanoparticles were characterized using dynamic light scattering and SEM. The nanoparticles were spherical in nature, having an average size lower than 200 nm. The uptake of nanoparticles with TAT by human brain endothelial cells and astrocytes was greater than that of the nanoparticles without TAT. Most importantly, the nanoparticles with TAT were able to cross the blood-brain barrier (BBB), and located around the cell nucleus of neurons. These nanoparticles may provide a promising carrier to deliver antibiotics across the BBB for the treatment of brain infection. However, the release process of the antibiotic molecules was found to have a rapid burst in the initial stage, in which 70% of drug was released within two hours. The best strategy to address this burst limit is to avoid the necessity of drug encapsulation by employing a nanoparticle system which can be antibiotics by themselves.

Therefore, an amphiphilic peptide, cholesterol-G3R6TAT, was synthesized and self-assembled into cationic micelles. These micelles are not only able to dock antibiotics to brain but also serves as a novel microbicidal against a broad spectrum of organisms ranging from Gram-positive bacteria to *Candida albicans* (yeast) and *Stachybotrys chartarum* (fungus). The antimicrobial micelles were spherical in nature and having an average diameter of about 300 nm with zeta potential of 92 mV. They displayed much stronger bactericidal and fungicidal ability than cationic peptides without micelle formation. The micelles simultaneously inhibited more than one target, which may make them capable of overcoming multi-drug resistance since mutations are required in all targets to confer resistance to the micelles. The cationic self-assembled peptide nanoparticles may provide a promising antimicrobial agent against brain infection.

Keywords: polymeric micelles; TAT peptide; blood-brain barrier; drug delivery; antibiotics; peptide microbicidal

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LIST OF ABBREVIATIONS

^1H -NMR: Proton Nucleic Magnetic resonance
AIDS: Acquired Immune Deficiency Syndrome
BBB: Blood-brain barrier
 CDCl_3 : Deuterium substituted chloroform
Chol: Cholesterol
CMC: Critical micelle concentration
D₂O: Deuterium substituted hydrogen dioxide
DCM: Dichloromethane
DI: De-ionized
DMEM: Dulbecco's modified Eagle's medium
DMF: N,N-Dimethylformamide
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DSC: Differential scanning calorimeter
EDC: 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
FBS: Fetal bovine serum
FDA: Food and Drug Administration
FITC: Fluorescein 5-isothiocyanate
FTIR Fourier transform infrared
GPC: Gel permeation chromatography
HPLC: High performance liquid chromatograph
MALDI-TOF: Matrix assisted laser desorption/ionization-Time of Flight
MES: 2-[morpholino]ethanesulfonic acid
M_w: Molecular weight
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NHSS: N-Hydroxysulfosuccinimide
PBS: Phosphate-buffered saline
PDLLA: Poly(DL-lactic acid)
PEG: Poly(ethylene glycol)
PEO: Poly(ethylene oxide)
PGP: P-glycoprotein
PLGA: Poly(lactide-co-glycolide)
PS: polystyrene
QDs: Quantum dots
RES: Reticuloendothelial system
SEM: Scanning electron microscopy
TAT: Transactivating-transduction
TEA: Triethylamine
TED: Transmission electron detector
TEM: Transmission electron microscopy
 T_f : Transferrin
 T_g : Glass transition temperature
TGA: Thermogravimetric analyzer
THF: Tetrahydrofuran
UV-VIS: Ultra-violet visible

CHAPTER ONE

INTRODUCTION

1 . Background and motivation

Encephalitis is an inflammation of the brain, usually caused by a direct viral infection or a hypersensitivity reaction to a virus or foreign protein. Brain inflammation caused by a bacterial infection is sometimes called cerebritis. When both the brain and spinal cord are involved, the disorder is called encephalomyelitis. An inflammation of the brain's covering, or meninges, is called meningitis. Acute neuroninflammatory diseases are lethal, and despite the availability of antibiotics, bacterial meningitis remains among the top ten infectious causes of death (Obermaier & Klein et al., 2006).

Choice of treatment for brain infection will depend on the cause. Viral encephalitis and meningitis are the least severe type of the disease. The symptoms are mild and patients usually recover with no long-term effects after treatment with antiviral drugs including acyclovir, ganciclovir, foscarnet, ribovarin, and zidovudine (AZT). Bacterial infection is far more serious and progress quickly. Antibiotics have been administrated for several decades and are capable of sterilizing the cerebral spinal fluid (CSF) relatively rapidly and reliably. Regardless, patients not only suffer high mortality rates ranging from 5 – 55 % depends on various etiologic microorganisms (Krcméry & Paradisi et al., 2000), about one-third of survivors retain speech loss, cognitive disabilities, seizures, memory loss and other permanent head damage (<http://www.ninds.nih.gov>). Important challenge remains to be solved, if not all, is the better strategies in transporting antimicrobial therapy beyond the blood brain barrier (BBB). The BBB resides at the level of the capillaries. At this level, four cell types converge: the endothelial cell, the pericyte, the endfeet of perivascular astrocytes; and the fine processes of the neuron. The distinct features of BBB, including tight junctions, a number of specific proteins and pharmaceutical application related transport systems, restrict antibiotics from the blood to brain (Aigner & Wolf et al., 1997). For example, all

betalactam antibiotics penetrate poorly into cerebral spinal fluid (CSF) (~0.5-2.0%) when the BBB is normal. One focus of this project is on the development of novel amphiphilic molecules, which can self-assemble into nanoparticles, facilitating penetration of antimicrobial agents across the BBB for brain infection treatment.

The high mortality and morbidity of brain infection in part is a result of growing prevalence of microbicidal-resistant strains of bacteria and fungi. Treatment of brain infection relies mainly on administration of third-generation cephalosporin and anaerobic cover drug. However, in response to the threat of extinction, pathogens have developed resistance to anti-microbial agent through mutations of target sites or the acquisition of novel biochemical functions. Moreover, resistance now includes potent antibacterial agents, which are used as a last resort, such as methicillin and vancomycin.

1.2 The problem: inadequacy of conventional drug delivery to brain

A few strategies have been carried out to avoid or disrupt the BBB for drug delivery. For example, trans-cranial approach using neurosurgical based intrathecal catheters direct injection, or via controlled release implantation has been tested. However, the slow rate of drug diffusion into brain parenchyma limited the application especially within the context of the relatively short windows of time for effective therapy in acute brain infection. Such invasive approach also cause penetration of non-targeted brain tissue and carry the risk of brain damage and bleeding.

Another often-contemplated approach to bypassing the BBB is through olfactory route. Trans-nasal delivery is based on that drugs can exit the submucous space of the nose and cross the arachnoid membrane, and enter into olfactory.CSF. From numerous animal studies,

the amount of drug transported to the central nervous system via this route is only 1% or less (Charlton & Davis et al., 2007). This is associated with similar tight junction existing in arachnoid membrane.

The disruption of BBB with intra-arterial injection of mannitol comes from the endothelial cells shrink and opening of the tight junctions. Likewise, solvents such as high-dose ethanol or dimethylsulfoxide (DMSO), alkylating agents like eopposide and melphalan, immune adjuvants and cytokines have all been used to disrupt the BBB (Pardridge, 2005). However, previous studies demonstrate increased BBB permeability may allow plasma protein, such as albumin which is toxic to brain cells into the brain.

1.3 The solution: novel nanoparticles for antibiotic delivery or as antimicrobial agent

In parallel with above-mentioned approaches, transvascular drug delivery to the brain *via* the use of carrier systems such as polymeric nanoparticles (Kataoka & Harada et al., 2001) has been suggested as a promising strategy. Polymeric micelles made from amphiphilic polymers possess a core-shell structure that can be modified superficially so that they can interact biologically without breaching the BBB. Polymeric micelles are small in size (<200 nm) and can solubilize hydrophobic drugs through hydrophobic interaction and/or hydrogen bonding, while exposing their hydrophilic shells to the external environment. This effectively protects the enclosed bioactive compounds against degradation and enables them to exhibit prolonged activity in the systemic circulation by avoiding the scavenging of the reticuloendothelial systems (RES). It was reported that core-shell poly(ethylene glycol) (PEG)-polycyanoacrylate nanoparticles penetrated into the brain to a larger extent than polysorbate 80 or poloxamine 908-coated polycyanoacrylate nanoparticles and uncoated

polycyanoacrylate nanoparticles because of the long-circulating characteristics of core-shell PEG-polycyanoacrylate nanoparticles (Calvo & Gouritin et al., 2001). Cationic albumin (Lu & Tan et al., 2005), OX26 (Aktas & Yemisci et al., 2005) and peptides such as RMP-7 (Zhang & Xie et al., 2003) and MMP-2200 derivatives (Costantino & Gandolfi et al., 2005) have been conjugated onto nanoparticles for drug delivery across the BBB. TAT (49-57, YGRKKRRQRRR) peptide is the protein transduction domain from the transcriptional activator Tat protein of the human immunodeficiency virus type-1 (HIV-1). It was conjugated to proteins with molecular weight ranging from 36 to 119 kDa (Schwarze & Ho et al., 1999), and the conjugates have been shown to cross the BBB. In addition, TAT can also transport quantum dots across the BBB to the brain parenchyma (Santra & Yang et al., 2005). The TAT-conjugated micelles may provide a promising carrier for the delivery of antibiotics across the BBB for brain infection.

Cationic antimicrobial peptides (Amps) represent good platform for novel antibiotic development. They are typically short (10 to 100 amino acids), with positive charge between +2 to +9, having a substantial hydrophobic residues (Jenssen & Hamill et al., 2006) and may have α -helical, β -sheet, loop, or extended types of structures (Hancock & Sahl, 2006). It is widely accepted that Amps act by electrostatic binding and subsequent compromising the structural and/or functional integrity of microbial membranes, but some may have additional or alternative modes of action. Therefore, the relatively nonspecific and involvements of a package of mechanisms make microbial resistance development unlikely. Common to the overwhelming majority of antimicrobial peptides, 11 amino acids TAT peptide (47-57) with electric charge $z_p = +8$ (Ziegler & Li et al., 2003), which can easily penetrate cell phospholipid membrane, may be served as antimicrobial agent. Fungicidal activity of TAT (47-58) peptide has been reported by Jung & Park et al. (2006).

Most peptides were designed using natural antimicrobial peptides as the template *via* the trial and error principle. Our approach is to form cationic micelles *via* self-assembly of amphiphilic peptides incorporated with TAT. The formation of TAT-anchored cationic micelles may increase antimicrobial activities. In addition, TAT molecules on the surfaces may be able to transport the micelles across the BBB.

1.4 Objectives

- To synthesize amphiphilic poly(ethylene glycol)-6-cholesterol(PEG-*b*-Chol)
 - To conjugate TAT peptide with PEG-*b*-Chol
 - To fabricate core-shell nanoparticles using TAT-PEG-6-Chol by self-assembly
 - To screen and encapsulate the selected potent antibiotic into the nanoparticles
 - To encapsulate FITC and quantum dots into TAT-PEG-*b*-Chol nanoparticles
 - To study the *in vitro* uptake of the nanoparticles by primary human brain microvascular endothelial cells and astrocytes
- To study biodistribution profile of nanoparticles in a rat model
- To design and synthesize novel peptidic micelles as powerful antimicrobe-agent capable of crossing the BBB
- To test pathogen susceptibility of the micelles
 - To investigate the penetration possibility of the peptidic micelles across the brain tissue in a rat model
 - To study the possible mechanism of action of the peptidic micelles

1.5 Anticipated technical challenges

Despite major advances in the design of TAT peptide derived Chol-b-PEG polymeric vector and CG3R6TAT peptidic antibiotic, several potential technical challenges need to be considered in advance.

First of all, the stability of the nanoparticles self-assembled from TAT-PEG-b-Chol and CG₃R₆TAT must be evaluated under *in vitro* and *in vivo* conditions. Critical micelle concentration (CMC) is a critical parameter to evaluate the stability of micelles in aqueous media and an indicator of whether micelles can be well maintained after administration. The relatively long hydrophilic PEG and TAT positive charge might render a high value of CMC. Amphiphilic molecules with high CMC values are restricted from their application in drug delivery, because the sink effect of our body fluid will dissociate the nanoparticles quickly even before they are degraded by proteolysis.

In addition, biocompatibility and cytotoxicity study should be carried out. Although the basic domain TAT₄₉₋₅₇ has been used in inhibiting neural death (Kilic & Dietz et al., 2002) and delivering large proteins, such as RNase, domain III of pseudomonas exotoxin A, horseradish peroxidase and β -galactosidase (Fawell & Seery et al., 1994) into intracellular space in addition to achieving biological effects (Nagahara & Vocero-Akbani et al., 1998), a very high dose of TAT₄₆₋₆₀ peptide was reported to be toxic (Sabatier & Vives et al., 1991). The cytotoxicity of PEG-b-Chol and TAT-PEG-b-Chol will be assessed against human brain microvascular endothelial cells and astrocytes. Cell viability will be measured by following a standard MTT assay procedure. The effect of TAT conjugation degree should be investigated and optimized.

An even more difficult task for this project is the specific brain targeting. It is known that at the physiological pH (i.e. pH 7.4), the luminal surface of the brain endothelium presents an overall negative charge (due in part to significant sialylation), and thus provides an environment more selective to positively charged substance (Hardebo & Kahrstrom, 1985; Vordbrodt, 1988). TAT (YGRKKRRQRRR) contains a highly cationic cluster of six arginines and two lysines, and an N-terminal alpha helical structure was identified as the protein transducing domain (Krama & Wunderli-Allenspach, 2003). For the static electric mediated transcytosis of peptide through the BBB, three steps have been proposed: 1) binding and internalization at the luminal side of endothelial cell membrane; 2) diffusion through the cytoplasm of endothelial cells; and 3) externalization at the basolateral side of endothelial cells. However, a variety of different cell surface has negative heparan sulfate proteoglycans (HSPG) (Tyagi & Rusnati et al., 2001), which cause cell internalization may not be specific to brain endothelia cells. Fluorescein 5-isothiocyanate (FITC), CdTe quantum dots (QDs) or nanogolds will be encapsulated into the nanoparticles as a label. Inverted fluorescence microscopy, confocal laser scanning microscopy as well as transmission electron microscopy may be utilized to visualize the localization of TAT-PEG-b-Chol nanoparticles in the rat's brain. The hypothalamus, occipital cortex, and hippocampus are considered as regions of the brain most permeable to TAT (Banks & Robinson et al., 2005), therefore, examination of tissue sections taken from these areas will be conducted to determine specific cell types which response more readily to TAT peptide. The study of the interactions of the nanoparticles with plasmatic components and brain blood vessel endothelial cells by TEM will be another important area of this research. The biodistribution of nanocontainers with respect to the brain, liver, kidney, heart and lung using the same rat model will be studied, and the mechanism by which these TAT-conjugated nanoparticles cross the BBB will be analyzed.

The major obstacles to the use of peptide-based anti-infectives as useful drugs are their toxicities, particularly if they are to be administered systemically and their short half-lives in the circulation. Several attempts have been made in this study to improve antimicrobial activity against bacterial cells while eliminating the cytotoxicity against host cells such as red blood cells by flexible spacer region, arginines chain length optimization, changing the net charge, and amphiphilicity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Infections in the brain and spinal cord continue to be important life-threatening diseases throughout the world. The prognosis mainly depends on rapid identification of the causative pathogens as well as to achieve microbicidal concentrations for specific agents in the cerebrospinal fluid (CSF).

The first part of this review provides an overview of classification of central nervous system (CNS) infection and a wide variety of associated microorganisms with an emphasis on bacterial meningitis, the most common and serious brain infection. It is important to note the structural differences between the gram-positive and gram-negative bacteria because these differences account for disease pathogenesis and it has also been possible to exploit these differences in developing a chemotherapy regimen.

The failure of brain infection treatment in many cases is attributed not to the potency of the drugs, but to the barriers that impair the efficient delivery of the drug to the brain. The following sections review the biology of blood-brain barrier (BBB), and introduce the invasive and non-invasive techniques, which are currently used to circumvent the BBB and deliver drugs into the brain. The use of nanocarriers, such as liposomes and polymeric micelles is especially discussed in details, providing evidence for their application as non-invasive antibiotic delivery vehicles across the BBB.

CNS infection treatment is frequently complicated by resistance of many microorganisms to the third-generation cephalosporins. This may necessitate the use of new antibiotics which are highly active and have novel and diversified mechanisms of action. One member of

antimicrobial **peptides** family, the **cationic peptide**, has been extensively studied and is the major focus of the third part of this review.

2.2 Central nervous system infection and common live pathogens

The CNS is especially sensitive to organism infection because it lacks normal host defense mechanisms, including antibody and complement activity. For example, the subarachnoid space has poor immunological defense, allowing rapid and relatively unhindered growth of bacteria in the CSF.

CNS infections may be classified according to the affected anatomical components:

- Meninges (meningitis)
- Brain parenchyma (encephalitis, abscess, granuloma, cyst)
- Extradural (abscess)
- Subdural (empyema)
- Spinal cord (myelitis)

Figure 2.1: Schematic representation of meninges and parenchyma structures of the central nervous system (Young & Fritz et al., 2000).

Transverse myelitis (MT) is a neurological disorder caused by an inflammatory process of the grey and white matter of the spinal cord. This demyelination occurs idiopathically following infection, or due to tuberculosis, cryptococcus and syphilis. One major theory of the cause is that an immune-mediated inflammation is present as the result of exposure to a viral antigen (Scotti & Gerevini, 2001).

Since the general focus of my study is on drug delivery across the blood-brain barrier for brain infection treatment, this review will concentrate specifically on the first four diseases: meningitis (detailed structure of meninge is shown in Figure 2.1), encephalitis and two kinds of brain abscesses.

Extradural and subdural empyema

Intracranial abscesses of extradural pus and subdural empyema are usually associated with bacterial inoculation from penetrating wound (after trauma or surgery to the skull), spread from adjacent infective focus (e.g. otitis media, frontal sinusitis (Goldberg & Oroszlam et al., 2001), or blood-borne spread from distant focus (e.g. endocarditic, lung abscess). Antibiotics and improved treatment of infection of the ear and sinus has decreased the incidence of intracranial abscess and empyema (Lindsay, 1997).

Meningitis

Meningitis is **an** acute infectious disease caused by several microorganisms, including viruses, bacteria, parasites, and fungi. The overall annual incidence of bacterial meningitis is about 2-3/100,000, with peaks of incidence in infants and adolescents. Fatality rates associated with this disease can be as low as 2%, and as high as 40% (Rosenstein & Perkins

et al., 2001), depends on patient age, infecting organisms involved, the severity of the illness, and how quickly treatment is given.

Viral meningitis is typically caused by enteroviruses that enter the body through the mouth and travel to the brain where they multiply. Other viruses that cause meningitis include *varicella zoster* (the virus that causes chicken pox and can appear decades later as shingles), influenza, mumps, HIV, and herpes simplex type 2. Viral meningitis can not be treated with antibiotics, but often resolves on its own in 14 days or fewer with no specific treatment except for fluids and pain relievers (BUPA's Health Information Team, 2004). Patients usually see some relief in 24-48 hours and can make a full recovery after receiving antiviral drug acyclovir (i.v.) treatment.

Bacterial meningitis (BM) is defined as an acute inflammation of leptomeninges (pia-arachnoid) caused by bacteria. BM is recognized as one of the top 10 cause of infection-related death and disability (Kim, 2006), especially in developing countries. The case fatality rate of BM remains around 10-40%. An additional 5-40% of cases have only partial recovery with late sequelae, such as hearing and/or speech loss, blindness, permanent brain and nerve damage, behavioral changes, cognitive disabilities, lack of muscle control, seizures, and memory loss. These patients may need long-term therapy, medication, and supportive care.

Several Gram-negative (g-) and Gram-positive (g+) bacterial species have been shown to be common causes of meningeal infection. *Haemophilus meningitis* was at one time the most prevalent form of bacterial meningitis. Fortunately, the *Haemophilus influenzae* (g-) b vaccine has greatly reduced the number of cases worldwide. *Neisseria meningitidis* (g-)

causes meningococcal meningitis frequently in children ages 2-18. *Escherichia coli* (g-) meningitis, which is most common in elderly adults and newborns, may be transmitted to a baby through the birth canal. Pneumococcal meningitis, the disease caused by the bacterium *Streptococcus pneumoniae* (g+), is the most common and the most serious form of meningitis at present. About 6,000 cases of pneumococcal meningitis are reported in the United States each year. Other forms of bacterial meningitis include *Listeria monocytogenes* (g+) meningitis, which can cross the placental barrier and cause a baby to be stillborn or die shortly after birth.

Gram stain is the widely used method of identifying bacteria and the cell wall plays an important role in the staining process. Gram-positive bacteria are encased in a plasma membrane covered with a thick wall of peptidoglycan which can retain crystal violet dye so they can be stained dark violet or purple. The Gram positive cell wall also contains teichoic acids, which are polymers of glycerol or ribitol joined by phosphate groups. Amino acids, such as D-alanine are attached. Teichoic acid is covalently linked to muramic acid and links various layers of the peptidoglycan mesh together. Gram-negative bacteria are more complicated than Gram-positive because they are encased in triple-layer. Outside of the central membrane is an open area called the periplasmic space. Beyond this is a thin layer of peptidoglycan. Finally, external to the peptidoglycan is an additional membrane called the outer membrane. The outer membrane of Gram-negative bacteria is composed of a high concentration of lipids, polysaccharides and proteins. The following image shows a cross-section of the two types of bacterial cell walls. Gram-negative and Gram-positive bacteria are found to display quite different sensitivities to antibiotic treatment.

Figure 2.2: Cell envelopes components of Gram-positive and Gram-negative bacteria, taken from http://filebox.vt.edu/users/chagedor/biol_4684/Methods/cellwalls.html.

Bacteria binding to and invasion of brain microvascular endothelial cells (BMEC) are considered important linked steps in the pathogenesis of meningitis (Kim & Kang et al., 2005). Routes of entry are diverse including paracellular and transcellular penetration. Some recent studies have implicated that meningeal pathogens invade BMEC via specific host cell signal transduction pathways affecting **actin** cytoskeleton rearrangements.

Parasitic meningitis usually is caused by a parasitic **helminth**, *Angiostrongylus cantonensis* found in contaminated water, food, and soil. Infected patients present with severe headaches, vomiting, paresthesia, weakness and sometimes visual disturbances and extraocular muscle paralysis. Only severe infection can lead to chronic, **harmful** disease and even death; Most patients have a full recovery (Pien & Pien, 1999).

Fungal meningitis develops in patients with conditions that compromise the effectiveness of their immune systems (e.g., HIV/AIDS, lupus, diabetes). Fungal meningitis occurs in 10% of

patients with AIDS and in patients undergoing neurosurgical procedures (6–17%) (Calabuig & Pemán et al., 2007). *Cryptococcus neoformans* (found mainly in dirt and bird droppings) and *Candida albicans* (*C. albicans*) are commonly involved in fungal meningitis. Although treatable, fungal meningitis often recurs in nearly half of affected persons. *C. albicans* is the predominant causative agent (60%) (Sanchez-Portocarrero & Perez-Cecilia et al., 2000) and also is responsible for significant morbidity and mortality if not recognized and treated effectively.

Encephalitis

Encephalitis can be caused by bacteria, fungus, parasite and, most often, by viral infections. Bacteria usually cause meningitis or abscess formation; with cerebritis, rather than encephalitis, describing the associated parenchyma inflammation (Laban & O'Neill, 2007). The most common bacteria that cause encephalitis are listed in Table 2.1.

Although almost any fungus may cause encephalitis, cryptococcal meningoencephalitis is most frequently seen, followed by aspergillosis and more rarely candidiasis (Kastrup & Wanke et al., 2005). *Aspergillus* is an agent for opportunistic infection of the CNS more frequently in bone marrow transplantation recipients than in AIDS patients (Coley & Jager et al., 1999). Cerebral candidiasis is usually preceded by a systemic candida infection and is frequently catheter related.

Parasite encephalitis induced by *Acanthamoeba* is rare but fatal. *Toxoplasma gondii* and *Plasmodium falciparum* are two other kinds of parasite involving the serious infection of parenchyma (Kim, 2006; Laban & O'Neill, 2007).

Acute infection of the brain parenchyma is usually caused by viruses. Following spread to the brain via a hematogenous route, viruses can enter brain cells by adhesion to specific cell surface receptors expressed by glia and neurons. Herpes simplex virus (HSV) I remains the most common cause of viral encephalitis (Schrnutzhard, 2001; Hinson & Tyor, 2001). Herpes simplex encephalitis (HSE) is responsible for about 10 percent of all encephalitis cases, with a frequency of about 2 cases per million persons per year. More than half of untreated cases are fatal. Most other viral encephalitis in the United States are caused by a bite from a rabid animal (rabies virus), or arboviruses, which are transmitted from infected animals to humans through the bite of an infected tick (Powassan encephalitis), mosquito (Equine encephalitis, *LaCrosse* encephalitis, St. Louis encephalitis, West Nile encephalitis) or other blood-sucking insect. However, the newly recognized viruses such as the Nipah virus and the previously unnoticed association of viruses like the human herpes viruses 6 or 7 (HHV 6, HHV 7) or enterovirus 71 with CNS infections underline that agents other than HSVI have to be considered in acute encephalitis. Even in immunocompetent adults, HHV 6 can cause chronic encephalitis. In immunocompromised patients the spectrum of possible causative agents is even broader.

The following table provides an overview of those organisms usually associated with meningitis, encephalitis and extradural abscess/subdural empyema.

Table 2.1 Pathogens commonly causing human brain infections

Brain infections	Organisms (selected examples)			
	Bacteria	Viruses	Fungus	Parasites
Meningitis	<i>Haemophilus influenzae</i>	Enterovirus	<i>Cryptococcus neoformans</i>	<i>Angiostrongylus cantonensis</i>
	<i>Streptococcus pneumoniae</i>	<i>Varicella zoster</i>	<i>Candida albicans</i>	
	<i>Neisseria meningitidis</i>	Mumps		
	<i>Escherichia coli</i>	HIV		
	<i>Mycobacterium tuberculosis</i>	Herpes simplex virus-2		
Encephalitis	<i>Mycobacterium tuberculosis</i>	Herpes simplex virus-1	<i>Cryptococcal</i>	<i>Acanthamoeba</i>
	<i>Listeria monocytogenes</i>	Rabies virus	<i>Aspergillosis</i>	<i>Toxoplasma gondii</i>
	<i>Treponema pallidum</i>	Arboviruses	<i>Candida</i>	<i>Plasmodium falciparum</i>
	<i>Nocardia</i>	Nipah virus		
	<i>Borrelia burgdorferi</i>	Human herpes viruses 6/7		
Extradural abscess/	<i>Neisseria meningitidis</i>			
subdural empyema	<i>Streptococcus pneumoniae</i>			
	Mixed anaerobes			
	<i>Staphylococcus aureus</i>			

2.3 Blood-brain barrier

2.3.1 Biology of blood-brain barrier

Figure 2.3: Schematic representation of cerebral capillary and the features of blood-brain barrier (Taken from 24).

The brain is residing within the bony skull, thus making direct delivery of drugs difficult. An obvious route of delivery would be via the blood vessels. Large arteries on the surface of the brain give off penetrating arteries that dive into the brain parenchyma, ending in extensive branches of capillary networks. The chief anatomical and functional site of the BBB is the brain endothelium: The endothelial cells (Figure 2.3) of the BBB are distributed along the capillary and completely encircle the lumen. The cytoplasm of the endothelial cells is uniformly thick with very few pinocytotic vesicles and no fenestrations. The cells are joined by tight junctions, which cement the capillary wall so that even ions have a hard time squeezing through. Additionally, the periendothelial accessory structures of astrocytes and pericytes add a lipid barrier to the system (Expert reviews in Molecular Medicine).

Astrocytes are thought to be responsible for signaling the cells forming the brain capillaries to "get tight" and are also believed to participate in the cross cellular transport of some substances. Furthermore, superfine filter function of the BBB is increased with P-glycoprotein (Pgp), an ATP-binding cassette family of active drug efflux transporter, which actively removes a broad range of molecules even including lipophilic drugs. Penetration rate and estimated bactericidal power of typical antibiotics for CNS infection treatment are listed in Table 2.2.

Table 2.2: Penetration and estimated bactericidal power of antibiotics used for treatment of bacterial meningitis (Sáez-Llorens & McCracken Jr, 2003)

Antibiotic	CSF penetration	Bactericidal power* against	
		β -lactam susceptible pathogens	β -lactam resistant pathogens
Penicillinampicillin	5-15%	1-10	<1
Chloramphenicol	>20%	>10	Not applicable
Cefotaxime/ceftriaxone	5-15%	>10	1-10
Cefepimelmeropenem	5-15%	>10	1-10
Vancomycin	<5%	1-10	1-10
Fluoroquionlones	>20%	>10	>10

*Concentration in CSF over the minimal bactericidal concentration against the isolated pathogen.

2.3.2 Transport routes across the blood-brain barrier

There are several transport routes by which solute molecules move across BBB (Neuwelt, 2004).

Diffusion of substances into the brain can be divided into paracellular (i.e. between cells) and transcellular (i.e. across cells). As illustrated in Figure 2.4A, small water-soluble molecules simply diffuse through the tight junction but not to any great extent.

Small lipid soluble substances like alcohol and steroid hormones penetrate transcellularly by dissolving in their lipid plasma membrane (Figure 2.4B).

However, for almost all other substances, including essential materials such as glucose, amino acids, require gated channels, ATP, selective membrane bound carriers or vesicular mechanisms to pass the BBB. In the case of facilitated diffusion (Figure 2.4C), there is a binding of a solute to a protein transporter on one side of the membrane that triggers a conformational change in the protein, resulting in transport of the substance to the other side of the membrane, from high to low concentration.

Receptor-mediated endocytosis (RME, Figure 2.4D) provides a means for selective uptake of macromolecules. Endothelial cells have receptors for the uptake of many different types of ligands, including growth factors, enzymes and plasma proteins. For example, insulin molecules first bind to receptors that collect in specialized areas of the plasma membrane known as coated pits. When bound to ligand these pits invaginate into the cytoplasm and then pinch free of the plasma membrane to form coated vesicles. After acidification of the endosome, the ligand will dissociate from the receptor and cross the other side of membrane.

Figure 2.4: Transport routes across the blood-brain barrier (Neuwelt, 2004).

Adsorptive-mediated transport (AME, Figure 2.4E) is triggered by an electrostatic interaction between a positively charged substance, usually a charge moiety of a peptide, and the negatively charged plasma membrane surface (i.e. heparan sulfate proteoglycans). AME has a lower affinity but higher capacity than receptor-mediated endocytosis. The development of many new drug delivery technologies focuses on AME (Pardridge, 1999). In this study, TAT peptide carrying positive charges is tagged onto the surface of the nanoparticles for facilitating cerebral transportation of antibiotics via adsorptive endocytosis.

Another significant transport mechanism at the BBB is carrier-mediated efflux (Figure 2.4F). This mechanism is involved in extruding drugs from the brain and is a major obstacle for many pharmacological agents, with the ATP binding cassette (ABC) transporter P-glycoprotein being the principle efflux mechanism of these agents (Condon-Cardo &

O'Brien et al., 1989). There also exist efflux transporters for organic anions, via multidrug resistance associated protein (MRP) (Kusuhara & Suzuki et al., 1998).

2.4 Various drug delivery approaches for the brain

A variety of strategies have been undertaken to manipulate the BBB to facilitate drug delivery, as briefly summarized below.

2.4.1 Modification of drugs and prodrugs

Only small, electrically neutral, lipid-soluble drugs (molecular weight less than 500 Da) can penetrate the BBB. Therefore, chemical modification of the drugs into more lipophilic and neutral form as well as a prodrug has been studied. This approach may bring in undesirable pharmacokinetic effects, such as increased uptake by the reticuloendothelial system (RES) and increasing non-specific plasma protein binding when administered intravenously (Greig & Daly et al., 1990).

2.4.2 BBB disruption

An often employed BBB opening practice is via arteries injection of hyperosmolar solution (e.g., mannitol, arabinose). The shrinkage of endothelial cells results in transient opening gaps between cells. One major limitation of this approach is its invasiveness, which requires considerable expertise. Bartus & Elliott et al. (1996) alternatively opened the BBB by intravenous injection of RMP7, which binds to receptors of endothelial cells and kicks off a biochemical cascade that loosens the tight junctions. But both methods are not specific

enough to exclude central nervous system (CNS) entry of toxins and other unwanted molecules.

BBB disruption by solvents, such as ethanol, dimethylsulfoxide (DMSO), or detergents (i.e. SDS, or Tween 80) has been reported in numerous literatures. When drugs are administered together with above mentioned solvents, there is a high risk of toxic side effect.

2.4.3 Trans-nasal drug delivery

The primary advantage of intranasal delivery is to deliver small molecules that would be rapidly removed from the bloodstream by hepatic first pass elimination. This non-invasive method is based upon drug molecules that are capable of moving from regions of the olfactory nerve dendrites in the olfactory epithelium, through the cribriform plate into the brain (Thorne & Emory et al., 1995). There are three points to consider when limited drug targeting was observed. First, any drug that enters into olfactory CSF will exit the CSF flow tracts and enter the peripheral blood stream. Secondly, the tight junction of arachnoid membrane, just like that of BBB endothelial cells, may prevent most molecules from entering olfactory CSF. The third difficulty is due to human nasal cavity which can only receive about 100 μl per nostril (in the absence of injury to the nose), much lower than the volume of drug administered ($\gg 200 \mu\text{l}$).

2.4.4 Trans-cranial drug delivery

Three neurosurgery based delivery approaches: intracerebral injection (or implantation), intracerebroventricular (ICV) infusion, and convection enhanced diffusion (CED) have been actively investigated for drug delivery to the CNS. To date, Gliadel[®] biodegradable polymer

wafer is approved for clinical use in U.S.A. The CED delivery system is used in two clinical trials. However, a fundamental problem is that diffusion rate for typical therapeutic drugs can be very slow compared with the high rate of CSF clearance. Additionally, the highly invasive procedure that requires neurosurgery and special equipment limit their application in high grade gliomas (Weaver & Laske, 2003).

2.4.5 Transport system mediated delivery

Attempt to find another entryway into the brain for CNS drugs involves combination of endogenous transport systems present in the BBB and surface enhanced nanoparticle delivery systems. As discussed in section 2.2.2, transporter proteins, specific receptors or adsorptive endocytosis can be used to realize drug delivery. For example, transferin and insulin receptors have been targeted utilizing antibody conjugation and chimeric peptide methods (Friden, 1994). This approach is promising with the remarkable development of nanotechnology and non-invasiveness, compared to other delivery methods listed above.

2.5 Nanoparticulate systems for brain drug delivery

Nanoscale drug carriers consist of particles in size below 1000 nm. Ideal properties of nanoparticles for drug delivery across the BBB are listed below (Koo & Reddy et al., 2006):

- Nontoxic, biodegradable, and biocompatible
- Stable in blood (no aggregation and dissociation)
- Prolonged blood circulation time
- BBB-targeted moiety (receptor or adsorptive mediated mechanism)
- Well maintained parent drug property
- Tunable drug release profiles

In general, such nanocarriers have been realized in polymers, inorganic clay minerals or ceramics, carbons, metals, and biological materials (i.e. lipids, nucleic acids, polysaccharides and viruses) (Yih & Al-Fandi, 2006; Hughes, 2005). A broad spectrum of nanoscale architectures have been documented including solid particles, core-shell spheres, capsules, tubes, porous particles and branched structures. The following table summarizes several subsets of nanocarriers and their advantages.

Nanocarriers	Size (nm)	Advantages
Polymeric biodegradable nanoparticles	10-1000	Sustained and localized drug release for weeks
Ceramic nanoparticles	<100	Easy preparation, water-soluble, stable
Metallic nanoparticles	<50	Small size with large surface area to carry large dose
Polymeric micelles	<100	Suitable carriers for water-insoluble drug
Liposome	50-100	Low system toxicity, well-established preparation process
Dendrimers	<10	Can carry both hydrophobic or hydrophilic drugs

Despite the large variety of the nanocarriers developed so far, it is noteworthy that only amphiphilic molecule-formed liposomes and polymeric nanoparticles have been extensively exploited for brain drug delivery (Garcia-Garcia & Andrieux et al., 2005), Several such systems are now in clinical trial for anticancer drug delivery. Other materials are still at relatively early development stages. Therefore, non-amphiphilic colloidal drug carrier systems such as dendrimers and microemulsions will not be covered in this review. In the following sections, liposomes and micelles, in terms of their structures and compositions, will be briefly introduced with emphasis of polymeric nanoparticles for drug delivery to the brain.

2.5.1 Supramolecular architectures of aggregated amphiphilic molecules

An amphiphile, by definition, is a chemical species having a “polar” (a hydrophilic) head group and "hydrophobic" tails. Amphiphiles interact very strongly with water. A small concentration of amphiphiles accumulate at the surface of the water because the free energy

of the air-water interface is reduced. The head group of the amphiphile points is adjacent to the water, and the hydrophobic tail group is adjacent to the air. Above certain critical concentration, structures from amphiphilic molecules may be formed in various morphologies, such as lamellar bilayer, micelles, rods, vesicles or larger hexagonal aggregates (Figure 2.5).

Figure 2.5: Schematic representation of typical aggregation topologies of amphiphilic molecules.

Vesicles are hollow, lamellar spherical structures. In cell biology, vesicles are relatively small and enclosed compartments, separated from the cytosol by at least one lipid bilayer. Their size ranges from 20 nm to 100 μm , whereas the thickness of the membranes is around 3.5 nm.

A micelle is basically a spherical aggregation of amphiphilic molecules floating in water – with their hydrophilic head groups on the surface of the sphere, and their hydrophobic tails collected inside. An important property of micelles is their ability to increase the solubility

and bioavailability of poorly soluble pharmaceuticals. However, micelles lack the shell-like character and encapsulated bulk solution phase of a vesicle.

Most work has been concentrated on micelles and bilayer vesicles due to their technologically useful compartment. These two systems are able to protect enclosed drug from chemical and biological degradation in the blood stream.

Multiple noncovalent interactions including hydrogen bonds, π -stacking, salt bridges and Van der Waals force play a critical role in the structure and function of amphiphilic macromolecule aggregates.

2.5.2 Classification of amphiphiles

2.5.2.1 Biological phospholipid

As shown in Figure 2.6, a typical phospholipid molecule has two long hydrophobic fatty-acid "tails" and a hydrophilic (polar) phosphate-containing "head." Phospholipids are principal backbone of biological membranes. Nature has long optimized the use of the amphiphilic molecules for a variety of applications. Relatively well-known examples include (I) the compartmentalization and segregation of different biological environments by using assemblies (such as bilayers) formed from phospholipids, and (II) the presentation of arrays of multivalent ligands at specific locations on the surfaces of cells to enable the passage of information (signal transduction) across cell membranes.

Figure 2.6: A membrane phospholipid molecule of palmitoyl-oleoyl-phosphatidyl-choline has a hydrophilic head and two hydrophobic tails.

Lipid **nanost**tructures that have been developed for drug delivery include lipid nanotubes (Cans & Wittenberg et al., 2003), lipid nanospheres (Fukui & Koike et al., 2003), lipid **nanoparticles** (Seki & Sonoke et al., 2004), and lipid emulsions (Seki & Sonoke et al., 2004). One of the most extensively studied lipid forms is the liposome. Liposomes are usually formed by the self-assembly of phospholipids in aqueous solution. Due to the existence of the aqueous core and the hydrophobic lipid bilayers, liposomes can accommodate both hydrophilic and hydrophobic drugs. They are classified according to their final size and preparation methods: small unilamellar vesicles (S W , 20-50 nm); large **unilamellar** vesicles (L W , 100 nm), reverse phase evaporation vesicles (REV, 0.5 pm); large multilammnellar vesicles (LMV, 2-10 pm).

The main challenge concerning liposomes as drug carriers is the easy disruption of the lipid bilayers and subsequent severe leakage of the entrapped.molecules in the biological systems.

For the present study, we will employ the liposomes as artificial bacteria to investigate the action model of antibiotics.

2.5.2.2 Synthetic amphiphiles

The autoassociated amphiphiles are successfully used by nature to form biological functional units. Therefore it has been tempting for a long time to mimic their design principles during fabrication of synthetic materials. In pharmaceutical applications, such as clinical nutrition and formulation of lipophilic drugs, lecithin-based emulsifiers are the most utilized natural lipids in preparing emulsions. However, such emulsions, possessing too lipophilic phosphatidylcholine, usually suffer from disadvantages, such as broad particle size distributions and particle fusion-mediated creaming (Shinoda & Carlsson et al., 1996). Alternatively, non-phospholipid-based synthetic vesicles/micelles (niosomes) (Uchegbu, 2000) broaden the range of properties through a variety of choices of amphiphile chemistry.

Block copolymers have the same basic amphiphilic property as lipids (Figure. 2.7) but they consist of distinct polymer chains covalently linked in a series of two or more segments (Foster & Zisenis et al., 1996).

Figure 2.7: Schematic representation of the block copolymer bilayer (b) compared with a bilayer made from typical lipids (a). Polymer vesicles (c,d) and micelle (e).

Amphiphilic block copolymers have recently emerged as a special class of materials. This is because polymer molecular weights can be orders of magnitude greater than those of lipids, self-assembled structures of which are rather stable. In addition, the diversity of block composition and block length offers the possibility of designing the most desirable drug delivery systems.

The commonly used hydrophobic core-forming polymers are poly(propylene glycol), poly(D, L-lactide) and poly(caprolactone) etc. Cholesterol is often included in membrane phospholipids to stabilize the membrane and reduce the membrane permeability towards encapsulated materials. In this context, cholesterol is an intriguing component of cell membranes because it both toughens and fluidizes the membranes (Edwards & Baeumner, 2006; Lipowsky & Sackmann, 1995). In this study, we employ cholesterol as the hydrophobic segment because cholesterol possesses good bio-compatibility and potential

interaction with cholesterol receptors on cell surface, and strong ability to drive self-assembly of cholesterol containing polymers (Liu & Pramoda et al., 2004; Wang & Gao et al., 2006). Akiyoshi and Kuroda (2002) reported that pullulan, a polysaccharide, modified with rigid and bulky hydrophobic cholesterol groups (CHP) showed a stronger tendency for self-association than the usual long alkyl chains. CHP formed stable nanoparticles with diameters of 20–30 nm in water via intermolecular self-association of cholesterol groups even in the lower content less than 5 cholesterol per 100 glucose residues. PEG has been widely used in the design of drug delivery systems. For example, PEG has been conjugated with Interferon to prolong the blood circulation of Interferon. PEG conjugated Interferon has been marketed in 2000 for treating Hepatitis C. PEGylated-recombinant methionyl human granulocyte colony stimulating factor (PEG-G-CSF) is used to prevent severe cancer chemotherapy-induced neutropaenia (marketed in 2002). In this study, cholesterol is conjugated to one end of PEG. We hypothesize that cholesterol-PEG ensures that hydrophobic drugs can be incorporated into the core through hydrophobic interactions, and the presence of PEG shell can prolong the blood circulation of the enclosed drug molecules.

2.5.3 Polymeric micelle preparation and drug loading methods

The commonly used methods of niosome preparation and drug loading can be divided in two groups. In the first group of techniques, the amphiphiles and drugs are dissolved in an organic solvent and mixed with an aqueous phase. The organic solvent is then removed as much as possible by dialysis, centrifuge or ultrafiltration. The second method involves the formation of a surfactant film by the evaporation of an organic solution of surfactant. This film is then hydrated with a solution of drug (Azmin & Florence et al., 1985). Drugs can be associated with polymeric carriers in several ways, including physical encapsulation within the hydrophobic inner lumen or between the hydrophobic tails. Other drug loading

mechanism involves covalent and electrostatic interactions with the lipophilic parts of polymers.

Figure 2.8 describes two types of processes for fabricating drug-loaded polymeric micelles through physical entrapment. In one process, polymer and drug are dissolved in an organic solvent, which is then placed in a dialysis membrane. The mixture is dialysed against an aqueous medium. During the dialysis process, the solvent is removed by frequently changing the aqueous phase with fresh medium. In another process, polymer forms micelles in water. Drug is dissolved in an organic solvent. The polymer micelle solution and drug solution are then mixed with stirring. The organic solvent is removed by evaporation.

The drug incorporation via the dialysis method has been shown to be affected by the properties of the organic solvent and of the hydrophobic segment of the copolymer.

Figure 2.8: Drug loading of polymeric micelles by the dialysis (a) and the oil-in-water method (b).

2.5.4 PEGylated polymer micelles for drug delivery across the BBB

Polymer micelles having a hydrophilic shell and a hydrophobic core are a promising carrier for incorporation of hydrophobic drugs to improve the bioavailability of the drugs and prolong their blood circulation. Uncoated nanoparticles, characterized by hydrophobic surfaces, are efficiently enveloped by specific plasma components (opsonins), such as immunoglobins (IgG), albumin, the elements of the complement system, fibronectin, and others, and then rapidly opsonised and removed by the macrophages in the liver (Kupffer cells, 60-90% of the injected dose), spleen (2-10%), lungs (3-20% and more), and bone marrow (>1%), which belong to the RES. The extent of this RES uptake depends also on particle size and surface charge.

In the early 1980s, Illum & Davis (1983, 1987) found that by coating of the nanoparticles with PEG-containing surfactants, such as poly (oxy-ethylene)-poly(oxy-propylene) block copolymers (poloxamer 338 = Pluronic[®]F108 and poloxamine 908 = Tetronic[®]), their blood circulation time could be prolonged and their uptake by the RES organs was reduced significantly. A highly flexible and hydrated PEG chain attached to the nanoparticle surface is assumed to have an effective protein-resistant property due to its steric repulsion effect. In particular, the amount of nanoparticles in the brain was increased with all surfactants up to 24 h. Polysorbate 80 coated poly(butylcyanoacrylate) (PBCA) nanoparticles were first investigated (Schroder & Sabel, 1996) *in vivo* to enhance the brain transport of the hexapeptide dalargin. The results showed that after *i.v.* injection, polysorbate 80 coating enabled the highest induction of analgesia in mice. Likewise, other drugs that normally do not penetrate the BBB, including tubocurarine, loperamide, 8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazino, quinoline-5-oxide choline salt (MRZ 2/576), and doxorubicin showed higher concentrations in the brain when associated with polysorbate 80-coated nanoparticles

(Blasi & Giovagnoli et al., 2007). The protein ApoE or B adsorption on the nanoparticles, followed by low density lipoprotein (LDL) receptor mediated endocytosis and transcytosis, is believed to facilitate the uptake of the nanoparticles by the brain (Figure 2.9).

Figure 2.9: A proposed mechanism of brain uptake of polysorbate 80-coated nanoparticles. 1. Apo adsorption on nanoparticle surface. 2. Nanoparticle binding to the LDL receptors of the BBB. 3. Drug release after endocytosis and transcytosis.

In addition to PEG-surfactant coating, micelles (i.e. nanoparticles) self-assembled from PEGylated polymers provide better choice due to chemical binding of PEG onto the nanoparticles. The PEG chains were first bound to poly(lactic acid) nanoparticles by Gref & Minamitake et al. (1994) and Bazile & Prudhomme et al. (1995). Later on PEG was chemically linked to poly(hexadecyl cyanoacrylate nanoparticles (PHDCA) (Peracchia & Vautier et al., 1997; Peracchia & Fattal et al., 1999). The preparation of the PEG-PHDCA copolymer was achieved by the synthesis of a cyanoacrylate monomer substituted with PEG and its co-polymerization with hexadecylcyanoacrylate in a 1:4 ratio. Both types of nanoparticles, the PEGylated poly(lactic acid) and poly(hexadecylcyanoacrylate) (PHDCA) nanoparticles, significantly prolonged the blood circulation time and reduced the liver uptake. Calvo et al. investigated the distribution of PEG-PHDCA nanoparticles in EAE rats (Calvo & Gouritin et al., 2001). Analysis by confocal microscopy evidenced that fluorescent PEG-PHDCA nanoparticles were present in the epithelial cells of the brain and spinal cord surface and in the ependymal cells of the choroid plexus. It was hypothesized that PEG-PHDCA nanoparticles reached the brain by two mechanisms: passive diffusion due to the increase of the BBB permeability and transport by nanoparticles-containing macrophages, which infiltrated these inflammatory tissues. The PEGylated PHDCA nanoparticles accumulated in the brain was at a 4-8 fold higher concentration than non-PEGylated PHDCA nanoparticles after intravenous injection into rats (Brigger & Morizet et al., 2002).

Therefore, the covalent attachment of PEG chains was a promising step forward. Other PEG-containing copolymers showing potential ability for drug delivery to the brain are depicted in Figure 2.10.

Figure 2.10: Examples of PEG containing block polymers for drug delivery.

2.5.5 Ligand bearing nanoparticles for improved brain uptake

In addition to providing steric stabilization, the presence of PEG on the surface of the micelles also allows for the preparation of bioactive polymeric micelles by attaching bioactive ligands on the surface for targeted delivery to the brain. There are evident reasons for the design of ligand coated long-circulating drug carriers:

- 1) Ligand (an antibody, protein, peptide, sugar moieties, folate or carbohydrate) attached to the carrier surface may increase the rate of its elimination from the blood

and uptake in the liver and spleen. However, the presence of the PEG protecting polymer may compensate for this effect;

- 2) Longevity of the specific ligand-bearing nanocarrier may allow for its successful accumulation in targets with diminished blood flow or with low concentration of the surface antigen.

Covalent linkage of targeting ligands to the nanoparticles is typically made by a simple coupling reaction between amine-functionalized nanoparticles and succinimidyl ester derivatives. To achieve better selective targeting, targetors were attached to micelles via the PEG spacer arm, so that the ligands are extended outside of the dense PEG brush excluding steric hindrances for their binding to the target receptors.

The successful strategy of the delivery of compounds that are unable to pass the BBB is the use of transport vectors, which activate natural transport routes. The endogenous carrier-mediated transport (CMT) for nutrients and receptor-mediated transcytosis (RMT) for peptides are portals of entry to the brain for circulating drugs.

The BBB expresses several transport systems for nutrients (Tsuji & Tamai, 1999), but the utilization of these transport systems for targeting the drugs into the brain is mainly limited to peptide drugs, which must have a molecular structure mimicking the endogenous nutrients. The prototypical example is levodopa, a lipid-insoluble precursor of dopamine that has been used for the treatment of Parkinson's disease because it contains the carboxyl and α -amino groups that allow it to compete for transport across the BBB by the large neutral amino acid carrier (Wade & Katzman, 1975). In addition, since nutrient carriers stay in the membrane of the cell, the size of the drugs must be close to that of the endogenous ligand if

they will be taken up and transferred into the brain. Most antibiotics do not have such characteristics, thus, the following review only covers receptor mediated drug transport across the BBB.

2.5.5.1 Endogenous receptor mediated BBB transport

Insulin Receptor

The insulin receptor is a large protein having a molecular weight of 300 kDa and is a heterotetramer of two extracellular alpha and two transmembrane beta subunits. Each beta chain contains a tyrosine kinase activity in its cytosolic extension. Basically, insulin binds to alpha chains and changes the shape of the receptor to form a tunnel, to allow for the entry of molecules such as glucose into the cells. The autophosphorylation carried out by beta chains is necessary for the receptor to be internalized into endosomes.

The use of insulin as a vector is problematical because the administration of a drug-insulin conjugate, would cause hypoglycemia by triggering insulin receptors in peripheral tissues. The use of IGF-1 or IGF-2 as a transport vector is difficult because of the very avid binding (>99%) of IGF by specific plasma binding proteins for these particular growth factors.

Transferrin Receptor

The most widely characterized receptor-mediated transcytosis systems for drug delivery across the BBB is the transferrin receptor (TfR), even though the intracellular trafficking of transferrin upon internalization via the TfR has not yet been elucidated. The TfR is a transmembrane glycoprotein consisting of two subunits of 90 kDa. A disulfide bridge links these subunits and each subunit can bind one transferrin molecule. The TfR is expressed mainly on hepatocytes, erythrocytes, intestinal cells, monocytes and endothelial cells of the

BBB. In the brain, the TfR is also expressed on choroid plexus epithelial cells and neurons. The TfR mediates cellular uptake of iron bound to transferrin. Da Cruz & Simoes 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. The use of transferrin as a delivery vector may not be advantageous owing to the very high concentration of endogenous transferrin in the circulation, which competes for BBB transferrin binding sites. The likely overdosing with iron when one tries to displace the endogenous transferrin with exogenously applied transferrin-containing systems is another limiting factor for TfR in vivo application.

LRP1 and LRP2 receptors

During the past few years, the low-density lipoprotein receptor related protein-1 (LRP1) and LRP2 receptors have been exploited to target drugs to the brain in a fashion similar to that used for the transferrin and insulin receptors (Figure 2.7).

Similar caveats should be considered with use of LRP-mediated vector design. The potential competition and limited transporter concentration may decrease the ability to deliver an adequate and consistent dosage of drug to elicit a pharmacological effect.

2.5.5.2 Peptide mediated BBB transport

In the past decade, a number of cell-penetrating peptides (CPPs) have emerged, facilitating the intracellular delivery of polar biomolecules *in vitro* and *in vivo*. While these individual peptides differ in length and sequence, they share a few common features, which include theoretical hydrophobicity and helical moment, the ability to interact with lipidic membranes, and to adopt a distinct secondary structure upon association with lipids (Deshayes & Morris

et al., 2005). The major dogma has been that CPPs enter cells by a receptor-independent process, although the exact mechanism(s) remain to be elucidated. Being peptidic by nature, they theoretically would be expected to be less able to affect the physicochemical properties of the active molecule. In any case, the ability of these short peptides to cross plasma membranes even when associated with hydrophilic cargoes, makes them a worthwhile technology for further study and development.

Table 2.3 summarizes the majority of cell penetrating peptides with their principal features. Primary attention is dedicated to Tat peptide.

Table 2.3 Principal features of the cell penetrating peptides (CCPs).

Peptide name	Sequence	Net charge	Cell lytic activity
MAP	KLALKLALKALKAAALKLA	+5	YES
pAntp ₄₃₋₆₈	RQIKIWFQNRRMKWKK	+8	NO
Transportan	GWTLSAGYLLGKINLKALAALAKKIL	+4	YES
SBP	MGLGLHLLVLAAALQGAWSQPKKKRKV	+6	—
FBP	GALFLGWLGAAGSTMGAWSQPKKKRKV	+6	—
Tat ₄₈₋₆₀	GRKKRRQRRRPPQ	+8	NO
SynB1	RGGRLSYSRRRFSTSTGR	+6	NO
SynB3	RRLSYSRRRF	+6	NO

MAP indicates model amphipathic peptide; Antp indicates Antennapedia; SBP: sequence signal-based peptide; FBP, fusion sequence-based peptide; Tat, transcription-activating factor. The peptide residues in this table were expressed with one-letter-code: K-lysine; L-leucine; A-alanine; R-arginines; Q-glutamine; I-isoleucine; W-tryptophan; F-phenylalanine; N-asparagine; M-methionine; G- glycines; S-serine; T-threonine.

A number of cell-penetrating peptides, such as MAP, transportan and different signal sequence-based peptides (SBP) are not derived from natural proteins but from the engineering of various short peptides. This class of peptides consists of amphipathic helical

or sheet structures, where lysine (L) is the main contributor to the positive charge. The unconventional internalization of these peptides seems to be neither saturable nor dependent on the cell type and could be related to their lipid-binding capacity.

The second class of peptides includes arginine (R)-rich peptides, such as SynB, penetratin and Tat.

SynB vectors, a family of CPPs originally isolated from the antimicrobial peptide protegrin-1 extracted from porcine leukocytes, are a third class of CPPs. Brain uptake of the enkephalin analogue, dalargin, a hexapeptide, was enhanced significantly when conjugated to SynB and injected intravenously in mice (Rousselle & Clair et al., 2003). Peptides reaching the brain tissue after systemic delivery, as done in this study intravenously, signal the potential for delivery of peptides for brain cancer.

Penetratin, a short peptide fragment from the third helix of the Antennapedia protein homeodomain, is able to penetrate into a variety of cells, such as lymphocytes or endothelial cells (Fenton & Bone et al., 1998). Early findings suggest that this entity translocates through plasma membranes without binding to a stereospecific receptor, most probably via interaction with the lipidic components of the plasma membrane.

In terms of the cellular uptake and cargo delivery kinetics, MAP has the fastest uptake, followed by transportan, TAT (48-60), and penetratin. Similarly, MAP provides the highest delivery efficiency, followed by transportan, Tat (48-60), and penetratin.

Tat is a HIV-transcription activating factor with 101 amino acids. The protein consists of five domains; the probably best-studied region of Tat is located in domain 4, which contains a highly basic region (with two lysines and six arginines in nine residues) involved in nuclear and nucleolar localization (Brigati & Giacca et al., 2003). While all CCPs listed above have been used for small cargoes such as peptide and oligonucleotides, Tat has been used more often for delivery of larger molecules such as proteins, one example is β -galactosidase (MW=540 kDa) (Loftus & Li et al., 2006).

Schwarze & Ho et al. (1999) synthesized full-length fusion proteins that contained a NH₂-terminal 11-amino acid protein transduction domain (PTD) from the HIV transactivator of transcription (Tat) protein. Transduction of the proteins evaluated was non-cell-specific, and was seen to occur even across the blood–brain barrier. Further proof of this mode of peptide delivery was attained by Cao et al. (Cao & Pei et al., 2002) who fused the antiapoptotic protein Bcl-xL to Tat and injected the construct intraperitoneally into mice that were affected by stroke. The Bcl-xL protein is expressed in adult neurons of the CNS and is believed to play an important role in the prevention of neuronal apoptosis that normally would occur during brain development, or results from varying stimuli leading to pathology, including cerebral ischemia. Protein transduction with this entity occurred in a rapid, concentration-dependent fashion, with entry into cells thought to occur via the lipid bilayer component of the cellular membrane. A study by Kilic & Dietz et al. (2002) using the same model showed that brain tissue was progressively transduced with Tat proteins within 3–4 h after intravenous delivery. Tat-Bcl-xL treatment reduced infarct volume and neurological deficits after long ischemic insults lasting 90 min, when applied both before and after ischemia. Dietz & Kilic et al., (2002) demonstrated that the same Tat-Bcl-xL construct, when injected into the eye, prevented 24% of mouse retinal ganglion cells from undergoing retrograde

neuronal apoptosis caused by optic nerve lesion. Thus, at least for neurological disorders, application of Tat fusion proteins may greatly facilitate protective therapy strategies.

Studies have also shown that even relatively large particles could be delivered into various cells by Tat vector. A biocompatible 45 nm particle with an iron core, a dextrane coating, and covalently linked Tat peptides are efficiently taken up by human hematopoietic CD34⁺ cells (Zhao & Kircher et al., 2002). Even liposomes having a diameter of 200 nm has been documented of cytoplasmatic uptake (Fretz & Mastrobattista et al., 2005; Torchilin & Rammohan et al., 2000).

Taking the Tat-mediated nanoparticles delivery approach a leap further, one of the most exciting demonstrations of the effectiveness of Tat-shuttled nanocarriers across the BBB was accomplished by Tat-conjugated CdS:Mn/ZnS quantum dots (Qdots) (Santra & Yang et al., 2005). Histological data clearly showed that Tat-Qdots migrated beyond endothelial cells and reached the brain parenchyma. TAT-mediated intracellular delivery of large molecules and nanoparticles was proved to proceed via the energy-dependent macropinocytosis with subsequent enhanced escape from endosome into the cell cytoplasm (Wadia & Stan et al., 2004). TAT promises a powerful delivery of giant cargo, and will be the focus of the current project.

2.6 CNS microbial therapy

2.6.1 General brain infection treatment

The term antibiotic in this review refers to any agent that inhibits or abolishes the growth of micro-organisms, such as bacteria, fungi and parasites. The therapeutics should be a

combination of agents chosen to be active against a broad spectrum of organisms and be able to reach adequate levels within the brain parenchyma.

The chief mechanisms by which antibiotics act are:

- Inhibition of cell-wall synthesis (penicillin and other β -lactam antibiotics; glycopeptides)
- Inhibition of folate metabolism (Sulfonamide, sulfone, trimethoprim)
- Binding to ribosomes and to prevent translation (ketolides, oxazolidinones, streptogramins)
- Interfering nucleic-acid synthesis (fluoroquinolones and nitroimidazoles)

Third-generation cephalosporins (ceftizoxime and ceftazidime, broad spectrum of activity and excellent CNS penetration) or penicillin plus metronidazole were the most common agents chosen for empirical therapy, followed by intravenous aminoglycosides (tobramycin and gentamicin) and piperacillin. However, Sims & Lim et al., (2005) doubted the usage of aminoglycosides, erythromycin, tetracyclines, and first-generation cephalosporins for brain abscesses treatment because these drugs do not cross the blood-brain barrier. Although Trimethoprim-sulfamethoxazole (TMP-SMX) has excellent CNS penetration and antimicrobial activity (Foster & Rhoney, 2005), TMP-SMX is generally regarded as a bacteriostatic agent and has been replaced by newer bactericidal agents in the treatment of severe infections. Other potential treatment options include fluoroquinolones. Amphotericin B is administered for *Candida*, *Cryptococcus*, and *Mucorales* infections; voriconazole for *Aspergillus*.

2.6.2 Antibiotic resistant

Unfortunately, in response to the threat of extinction, pathogens have developed resistance to every anti-microbial agent used to date through mutations of target sites or the acquisition of novel biochemical functions. Brain infection treatment is also complicated by resistance of species to third-generation cephalosporins.

For example, *Enterobacter* species (*E. cloacae* and *E. aerogenes*) commonly contain chromosomally mediated, depressed type I β -lactamases that cleaves the β -lactam ring rendering third-generation cephalosporins inactive. Methicillin-resistant *S. aureus* (MRSA) has emerged as an important cause of hospital-acquired CNS infections. Many MRSA strains are also resistant to several other antibiotics including all other β -lactam antibiotics, macrolides and lincosarnides while usually being highly susceptible only to vancomycin and teicoplanin. In these circumstances, the two glycopeptides agents vancomycin and teicoplanin remain as major choices (Arda & Yamazhan et al., 2005). The presence of *S. pneumoniae* species with diminished susceptibility to penicillin and other agents is now recognized worldwide. It is accepted that penicillin therapy is inappropriate for the treatment of penicillin-resistant pneumococcal meningitis.

In response to this medical challenge, the search for new antibiotics has been intensified. For example, Tsushima et al. have synthesized new cephalosporin-based antibiotics by introducing bicyclic heterocycles at the C-3 position and the activity against MRSA has been confirmed to be high. Likewise, Ohtaka and colleagues have developed novel dithiocarbamate carbapenems that are at least as active as vancomycin against MRSA. In many cases, new drugs are analogues of existing antibiotics and they could, therefore, easily encounter bacteria that already have a defense against them or that could rapidly adapt to

accommodate the new derivatives (Tan & Tillett et al., 2000). Regarding the three kinds of new antibiotics (cyclic lipopeptides, glycyclines, and oxazolidinones) recently in clinical use follows a 40-year hiatus in discovering new compounds to counteract the bacterial resistance to existing antibiotics, only oxazolidinones (linezolid and eperezolid), used for gram-positive infections, can be truly regarded as structurally novel inhibitors.

With the steady rise in the number of antibiotic-resistant pathogens, it has become increasingly important to search new therapeutic agents, which have novel and diversified mechanisms of action. Among the possible candidates, the antimicrobial peptides have attracted increasing research and clinical interest.

2.6.3 Antimicrobial peptides (AMPs)

AMPs are important components of innate host defense in species from microorganism to arthropods, vertebrates and mammals including humans. A full and detailed account of these peptides is beyond the scope of this review and we refer the reader to the websites such as AMSDb (<http://www.bbcm.univ.trieste.it/~tossi/pag1.htm>) and ANTIMIC (Brahmachary & Krishnan et al., 2004), which store a large number of AMPs data.

2.6.3.1 Antimicrobial peptide groups

The diverse ribosomally-synthesized AMPs, containing only natural amino acids can be classified into four groups on the basis of their amino acid composition, charge and secondary structure. One group contains anionic peptides, produced in mM concentrations and requires Zn^{2+} or Ca^{2+} as a cofactor for antimicrobial activity (Dennison & Howe et al., 2006). The second group peptides are short (<40 amino acid residues), linear, positively

charged and adapt to α -helical structure in the presence of sodium dodecyl sulphate (SDS) micelles or phospholipid vesicles and liposomes (Tossi & Sandri et al., 2000). The third group contains cationic peptides that are rich in certain amino acids such as proline, arginine, phenylalanine and tryptophan but lack cysteine residue. They are usually linear, although some form extended coils. Peptides in the fourth group contain cysteine residues therefore form disulphide bonds and stable β -sheet (Ganz, 2002). The last group contains some fragments of larger proteins that are similar in composition and structure to the peptides described above.

There are also a large number of peptides with potent antimicrobial activity that are synthesized extra-ribosomally or contain substantial post-translational modifications, for example lipopeptides (polymyxin, dermaseptin) and the lantibiotics, which contain non-native amino acids and /or non peptide backbone structures. In addition, a wide variety of synthetic AMPs have been developed employing either a combinatorial synthesis approach or through modification of existing AMPs. These include peptides such as cyclic peptides containing a combination of D- and L-amino acids, the effective bactericidal-fungicidal decapeptide, KSL, and the ornithine-based lipopeptide MSI-843 (Sato & Feix, 2006).

2.6.3.2 Antimicrobial mechanisms of peptides

Despite the substantial diversity in the amino acid composition and the secondary structures (i.e. β -sheets, α -helices, loops and extended structures) of antimicrobial peptides, most peptides in their final folded three-dimensional conformations have patches of hydrophobic and charged residues that permit the peptides to interact strongly with membranes.

The most studied group within antimicrobial peptides contains short, linear, positively charged, amphipathic peptides, whose activities and possible modes of action have been reviewed in detail (Straus & Hancock, 2006; Hancock & Sahl, 2006; Zasloff, 2002). Peptides in this group fold into amphipathic three-dimension arrangement, with one face being hydrophobic, while the other is hydrophilic and positively charged. It is believed that peptides target the negatively charged outer leaflet membrane of bacteria directly, rather than acting via a specific receptor. A model that explains the initial interactions of peptides with membranes has been proposed by Shai-Matsuzaki-Huang (Shai, 1999; Matsuzaki, 1999; Yang & Weiss et al., 2000). The peptides start off being unstructured in solution. Upon interaction with the membrane, they adopt a three-dimensional structure such that the molecule becomes amphiphilic, with the positively charged side interacting directly with the lipid headgroups. The peptide then integrates into the outer half of the membrane, leading to thinning of the outer leaflet. After this step, pore/channel formation, membrane perturbation, leakage of cellular components and cell death can occur. A number of different models termed the barrel-stave, the toroidal pore, the carpet, and sinking raft model have been proposed as illustrated in Figure 2.11.

Works on anionic peptides suggested that they target and depolarize the bacterial membrane in a manner somewhat reminiscent of the cationic peptides. Anionic antimicrobial peptides possess an asymmetric distribution of hydrophobicity along the alpha-helical long axis, which can penetrate membranes at shallow angle, thereby disturb the integrity of membrane lipid (Goumon & Lugardon et al., 1998; Lorin & Thomasa et al., 2006).

Figure 2.11: The models of the mechanism of action of α -helical cationic peptide. a (barrel-stave model), the attached peptide aggregate and insert into the membrane bilayer with the hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide regions form the interior region of the pore. b (toroidal model), the induced lipid monolayer bending is continuous and the water core is lined by both the inserted peptides and the lipid head groups. c (carpet model), high concentration peptide forming an extensive layer or carpet to the surface of the lipid bilayer. d (sinking raft model), the gravity of aggregated peptide molecules leading to curvature strain and disruption of the membrane. Hydrophilic regions of the peptide are shown in red color; hydrophobic regions are in blue color.

On the other hand, disulfide bridged antimicrobial agents pre-form their secondary and tertiary structures in free solution. Most peptides predominantly have β -sheet or β -hairpin structures and share the same characteristics with other cationic peptides in disrupting the cell membrane. The amphiphilicity of the β -hairpin and total number of positively charged residues impact significantly on their antimicrobial activity (Hunter & Fulton et al., 2002).

AMPs in certain cases can enter through the pores and killing occurs by electrolyte disturbance and sabotage of the mitochondria. Some other modes of intracellular killing hypotheses have been presented, which include: flocculation of intracellular contents by anionic peptides; alters cytoplasmic membrane septum formation; inhibits cell-wall synthesis; binds nucleic acids; inhibits nucleic-acid synthesis; inhibits protein synthesis and inhibits enzymatic activity (Brogden, 2005). Simultaneous inhibition of more than one target renders the emergence of resistance less likely because mutations are required in all targets to confer resistance to the drug.

The unique mechanism of action of these peptides relies on their interaction with the membrane bilayer, via charge–charge and hydrophobic interactions, resistance is curtailed because it would be too costly or require too many mutational events for a microbe to change the composition or organization of its lipids in order to weaken these interactions. In addition, the large variability in peptide sequence found for these peptides ensures that there is no unique recognition site for protease cleavage. Another important factor in curbing resistance is the existence of secondary targets, i.e., although cationic peptides preferentially bind to specific targets, they can also interact with other targets in the bacterial cell and affect processes such as cell wall synthesis or degradation, cell division, or macromolecular synthesis. Finally, it should be noted that multicellular organisms often attack bacteria by using more than one cationic peptide, thereby minimizing the chances of antibiotic resistance.

CHAPTER THREE

EXPERIMENTAL MATERIALS

AND METHODS

The materials and methodology used in the course of the research project are presented as follows. Included are the methods for amphiphilic molecules synthesis and characterization, fabrication and characterization of micelles (i.e. core-shell nanoparticles), drug loading, and in vitro as well as in vivo blood-brain barrier transportation.

3.1 Materials

Cholesteryl chloroformate (purchased from Sigma-Aldrich) was used as received. Heterobifunctional H_2N -PEG-COOH (PEG: Mw 5000 and 3000) was purchased from Nektar, USA. Ciprofloxacin lactate, penicillin G potassium and doxycycline monohydrate were bought from Ningjiang Pharmaceutical & Chemical Corp., P. R. China. TAT peptide (NH_2 -YGRKKRRQRRR) was ordered from GL Biochem Ltd. (Shanghai, P.R. China). Fluorescein 5-isothiocyanate (FITC), dichloromethane (DCM) and dimethylformamide (DMF) were purchased from Sigma-Aldrich, and used as received. Dimethyl sulfoxide (DMSO) and triethylamine (TEA) were purchased from Fluka. N-Hydroxysulfosuccinimide (NHSS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and BupH MES buffered saline packs were obtained from Pierce. Primary human brain microvascular endothelial cells (ACBRI 376) were purchased from Applied Cell Biology Research Institute (USA). Fetal serum albumin (FBS) was supplied from Invitrogen Corporation. Glutathione-capped CdTe quantum dots were synthesized according to the protocol reported previously. For $\text{G}_3\text{R}_6\text{TAT}$ peptide synthesis, all amino acids, Fmoc-Arg(Pbf)-Rink Amide-MBHA resin and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from LC Sciences (U.S.A.). Piperidine and trifluoroacetic acid (TFA) were bought from Merck. Diethyl ether was purchased from Sigma-Aldrich. All other chemicals were of analytical grade, and used as received.

3.2 Synthesis and characterization of cholesterol-terminated PEG (PEG-b-Chol)

The PEG-b-Chol molecules with different molecular weight of PEG were synthesized (Figure 3.1). Cholesteryl chloroformate (0.352g, 0.78 mmol) in 20 mL of DCM was slowly added to a solution of TEA (100 μ L) and NH₂-PEG5k-COOH (1 g, 0.2 mmol) dissolved in 20 mL of DCM at room temperature with vigorously stirring. The mixtures were left to react for 2 days. The crude product was purified by dialysis against DCM for 6 days using membrane with a molecular weight cut-off of 2,000 Da. DCM was then removed by vacuum drying to yield a final product. Similar process was employed to fabricate PEG3k-b-Chol.

Figure 3.1: Synthesis route of PEG-b-Chol.

The ¹H-NMR spectra of PEG and PEG-b-Chol were studied using a Bruker Avance 400 spectrometer (400 MHz), and chloroform-d (CDCl₃) was used as the solvent.

3.3 Synthesis and characterization of TAT-PEG-b-Chol

TAT peptide (Figure 3.2) was conjugated to PEG via EDC/NHSS chemistry. Briefly, EDC and NHSS were dissolved in 0.1 M MES buffer to get 100 mM and 40 mM stock solutions, respectively. TAT peptide stock solution was prepared in PBS at a concentration of 1 mg/mL. PEG-*b*-Chol (5 mg) were dissolved in 400 μ L of 0.1 M MES buffer, and incubated with 40 μ L of EDC and NHSS solutions for 1 hour at 4°C. TAT peptide solution (250 μ L) were then added to the activated PEG solution, and incubated for 3 hours at room temperature. The reaction mixture was dialyzed against de-ionized (DI) water to remove byproducts and unreacted TAT peptide molecules by using the membrane with a molecular weight cut-off of 3,500 Da. The final product was freeze-dried for 2 days prior to use.

Figure 3.2: Molecular structure of TAT peptide ~~with~~ amino acid sequence of YGRKKRRQRRR..

The ^1H -NMR spectrum of TAT-PEG-*b*-Chol was studied in comparison with TAT and PEG-*b*-Chol using a Bruker Avance 400 spectrometer (400 MHz), and D_2O was used as the solvent.

Molecular weights of PEG5k-*b*-Chol and TAT-PEG5k-*b*-Chol were measured by gel permeation chromatography (GPC) (Waters 2690, MA, USA) with a Differential

Refractometer Detector (Waters 410, MA, USA). The mobile phase used was THF with a flow rate of 1 mL/min. Weight average molecular weights were calculated from a calibration curve using a series of polystyrene standards (Polymer Laboratories Inc., MA, U.S.A., with molecular weight ranging from 1350 to 151,700). TAT-PEG3k-b-Chol synthesis was confirmed by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (Autoflex II, Bruker Daltronics).

Thermogravimetric analysis (TGA) was carried out on Pyris 1, (Perkin Elmer) to reveal the weight loss process and the thermal stability of the synthesized TAT-PEG-b-Chol molecules. Samples were heated at 10°C/min from 25°C to 700°C in air (flow rate = 30 mL/min).

A differential scanning calorimeter (DSC), model TA 2920 (TA Instruments, New Castle, DE, USA) was used under nitrogen gas with a flow rate of 50 mL/min at a heating rate of 10°C/min. The samples were sealed in an aluminum pan. A sample with an accurate weight of 2 to 3 mg was subjected to the DSC run over the temperature range of 20 to 100°C. The temperature was calibrated using pure indium with a melting point of 156.60°C. An empty pan was used as a reference. The melting points of polymers were determined from the second heating run.

3.4 G₃R₆TAT peptide synthesis and cholesterol conjugation

GGGRRRRRRRYGRKKRRQRRR (G₃R₆TAT) was synthesized according to the 9-fluorenylmethoxycarbonyl (Fmoc) approach using an Apex 396 peptide synthesizer (Aapptec, U.S.A.). The peptide was assembled on Fmoc-Arg(Pbf)-Rink Amide-MBHA resin (LC Sciences, U.S.A.) at 0.1 mmol scale using a double coupling method. Briefly, resin was reacted with 5 equivalents of amino acids, 5 equivalents of activator reagents, benzotriazol-1-yl-oxytripyrrolidinophosphoniumhexafluorophosphate (PyBOP, LC Sciences, U.S.A.) and

10 equivalents of base, N-methylmorpholine (NMM, Merk). The Fmoc group was removed by gentle agitation in 20% of piperidine (Merk) in dimethylformamide (DMF, Sigma-Aldrich). After peptide synthesis, cleavage of the peptides from the resin was carried out with a mixture of trifluoroacetic acid (TFA, Merk), triisopropylsilane (TIS, Merk) and water in a ratio of 95:2.5:2.5 for 4-6 hours. The solution was concentrated by rotary evaporation, followed by precipitation in cold diethyl ether (Sigma-Aldrich). The crude peptide was collected by filtration and dried under vacuum. The crude peptide was further purified using high performance liquid chromatography (HPLC) consisting of a Waters 2767 sample manager, Waters 996 PDA detector (Waters Corporation, U.S.A.) and a GraceVydac C₁₈ column (10x250 mm). The mobile phase was composed of 0.1% TFA and acetonitrile, and the percentage of acetonitrile was gradually increased from 5% to 40% in 20 minutes at a flow rate of 8 mL/min. The peptide was characterized by analytical reverse phase HPLC and matrix-assisted laser desorption ionization of time-of-flight (MALDI-TOF) mass spectrometry (Autoflex II, Bruker Daltronics). The purity of peptide was found to be about 95% according to HPLC analysis.

Figure 3.3: Synthesis route of CG₃R₆TAT.

CG₃R₆TAT was obtained by grafting cholesteryl chloroformate onto G₃R₆TAT via the N-terminus of G. Cholesteryl chloroformate (Sigma-Aldrich, 148 mg) dissolved in 15 mL of DMF was slowly added to 5 mL of DMF containing 70 μ L of triethylamine (Fluka) and 88 mg of G₃R₆TAT at 0°C with stirring. After 24 hours of reaction, DMF was removed from the mixture by purging dry nitrogen gas, and the mixture was then rinsed with diethyl ether for three times to remove unreacted cholesteryl chloroformate. The crude product was further purified by dialysis against DMF for six days using a membrane with a molecular weight cut-off of 1,000 Da. DMF was then removed by vacuum drying to yield a final product. The successful synthesis of CG₃R₆TAT was evidenced by MALDI-TOF and ¹H-NMR analyses.

3.5 Antibiotic screen

Bacillus subtilis (ATCC23857) and *Escherichia coli* (ATCC25922) were grown in tryptic soy broth (TSB) at 37°C. All microorganisms were cultured with 200 rpm shaking to facilitate the growth. Ten μ L of fresh overnight cultures of bacteria and yeast were inoculated into 10 ml of respective broth and cultured till reaching mid-logarithmic-phase for antimicrobial susceptibility testing. The minimum inhibition concentration (MIC) was determined using the method of broth microdilution. Briefly, 50 μ L of twofold serial dilution (25 to 500 ppm) of antibiotic solution was dispensed into wells of 96-well plates. 50 μ L of diluted organism suspension were added to each well of the microplate, giving optical density reading 0.1 to 0.2 at 600 nm. The cell cultures were then incubated for 16 hrs. Broth containing cells alone was used as control to validate the assays. The MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism. The tests were repeated on three different days.

3.6 Preparation and characterization of micelles

3.6.1 Critical micelle concentration (CMC)

CMC is an important parameter, above which an amphiphilic copolymer forms core-shell structured micelles. The CMC values of TAT-PEG-h-Chol and CG₃R₆TAT in DI water were estimated by fluorescence spectroscopy using pyrene as a probe. Fluorescence spectra were recorded by a Fluorolog fluorescence spectrometer (Jobin Yvon Horiba) at room temperature. Pyrene was first dissolved in methanol and then diluted by polymer solutions. Polymer solutions (4 mL) with different concentrations ranging from 0.05 ppm (mg/L) to 400 ppm were added to the pyrene/methanol solution. The final pyrene concentration was 6×10^{-7} M. The solutions were equilibrated for 24 hours at 35°C. The excitation spectra were recorded from 300 to 360 nm with an emission wavelength of 395 nm. Both excitation and emission bandwidths were set at 2.5 nm. The intensity ratios of I₃₃₇ to I₃₃₄ were plotted as a function of logarithm of polymer concentration. The CMC value was taken from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentrations.

3.6.2 Preparation of ciprofloxacin-loaded micelles and its encapsulation efficiency

Ciprofloxacin-loaded micelles were prepared by a membrane dialysis method under various conditions. Briefly, the polymer (5 mg) was dissolved in 3 mL of DMSO. A certain amount of ciprofloxacin (3 mg to 8 mg) was then added. The solution was dialyzed against DI water at 10°C or room temperature (22°C) for 48 hours using a dialysis membrane with a molecular weight cut-off of 2,000 (Spectra/Por 7, Spectrum Laboratories Inc.). The water

was replaced overnight. After dialysis, the solution in the dialysis bag was collected and freeze-dried for two days.

To determine the loading level of ciprofloxacin, the drug-loaded micelle solution (30 μ L) was first added in 1.77 mL of DMSO, and measured with a JASCO UV/VIS/NIR spectrophotometer using an established calibration curve. The detection wavelength was set at 286 nm, and 310 nm was used as the base line. A calibration curve was constructed in the concentration range of 1 to 25 ppm. The loading level of ciprofloxacin was calculated based on the following formula: Actual loading level (wt%) = (mass of ciprofloxacin in micelles/mass of ciprofloxacin-loaded micelles) x 100 %.

3.6.3 Release of drug in PBS solution

In vitro release of ciprofloxacin lactate was measured in PBS at 37 °C. Ciprofloxacin-loaded nanoparticles solution (20 mg in 5 mL of PBS, pH 7.4) was put in a dialysis membrane with a molecular weight cut-off of 2,000, and the external PBS buffer was shaking at 80 rpm. Samples were withdrawn at predetermined time intervals and analyzed by JASCO UV/VIS/NIR spectrophotometer using a generated standard curve. The detection wavelength was set at 270 nm, and 310 nm was used as the base line. The standard curve was constructed in the concentration range of 2.5 to 20 ppm. The sample was put back to maintain the PBS volume of 50 mL after testing. Percentage of drug released was calculated based on the following formula: Drug release (wt%) = (mass of ciprofloxacin in PBS solution/mass of ciprofloxacin in ciprofloxacin-loaded micelles) x 100 %.

3.6.4 Preparation of FITC and quantum dots-loaded micelles

FITC or CdTe quantum dots (QDs) loaded TAT-PEG-b-Chol and CG₃R₆TAT micelles were prepared by a similar dialysis method used for ciprofloxacin loading. Briefly, 0.15 mg of FITC or QDs and 5 mg of polymer were dissolved in 3 mL of DMSO, which was then placed in a dialysis bag with a molecular weight cut-off of 2000 for dialysis against DI water for two days.

3.6.5 Particle size analyses

The particle sizes of blank, drug-, FITC- and QDs-loaded micelles in DI water were measured by ZetaPALS/BI-MAS (Brookhaven Instruments Corporation) equipped with a He-Ne laser beam at 658 nm (scattering angle: 90°). The concentration of micelles in DI water was about 0.6 mg/ml. Each measurement was repeated 5 times, and an average value was obtained and used. The size measurements were performed by multimodal analysis.

3.6.6 Zeta potential test of blank, drug- and FITC- loaded micelles

Zeta potential values of blank, drug- and FITC-loaded micelles in DI water were measured using ZetaPALS/BI-MAS. The zeta potential measurement was repeated for 5 runs per sample, and an average value was reported.

3.6.7 Morphology

The morphology of the blank TAT-PEG5k-b-Chol blank micelles was analyzed by transmission electron microscope (TECHNAI G2 F20, FEI Company). 20 μ L of the micelles solution was placed on a copper grid coated with carbon film and air-dried at room

temperature. The TEM observations were carried out with an electron kinetic energy of 300 k eV.

The morphology of the blank micelles as well as ciprofloxacin-loaded micelles was observed using a field emission scanning electron microscope (JEOL JSM-7400F) operated at 5.0 kV accelerating voltage. To prepare SEM samples, 20 μL of the micelles solution were placed on a silicon wafer chip, and air-dried at room temperature. The silicon chip was then coated with Pd film and mounted on SEM stud for visualization.

3.7 Minimal inhibitory concentration (MIC) determination of CG₃R₆TAT micelles

Bacillus subtilis, *Candida albicans* and *Stachybotrys chartarum* (ATCC) were grown in tryptic soy broth at 37°C, yeast mold broth at 24°C and in tryptic soy broth at 26°C respectively. The MICs of the peptides or peptide nanoparticles were measured using a broth microdilution method. Briefly, 50 μL of peptide and peptide nanoparticle solutions with a concentration of 7.1 to 142 μM was placed dispensed into each well of 96-well plates. 50 μL of microorganism solution was added to each well to give an optical density reading of 0.1 to 0.2 at 600 nm. The cell cultures were then incubated for 15, 12 and 170 hours for *Bacillus subtilis*, *Candida albicans* and *Stachybotrys chartarum* respectively, and the MIC was taken at the concentration at which no growth was observed. Broth containing cells alone was used as control. The tests were repeated three times.

3.8 Cellular uptake study

The cell penetration tests of micelles with and without TAT were performed in human microvascular endothelial cells (ACBRI 376) and astrocytes by labeling the micelles with FITC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, ATCC, USA) supplemented with 10% FBS, 1% penicillin-streptomycin and incubated at 37°C, 5% CO₂, and seeded onto 4-well plates (Invitrogen) at a density of 10,000 cells per well. After one day of incubation, FITC-loaded micelles with or without TAT were added to the growth media. The growth media were removed after one hour, and the cells were washed with PBS for three times. The cells were then observed under a Leica DMI6000B inverted fluorescence microscope. One hour of incubation time was chosen since TAT was reported to penetrate cell membrane within 4 minutes.

3.9 In vivo animal test of BBB transportation

All procedures involving animals were approved by the DSO IACUC committee and performed according to the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications NO. 85-23, revised 1996). SD adult rats (250 g in weight) of 10 weeks old were injected with micelle solution via tail vein. Animals were sacrificed at 4 h post-injection. They were perfused with Ringer's solution followed by 4% paraformaldehyde (pH 7.4). Following the perfusion, the brains were removed and kept in a similar fixative for 2 h. They were then kept in 0.1M phosphate buffer containing 20% sucrose overnight at 4°C. Frozen coronal sections of the cerebrum of 30 µm thickness were cut and rinsed in PBS with a cryostat and mounted on slides using a fluorescence mounting medium containing DAPI for nuclear staining. The specimens were observed with a laser-scanning confocal microscope (Olympus Fluoview™1000).

3.10 Scanning electron microscopy study of micoorganisms treated with CG₃R₆TAT micelles

The microorganisms grown in broth alone or incubated with peptides or peptide nanoparticles were harvested by centrifugation at 2500g for 10 minutes. Cells were washed with phosphate-buffered saline (PBS) for three times and then fixed in PBS containing 5% formaldehyde for one day. The cells were further washed with DI water before being dehydrated using a series of ethanol washes and dried in a critical point dryer (Autosamdri-815, Tousimis Research Corporation, U.S.A.) and mounted onto aluminum stubs. The samples were coated with platinum and mounted on aluminum studs prior to SEM analyses.

3.11 Hemolysis assays

Figure 3.4: The optical absorbance spectrum of hemoglobin had two peaks in the range of 500 nm to 600 nm. The absorbance at 576 nm was chosen as all starting solutions have no absorbance at this wavelength.

Fresh rat red blood cells were washed with PBS for three times. 100 μ L of red blood cells suspended in PBS (4% in volume) was placed in each well of 96-well plates and 100 μ L of

peptide nanoparticle or amphotericin B solution was added to each well. Plates were incubated for one hour at 37°C. The cell suspensions were taken out and centrifuged at 1000g for 5 minutes. Aliquots (100 μ L) of supernatant were transferred to 96-well plates, and hemoglobin release was monitored at 576 nm using a microplate reader (Bio-Teck Instruments, Inc). Percentage of hemolysis was calculated using the following formula: Hemolysis (%) = [(O.D._{576nm} in the nanoparticle solution - O.D._{576nm} in PBS) / (O.D._{576nm} in 0.1% Triton X-100 - O.D._{576nm} in PBS)] $\times$ 100.

3.12 Peptide secondary structure analysis by circular dichroism

Circular dichroism (CD) spectra of TAT, (WA)₆H₅S₅TAT, A₁₂S₅TAT and CG3R6TAT in DI water were analyzed using a Jasco J-720 spectropolarimeter (Tokyo, Japan). The spectra in the far-UV region (185–260nm) were measured using a cylindrical quartz cuvette with 0.1 mm path length. The baseline was recorded, and subtracted from the measured spectra. The spectra shown were averages of four scans.

CHAPTER FOUR

RESULTS AND DISUCSSIONS 1:

Biologically Active Core/Shell Nanoparticles Self-Assembled from Cholesterol-Terminated PEG-TAT for Drug Delivery across the Blood-Brain Barrier

4.1 Synthesis and characterization of TAT-PEG-b-Chol molecules

Figure 4.1: ^1H NMR spectrum of PEG5k-b-Chol in CDCl_3 .

From the ^1H NMR spectrum of PEG5k-b-Chol (Figure 4.1), PEG5k-b-Chol was successfully synthesized. The multiple peaks at δ 3.5–3.7 were attributed to the protons of $-\text{CH}_2-$ groups in PEG, and the weak and multiple peaks at δ 0.68–1.02 were from cholesterol (Wang and Gao et al., 2006). Figure 4.2 shows the spectra of TAT-PEG5k-b-Chol, TAT and PEG5k-b-Chol in D_2O . The weak and broad peak at δ 4.0–4.2 (signal a) was attributed to the protons of $-\text{NH}-\text{CH}(\text{CH}_2)-\text{CO}-$ in TAT. The peaks at δ 2.52–2.58 (signal b) came from the protons of $-\text{CH}_2-\text{NH}-\text{NH}-\text{NH}_2$ in arginine. The peaks at δ 6.60–6.66 (signal c) and at δ 6.95–7.0 (d) were attributed to the protons from the benzene ring in tyrosine. From the ratio of the peak area of signal c from TAT to the peak area of signal e from PEG5k and the number of their

representative protons, the molar ratio of TAT to PEG in the polymer was estimated to be about 0.9 to 1.0.

Figure 4.2: ^1H NMR spectra of PEG5k-b-Chol, TAT-PEG5k-b-Chol and TAT in D_2O .

Figure 4.3: Gel permeation chromatography (GPC) graphs of PEG5k-b-Chol and TAT-PEG-b-Chol.

After cholesterol and TAT conjugation, the increase in weight average molecular weight from 4.96 to 6.72 kDa further proved that synthesis of TAT-PEG5k-*b*-Chol was successful.

Figure 4.4: ^1H NMR spectra of TAT-PEG3k-*b*-Chol and TAT in D_2O .

From the ^1H NMR spectra of TAT and TAT-PEG3k-*b*-Chol (Figure 4.4) in D_2O , TAT-PEG3k-*b*-Chol was successfully synthesized. The multiple peaks at δ 3.8-3.4 (signal b) were attributed to the protons of $-\text{CH}_2-$ groups in PEG, and the weak and multiple peaks at δ 1.1-0.7 (signal a) were from cholesterol. The peaks at δ 6.80-6.75 (signal c) and at δ 7.20-7.15 (d) were attributed to the protons from the benzene ring in tyrosine of TAT. From the ratio of the peak area of signal c to the peak area of b and the number of their representative protons, the molar ratio of TAT to PEG in the polymer was estimated to be about 0.9 to 1.0.

TAT-PEG3K-b-Chol: Mw=5400;

PEG: Mw=3390

Cholesteryl chloroformate: Mw=449;

TAT peptide: Mw=1561

Figure 4.5: TAT-PEG3k-b-Chol synthesis confirmed by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.

The molecular structure of TAT-PEG3k-b-Chol was characterized by MALDI-TOF mass spectrometry. The analysis was performed by the dried-droplet method with 2, 5-dihydroxybenzoic acid as matrix. All samples were run in reflectron positive ionization

model. The increase in molecular weight from around 3 to 5 kDa proved that synthesis of TAT-PEG3k-6-Chol was successful (Figure 4.5).

Figure 4.6: Weight loss of PEG5k-b-Chol, TAT and TAT-PEG5k-b-Chol as a function of temperature.

The thermal stability of TAT-PEG-*b*-Chol was evaluated using TGA. Figure 4.6 shows the TGA scan results for TAT-PEG5k-*b*-Chol compared with precursors PEG-*b*-Chol and TAT. The degradation of PEG-*b*-Chol was characterized with two phases. The first phase was between 150°C and 200°C, and the second phase from 200°C to 445°C. TAT is hygroscopic and quickly absorbs water from the atmosphere. The initial weight loss stage occurred from 25°C to 120°C, resulting from the evaporation of absorbed water. It then underwent three-phase degradation with the first one occurring between 120°C and 256°C and the second one starting from 256°C to 480°C and the third one between 480°C and 610°C. Three-phase weight loss curve was also observed for TAT-PEG-*b*-Chol. The weight loss at 250 °C may be attributed to the removal of ammonia molecules. Decomposition of the main carbon chain starts above 256°C with the evolution of carbon dioxide. This decomposition process continues up to 600°C with the removal of almost all the compound as gaseous products.

These findings further demonstrate that the presence of TAT peptide did exert an improved effect on the thermal stability of the final products. This indicates that the storage and

transportation conditions should not be stringent. TAT-PEG3k-b-Chol molecules display the same property.

Figure 4.7: Calorimetric curves of PEG-b-Chol and TAT-PEG-b-Chol.

DSC thermograms of PEG-*b*-Chol and TAT-PEG-*b*-Chol were made as shown in Figure 4.7. DSC thermograph of PEG containing molecules showed a single endothermic effect, with a peak at 47°C for TAT-PEGSk-*b*-Chol and 54°C for PEG5k-*b*-Chol, corresponding to the melting point of the polymers (Figure 4.7a). The melting points of PEG3k-*b*-Chol and TAT-PEG3k-*b*-Chol were determined to be 46 and 42°C respectively (Figure 4.7b). The decrease in melting point is attributed to the TAT peptide which disrupted the alignment of PEG molecules and jeopardized the crystallinity of PEG-*b*-Chol. The difference in melting points between PEG-*b*-Chol and TAT-PEG-*b*-Chol indicates that TAT was successfully conjugated to PEG-*b*-Chol.

4.2 Screening of antibiotics

Figure 4.8: Minimal inhibition concentration (MIC) assays of ciprofloxacin lactate, doxycycline monohydrate and penicillin G potassium to gram-positive *B. subtilis* (A) and to gram-negative *E. coli* (B).

After 1990s, the leading causes of bacterial meningitis are gram-positive *Streptococcus pneumoniae*, gram-negative *Escherichia coli* and *Neisseria meningitides*. Ciprofloxacin is the most potent generation of fluoroquinolone and has a wide spectrum of bactericidal activity through inhibition of bacterial chromosome replication. For example, an *E. coli*

O18:K1:H17 susceptibility test revealed that the minimum inhibition concentration of ciprofloxacin was 0.03 ppm, whereas ampicillin and streptomycin were 8 and 32 ppm, respectively. In our study (Figure 4.8), ciprofloxacin lactate displayed the lowest MIC to Gram-positive *B. subtilis* and Gram-negative *E. coli* (0.46 μM), compared with penicillin G potassium (933 μM) and doxycycline monohydrate (15 μM) respectively. When formulating drug nanoparticles, it should always be kept in mind that generally as high as 90% of the material to be administered will likely be nanoparticle excipients with their potential toxicity. Drug with high intrinsic pharmacological activities is preferred to avoid the administration of massive dose of nanoparticle material. Therefore, ciprofloxacin lactate has been chosen as a drug model in the following studies.

4.3 CMC of polymers, size and morphology of blank and ciprofloxacin-loaded micelles

The amphiphilic polymers consisting of hydrophilic and hydrophobic segments can self-associate to form micellar structure in aqueous solutions. The formation of micelles with PEG-b-Chol and TAT-PEG-b-Chol was examined by the detection of CMC values using a fluorescence technique, where pyrene was chosen as a fluorescent probe. The gradual shift of the third peak in the excitation spectra of pyrene from 334 nm to 339 nm was observed (data not shown), indicating the change in vibration structure of pyrene emission. The peak intensity increased markedly when the polymer concentration was above 1 mg/L for PEG-b-Chol or 10 mg/L for TAT-PEG-b-Chol. At low concentrations, the intensity ratio of I_{339} to I_{334} changed slightly. However, as polymer concentration increased, the intensity ratio increased sharply, indicating the partitioning of pyrene into the hydrophobic core of the micelles. As shown in Figure 4.9, the CMC values of PEG5k-b-Chol and TAT-PEG5k-b-

Chol, PEG3k-*b*-Chol and TAT-PEG3k-*b*-Chol in DI water were about 7.9, 12.6, 6.6 and 11.2 mg/L respectively. The low CMC value indicates that the micelles can be formed at low concentrations because of the strong hydrophobicity of cholesterol, allowing their use in very dilute medium such as body fluids. The presence of TAT and the longer PEG length led to a slight increase in CMC. This is because more cholesterol molecules were needed to interact and to form the core of micelles due to the strong interactions between TAT and water molecules, and the repulsion force between TAT molecules.

Figure 4.9: Plot of I_{339}/I_{334} ratio as a function of logarithm of polymer concentration ($\text{Log } C$) in DI water. (a) PEG5k-b-Chol; (b) TAT-PEG5k-b-Chol; (c) PEG3k-b-Chol; (d) TAT-PEG3k-b-Chol.

We dissolved PEG5k-b-chol molecules in organic solvent and dialyzed them against water to get micelles. We have optimized solvent, water changing time and dialysis temperature to get acceptable nanoparticles with 180 nm in diameter under the condition listed in Table 4.1.

The dynamic light scattering results indicate there were two populations of nanoparticles. Their diameters were around 40 and 350 nm respectively. TEM images further confirm the DLS results (Figure 4.10).

Table 4.1 TAT-PEG-b-Chol blank micelle fabrication and optimization conditions.

Particle Size	Solvent	H2O change time	Temperature
No micelle formation	DMF	10 min.	Room temperature
348.9 nm	DMF	1 hour	Room temperature
273.3 nm	DMF	1 hour	10 °C
146.5 nm	DMAC+H2O	1 hour	10 °C
362.7 nm	DMSO+TEA+NaOH	24 hours	10 °C
180 nm	DMSO	24 hours	10 °C

Figure 4.10: Blank TAT-PEG5k-b-Chol micelle size distribution and typical TEM images.

To further confirm the nature/type of cholesterol-terminated PEG-TAT molecules formed object, the particle solution was diluted below the CMC to check if the micelles were reversible. Dynamic light scattering is another well studied technique for CMC determination. In the results summarized in Figure 4.11, measurements were made of different concentrations of TAT-PEG-*b*-Chol prepared in DI water. Below the CMC, the intensity of scattered light detected from each concentration was similar to that obtained from water. In addition, the autocorrelation functions obtained showed very poor signal to noise ratios i.e. very low intercepts and no size distribution information. However, once the CMC was reached, the intensity of scattered light (in kilo counts per second) increased due to the presence of micelles. The data showed the particles were dissolved by diluting, suggesting that dynamic micelles instead of solid core nanoparticles were formed from TAT-PEG-*b*-Chol molecules.

Figure 4.11: Plot of the intensity of scattered light (in kilo counts per second) as a function of TAT-PEG5k-*b*-Chol concentration (ppm).

Under the fabrication conditions as described in Section 3.6.2, the average diameter of the blank, drug-loaded, and QDs-loaded as well as FITC-loaded micelles was 180, 190, 170 and 199 nm respectively. The blank and drug-loaded micelles were spherical in nature, and the size of the micelles increased slightly after drug and FITC loading. The detailed populations of micelles for each type of samples were indicated in Figure 4.12 by multimodal analysis. Drug-, QDs- and FITC-loaded nanoparticles had a similar size distribution, which was characterized by two populations (one population with a size ranging from 80 to 140 nm, and the other from 300 to 490 nm).

Figure 4.12: Comparison of particle size population in drug, FITC- and QDs-loaded TAT-PEG5k-b-Chol micelles.

Blank micelles

Run	Mobility	Zeta Potential [mV]	Rel. Residual
1	1.67	21.34	0.0185
2	1.72	22.04	0.0184
3	1.52	19.44	0.0188
4	1.70	21.80	0.0156
5	1.93	24.64	0.0229
Mean	1.71	21.85	0.0189

Drug-loaded micelles

Run	Mobility	Zeta Potential [mV]	Rel. Residual
1	1.14	14.61	0.0404
2	1.63	20.84	0.0557
3	1.88	24.09	0.0246
4	1.96	25.10	0.0189
5	1.98	25.40	0.0182
Mean	1.72	22.01	0.0315

FITC-loaded micelles

Run	Mobility	Zeta Potential [mV]	Rel. Residual
1	1.42	18.16	0.0279
2	1.33	17.07	0.0288
3	1.60	20.49	0.0251
4	1.66	21.26	0.0306
5	1.37	17.59	0.0197
Mean	1.48	18.91	0.0264

Figure 4.13: Surface charge comparison in blank, drug- and FITC-loaded micelles.

In order to be sure that drug loaded nanoparticles would have similar surface charge with fluorescence material-loaded nanoparticles as it may influence *in vitro* and *in vivo* distribution of the nanoparticles, zeta potentials of blank, drug- and FITC-loaded micelles were determined to be 21.9, 22.0 and 18.9 mV, respectively (Figure 4.13). These findings prove that QDs- and FITC-loaded micelles can represent drug-loaded micelles for the study of cellular uptake and *in vivo* biodistribution.

Figure 4.14: Comparison of particle size in drug and FITC -loaded TAT-PEG3k-b-Chol micelles.

Similarly, the drug-loaded and FITC-loaded TAT-PEG3k-b-Chol micelles were well correlated as indicated in Figure 4.14. They were around 170 nm in diameter and their surface charges were determined around 30 mV.

Figure 4.15 shows typical SEM images of drug-loaded TAT-PEG-b-Chol micelles. The size of the micelles showed in the SEM images was slightly smaller than the results obtained from light scattering possibly because of the shrinking of the shell of the micelles during the process of vacuum drying required for sample preparation and SEM operation.

Figure 4.15: Typical SEM images of drug-loaded TAT-PEG5k-b-Chol micelles (A & B) and drug-loaded TAT-PEG3k-b-Chol micelles (C & D). Size of the bar: 1 μ m for A, C; 100 nm for B, D.

4.4 Drug loading

Ciprofloxacin was loaded into the TAT-PEG5k-b-Chol micelles at different initial drug loadings (i.e. 3, 5 and 8 mg) and at room temperature. Increasing initial drug loading led to an increased actual drug loading level, i.e. 2.3%, 2.7% and 3.2% respectively. However, the particle size increased as a function of initial drug loading, reaching up to 297 nm at the initial loading of 8 mg. This size might be too high to yield a prolonged blood circulation of the micelles. The ciprofloxacin-loaded micelles were also fabricated at 10°C with an initial drug loading of 3 mg, and an increased drug loading level was achieved (3.7% vs. 2.7%). This may be due to the fact that the solvent removal rate was lower at 10°C so that the drug molecules had more time to assemble into the nanoparticles. In addition, the particle size of the drug-loaded micelles fabricated at 10°C was smaller (190 nm vs. 208 nm). Therefore, loading of FITC or QDs into micelles was conducted at 10°C and low initial loading (i.e. 0.15 to 0.75 mg) to yield particles of small size.

Ciprofloxacin was loaded into the TAT-PEG3k-b-Chol nanoparticles at different initial drug loadings; say 3 mg, 6 mg, and 12 mg at 10 °C. Mixed solution of polymers, drug and DMSO was dialyzed against mixture of DI water and drug stock solution. The addition of drug outside the dialysis bag is employed to decrease the diffusion gradient of ciprofloxacin lactate. With shaking speed of 60 rpm, drug loading dialysis was first allowed to last for 4 hours and then the dialysis bags was transferred to 500 ml DI water to remove free drugs. Diffusion of free drug from dialysis bag to water lasted overnight with shaking speed of 60 rpm. The loading level detected in this case was 1.7%, 2.3%, and 2.2% respectively. However, when the dialysis was allowed to last overnight and the diffusion lasted for 4 hours with shaking speed of 100 rpm, the loading level increased to 3.1%, 7.2%, and 7.3% respectively. This is because 4 hours is insufficient for the encapsulation of drug to be

completed. According to the free drug release profile, 4 hours is needed for all 6.0 mg ciprofloxacin lactate to diffuse out of the dialysis bag. The loading level almost increased two folds compared to using TAT-PEG5k-*b*-Chol (7.3% vs 3.7%). The small difference in loading efficiency between combinations using 6 mg drug (7.3%) and 12mg drug (7.2%) may be due to the increased diffusion gradient between inside and outside the dialysis bags. Thus the fabrication condition can be further modified to increase the loading level.

4.5 In vitro drug release

Figure 4.16: Release profiles of ciprofloxacin from TAT-PEG5k-*b*-Chol micelles (a) and TAT-PEG3k-*b*-Chol micelles (b) in PBS (pH 7.4) at 37°C.

As sustained release has been shown a most successful strategy to improve drug efficacy and/or reduce drug toxicity due to the alteration of its tissue distribution and pharmacokinetics, the potential of using the micelles to sustain ciprofloxacin lactate release was investigated. Figure 4.16 exhibits the *in vitro* release profiles obtained in PBS (pH 7.4) at 37°C. There was a fast release in the first 2 h, followed by a constant rate slow release for the next 4 h. An initial fast release was also observed for lidocaine molecules encapsulated into PEG-PLGA nanospheres (Peracchia & Gref et al., 1997). The rapid initial burst release could be explained by the relative high water solubility of ciprofloxacin lactate (~ 100 mg/L). The drug molecules exhibit a tendency to migrate to the aqueous medium, thereby concentrating at the surface of the particles and involving the burst effect.

Bulk flow of the CSF across the choroid plexus and into the systemic circulation has been proposed as a prominent mechanism of drug removal from the CSF, with drug being removed along with the CSF. In an adult, CSF is formed at a rate of approximately 500 mL/d, resulting in the complete turnover of CSF between three and four times per day (Luer & Hatton, 1993). This turnover rate would contribute to the overall clearance of the micelles from the CSF. Without special design to avoid mononuclear phagocyte system (MPS) uptake, blood half-lives of nano drug carriers are generally as short as 2-3 min. (Li & Pei et al., 2001). Minchin (2008) reported gold-dendrimer nanoparticles disappeared from the circulation rapidly, with a half-life of two hours or less. In contrast, the blood half-lives of PEGylated nanoparticles could vary from 5 min, 15 min, 30 min, to 270 min and 6 h even with the same PEG block molecular weight of 5000 (Oliver, 2005). Therefore, a quick yet sustained release of the drug before elimination may be one of the advantages of this study.

4.6 Transport of TAT-PEG-b-Chol into cells

As shown in Figure 4.17, the PEG5k-*b*-Chol micelles with and without TAT peptide could be taken by cells after 1 hour of incubation. However, it seemed that the uptake of the nanoparticles with TAT peptide was higher as the fluorescent intensity of the cells incubated with the micelles with TAT peptide was stronger when compared to that of the cells incubated with the micelles without TAT peptide. This is because the presence of the cell penetrating peptide on the surfaces of the micelles promoted their cellular uptake. While FITC encapsulated in PEG3k-*b*-Chol was poorly detected in Figure 4.18B, FITC encapsulated in TAT-PEG3k-*b*-Chol micelles unambiguously internalized the astrocytes as show in Figure 4.18A. Internalization follows a dose-dependent curve as observed by the intensity of the recorded signals in Figure 4.19. We attempted to study the time dependence of live cell internalization by using inverted fluorescence microscope. However, no significant fluorescence intensity difference was observed for endothelial cells incubated with TAT-PEG5k-*b*-Chol micelles for 15, 30 and 60 min. The *in vitro* kinetics of other cells internalization of TAT peptides derivatives have been well studied. The TAT peptide uptake is extremely rapid. Ziegler et al. (2005) reported the translocation of fluorescence derivative of TAT (Fg-TAT) across the cell membrane started in some fibroblasts as early as 4 s after the administration of the peptide. Vives & Brodin et al. (1997) investigated the kinetics of internalization by incubating the cells in peptide solution from 15 s to 1 h and peptide was detected in the cell after as little as 1 min of incubation. We may assume that TAT conjugated micelles enter into endothelial cells within 15 min.

Figure 4.17: Fluorescence microscopy images of viable cells incubated with the FITC-loaded PEG5k-b-Chol micelles without TATpeptide (A) and with TATpeptide (B) for 1 hour at 37°C.

Figure 4.18: Confocal fluorescence microscopic images of astrocytes incubated with FITC-loaded TAT-PEG3k-b-Chol micelles (A) and FITC-loaded PEG3k-b-Chol (B) for 1 hour at 37°C. Size of the scale bar: 100 μm for A and B.

Figure 4.19: Dose-dependent fluorescence signals of FITC-loaded TAT-PEG3k-b-Chol micelles. 10^4 cells/well were incubated with 10(a), 50(b) and 100(c) μL of micelle solution in 1 mL DMEM medium. All pictures were taken with the same camera acquisition parameters.

4.7 Transport of TAT-PEG-b-Chol micelles into rats

To determine whether the micelles crossed the blood-brain barrier, the distribution of FITC and QDs in hippocampus brain sections of rats was observed at 4 hours after *i.v.* injection of FITC, QDs, FITC- or QD-loaded TAT-PEG-b-Chol micelles. QDs or FITC were unable to cross the BBB, which only existed in blood vessels (Figures 4.20A and 4.21A, white arrows). In contrast, FITC- or QD-loaded TAT-PEG-b-Chol micelles crossed the BBB (Figure 4.20B and 4.21C), principally surrounding the nuclei of neurons (Figure 4.21C, white arrows). This finding is in agreement with the results reported in the literature (Santra & Yang et al., 2005) in which TAT conjugated QDs were detected in the rat brain after 5 minutes of QD injection. The QD-loaded micelles were not detectable in the neuron nucleus possibly because of the large size of the micelles. It is known that physiological pH values of the luminal surface of the brain endothelium presents an overall negative charge (due in part to significant sialylation), thus creates a more selective environment to positively charged substances. The TAT micelles are positively charged. This net positive charge is likely to play a key role in the adsorptive-mediated endocytosis process wherein electrostatic interaction of the peptide vector with the surface of endothelial cells may mediate surface binding and subsequent internalization of the peptide vectors into the brain capillaries. For the transcytosis of peptides through the BBB, three steps have been proposed: 1) binding and internalization at the luminal side of endothelial cell membrane; 2) diffusion through the cytoplasm of endothelial cells; and 3) externalization at the basolateral side of endothelial cells. FITC-loaded TAT-PEG3k-b-Chol micelles were also able to cross the BBB as shown in Figure 4.22. In addition, the sections of liver, kidney, and spleen tissues were analyzed for FITC distribution (Figure 4.20). FITC was localized across the tissue sections at 2 hours after *i.v.* injection. Our histological results suggest that the TAT conjugated micelles have passed into most other tissues as observed by Schwarze et al. (1999).

Figure 4.20: Transport of FITC and FITC-loaded *TAT-PEG5k-b-Chol* micelles across the blood-brain **barrier**. Hippocampus brain sections of rats at 2 hours after *i.v.* injection. Noted that in the control group (A), no cells were labeled with FITC, whereas in the group treated with FITC-loaded micelles (B), some neural cells were labeled with the micelles (white arrows). In both groups, many cells in liver, kidney and spleen were labeled with FITC.

Figure 4.21: *Transport of QDs (A) and QD-loaded TAT-PEG5k-b-Chol micelles (B) across the blood-brain barrier. (C) A higher magnification of the boxed area in (B).*

Hippocampus brain sections of rats at 4 hours after i.v. injection. DAPI-stained nuclei in blue; Red dots-QDs. Noted that in (A), QDs were confined to the blood vessels (arrows in A), whereas in (B & C), QDs were localized in the neuronal soma in the perikaryal region (arrows in C) and in neuropil.

Figure 4.22: Transport of FITC-loaded PEG3k-b-Chol micelles (A) and FITC-loaded TAT-PEG3k-b-Chol (B) micelles across the blood-brain barrier.

4.8 TAT anchored nanoparticle uptake mechanism

The mechanism of in vivo TAT modified nanoparticle BBB transportation remain poorly understood. A recent mechanism proposed for TAT-conjugated liposome and micelle uptake is macropinocytosis. Macropinocytosis is a non-specific form of cellular uptake, brought about by large vesicles, known as micropinosomes, which are generated from actin filaments. The high density of basic amino acid residues (Arg and Lys) in TAT contribute to a tight ionic interaction between the peptide chains and the negative charges of the phospholipid heads of vesicles could induce a local invagination of the plasma membrane. The local reorganization of the phospholipid bilayer would then lead to TAT-conjugated nanoparticles to the cytoplasmic release. So, it looks like the adsorptive-mediated uptake (Wadia & Stan et al., 2004). The schematic pattern of such mechanism is shown in Figure 4.23.

Figure 4.23: Adsorptive macropinocytosis route across the blood-brain barrier.

4.9 Conclusion

Polymer micelles, the surface of which is anchored with TAT molecules, have been successfully synthesized for drug delivery across the BBB. Ciprofloxacin as a model antibiotic was loaded into the micelles. The size of the drug-loaded micelles was less than 200 nm. The presence of TAT on the surfaces of the micelles promoted their uptake by human microvascular endothelial cells. The TAT-PEG-b-Chol micelles crossed the BBB, and entered the cytoplasm of neurons. The successful transport of TAT-PEG-b-Chol micelles into the brain opens new opportunities for the treatment of infectious diseases.

However, ciprofloxacin was incorporated into the hydrophobic core of micelles by weak hydrophobic interaction and a first initial burst release occurred. A number of other antibiotics, such as doxycycline monohydrate and penicillin G potassium which has higher water solubility of 630 mg/L and 210 mg/L, respectively, are susceptible to be easily and rapidly released too. Moreover, because intravenous injections of micelle solution are extremely dilute by blood, polymer micelles are easily disassembled which result in the leakage of loaded drugs in vivo. A better strategy should be employed to overcome the drug burst release and drug leakage limitation of TAT-PEG-b-Chol micelles.

CHAPTER FIVE

RESULTS AND DISUCSSIONS 2:

Cationic Core/Shell Nanoparticles self-assembled from Amphiphilic Cell-penetrating Peptide as an Antimicrobial Agent Capable of Crossing the BBB

5.1 Introduction

In order to reduce the drug rapid release *in vitro* and drug leakage during *in vivo* transport process, a number of approaches are used including the drug conjugation with polymer chains (Kataoka & Harada et al., 2001) and the cross-link of the shell of micelles (Hu & Ren et al., 2006). However, these strategies are complicate and would change the pharmacological activities of drugs. Inspired by naturally occurring positive antibiotic peptides, we are engaged in fabricating a potent antimicrobial micelle system, which can exclude the necessity of clinical antibiotic encapsulation.

TAT (YGRKKRRQRRR) peptide is the protein transduction domain from the transcriptional activator TAT protein of the human immunodeficiency virus type-1 (HIV-1). After conjugation with TAT, proteins with molecular weight ranging from 36 to 119 kDa and quantum dots were able to cross the BBB. Meanwhile, TAT peptide with electric charge +8 mV and can easily penetrate cell membrane, may be an effective antimicrobial agent. The fungicidal effect of TAT against *Candida albicans* was studied by Jung & Park et al. (2006). They used MTT assay to get MIC value of 40 ppm. In our earlier study discussed in Chapter 4, TAT peptide was chosen as a biologically active ligand to facilitate brain drug delivery only. In this study, we design an amphiphilic peptide (CG₃R₆TAT), which contains a hydrophilic block of cell-penetrating peptide TAT and six arginine residues (R₆), and a hydrophobic block of cholesterol (C) (Fig. 5.1). A spacer of three glycine moieties (G₃) is built in between to separate the two blocks. This peptide can easily form core/shell structured nanoparticles (i.e. micelles) having a hydrophobic cholesterol core and a hydrophilic cationic peptide shell with TAT molecules arranged towards the surrounding environment. Brain inflammatory diseases may be induced by bacteria such as *Bacillus anthracis* and *Bacillus subtilis* or fungi or virus. HIV-infected patients can easily contract fungi due to their

damaged immune systems, and *Candida albicans* is the most frequently found fungus in meningitis. Satratoxin G from *Stachybotrys chartarum* was also reported to cause brain inflammation (Islam & Harkema et al., 2006). The formation of nanoparticles is expected to increase local density of positive charge, enhancing antimicrobial activities of the cationic peptide against a broad-spectrum of pathogenic microorganisms. In addition, the presence of TAT molecules on the surfaces renders these nanoparticles capable of crossing the BBB for the treatment of brain infection.

Figure 5.1: Rational peptide design (a) with cholesterol, glycine (G), arginine (R) and TAT. The amphiphilic molecules self-assembled into peptidic micelles as simulated with software (b).

5.2 Synthesis and characterization of CG₃R₆TAT molecules

G₃R₆TAT was synthesized by a solid-phase method. CG₃R₆TAT was obtained by grafting cholesteryl chloroformate onto the N-terminus of G₃R₆TAT.

Figure 5.2: MALDI-TOF mass spectra of G₃R₆TAT and CG₃R₆TAT. The spectrum of G₃R₆TAT shows its theoretical molecular weight at 2667 Da, indicating the successful synthesis of the peptide. The theoretical molecular weight of CG₃R₆TAT was 3080 Da, which appears in the spectrum of CG₃R₆TAT, indicating successful conjugation of cholesterol.

Figure 5.3: ^1H -NMR spectra of $\text{CG}_3\text{R}_6\text{TAT}$ and $\text{G}_3\text{R}_6\text{TAT}$ in d -DMSO. The weak and multiple peaks at δ 0.6-1.1 (signal a) were from cholesterol. The multiple peaks at δ 6.6-8.5 (signal b) were attributed to the protons from the benzene ring of tyrosine in the peptide. These findings further prove successful conjugation of cholesterol onto the peptide.

In the ^1H -NMR spectra, $\text{CG}_3\text{R}_6\text{TAT}$ molecules presented characteristic peaks from both cholesteryl and $\text{G}_3\text{R}_6\text{TAT}$. The methyl groups (CH_3 -18 and CH_3 -19) of cholesteryl appeared at 0.65 ppm and 0.89 ppm, respectively, as singlets in $\text{CG}_3\text{R}_6\text{TAT}$ spectrum. The methyls at the 26 and 27 positions appeared as doublets at 0.86 and 0.87 ppm, with 6.6-6.8 Hz coupling constants. The 21 methyl was a doublet at 0.90 ppm with a 6.5-6.7 Hz coupling constant. Additionally, characteristic peaks from $\text{G}_3\text{R}_6\text{TAT}$ peptide appeared from 4 to 8 ppm. The weak and broad peak at δ 4.0-4.2 was attributed to the protons of $-\text{NH}-\text{CH}(\text{CH}_2)-\text{CO}-$ in TAT. The peaks at δ 6.60-6.66 and at δ 6.95-7.0 were attributed to the protons from the benzene ring in tyrosine.

5.3 CMC of CG₃R₆TAT, size and morphology of CG₃R₆TAT micelles

CMC is an important parameter, above which an amphiphilic macromolecule forms core/shell structured nanoparticles (i.e. micelles). The CMC value of CG₃R₆TAT in DI water was estimated by fluorescence spectroscopy using pyrene as a probe. Fluorescence spectra were recorded by a Flourollog fluorescence spectrometer (Jobin Yvon Horiba) at room temperature. Aliquots of pyrene solutions in acetone were added to 15 mL volumetric flasks, and the acetone was allowed to evaporate. Peptide solutions (4mL) with different concentrations ranging from 0.1 to 200.0 ppm were then added to the flasks. The final pyrene concentration is 6.16×10^{-7} M. The solutions were equilibrated for 24 hours at 35°C. The excitation spectra were recorded from 300 to 360 nm with an emission wavelength of 395 nm. Both excitation and emission bandwidths were set at 2.5 nm. The intensity ratios of I_{339} to I_{334} were plotted as a function of logarithm of peptide concentration. The CMC value was taken from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentrations. This peptide had a critical micelle concentration (CMC) of 31.6 mg/L (i.e. 10.1 μ M) in de-ionized (DI) water (Figure 5.4).

Figure 5.4: Plot of I_{339}/I_{334} ratio as a function of logarithm of peptide concentration ($\text{Log } C$, mg/L) in DI water.

At concentrations above the CMC, it can easily self-assemble to form core/shell nanoparticles. Briefly, 10 mg of CG3R6TAT was dissolved in 3 mL of dimethyl sulfoxide (DMSO), and dialyzed against 500 mL of DI water at room temperature (22°C) for 24 hours using a dialysis membrane with a molecular weight cut-off (MWCO) of 1,000 (Spectra/Por 7, Spectrum Laboratories Inc.). The external water phase was replaced every 6 hours. The resulting nanoparticles were characterized using a zeta potential analyzer with dynamic light scattering capability (ZetaPlus, Brookhaven, U.S.A.). Their effective diameter and zeta potential were 300 nm with polydispersity index of 0.25 and 92 ± 2 mV respectively (Figure 5.5). Smoluchowsky factor ($f(ka) = 1.5$) is selected for the calculation method, the mean and combined values for mobility and zeta potential agree to about the standard error of the measurement, therefore the results are reliable. The size distribution is multimodal with the prominent mode appear at approximately 180 nm diameter and a minor population at approximately 600 nm, indicating the presence of secondary aggregation. TED analysis confirmed the bimodal distribution (Figure 5.6 a). In addition, the major mode located at approximately 180 nm was also confirmed by the higher resolution of SEM Figure 5.6b. The nanoparticles were spherical in nature and had a size less than 150 nm after self-drying under air.

CG₃R₆TAT micelle

Effective Diameter: 300.1 nm
Polydispersity: 0.250
Avg. Count Rate: 1.4 Mcps
Baseline Index: 4.8

Run	Mobility	Zeta Potential(mV)	Rel. Residual
1	7.80	99.80	0.0237
2	7.45	95.35	0.0311
3	7.01	89.72	0.0272
4	7.31	93.52	0.0305
5	6.44	82.43	0.0369
6	6.88	88.00	0.0481
7	7.14	91.40	0.0464
8	7.70	98.52	0.0433
9	7.24	92.65	0.0172
10	7.23	92.57	0.0270
Mean	7.22	92.40	0.0331
Std. Error	0.12	1.60	0.0032
Combined	7.22	92.37	0.0269

Figure 5.5: Size distribution and zeta potential of CG₃R₆TAT micelles characterized by ZetaPALS/BI-MAS.

Figure 5.6: TED image ofpeptidic micelles (a) clearly shows bimodal distribution of the nanoparticles. Scanning electron micrographs (b and c) indicate the majority of micelles fell in the range about 180 nm. Scale bar: 1 µm for b; 100 nm for c.

5.4 Antimicrobial activities related with cationicity and amphiphilicity

In clinical practice, patients with brain infection are empirically treated with antibiotics of broad-spectrum antimicrobial activities prior to identifying specific pathogens as any delay in treatment may cause mortality. Therefore, peptides as potential antimicrobial agents for combating brain infections must be able to kill various microbes such as bacterium, fungus and yeast. We evaluated minimal inhibitory concentrations (MICs) of peptides and cationic peptide nanoparticles against *Escherichia coli* (gram-negative), *Bacillus subtilis* (gram-positive bacterium), *Candida albicans* (yeast) and *Stachybotrys chartarum* (fungus). The incubation time of each microorganism was chosen based on its growth curve (Figure 5.7), i.e. incubation was terminated once bacteria and yeast growth reached the stationary phase; a popular yet complex method for detecting the pure *Stachybotrys chartarum* in culture includes isolating DNA from a sample and further subject to polymerase chain reaction amplification (PCR) (United States Patent 20040157260; Cruz-Perez & Buttner et al., 2001). We simplified the quantification by centrifuging the mold at 1500 rpm for 15 minutes to exclude the hyphae precipitation then resuspending the spores into 96-well plate to monitor the growth at 600 nm. The incubation was terminated at log growth phase in order to avoid the effect of bulky hyphae and broth evaporation.

The nanoparticles possessed a strong ability against gram-positive bacteria, fungi and yeast, and their MIC was 10.7, 10.8 and 11.0 μM respectively (Figure 5.8), which was close to the CMC of peptide. Below the MIC, the peptide was unable to suppress the growth of microorganisms. However, it was efficiently inhibited at the MIC due to the formation of nanoparticles and the increased local density of positive charge.

Figure 5.7: Growth of *Bacillus subtilis* (A), *Candida albicans* (B) and *Stachybotrys chartarum* (C) as a function of incubation time. Incubation of *Stachybotrys chartarum* was ended at the logarithmic phase to avoid inaccurate O.D. readings caused by broth evaporation and formation of bulky hyphae.

Figure 5.8: Dose-dependent growth inhibition of *Bacillus subtilis* (A, MIC: 10.7 μM), *Candida albicans* (B, MIC: 10.8 μM) and *Stachybotrys chartarum* (C, MIC: 11.0 μM) in the presence of peptide nanoparticles and conventional antifungal agents. The incubation time of each microorganism was chosen based on its growth curve, i.e. incubation was terminated once cell growth reached the stationary phase.

G₃TAT had a low antimicrobial activity and its MIC against *Bacillus subtilis* and *Candida albicans* was 290.0 and 289.0 μ M respectively (Figure 5.9).

Figure 5.9: Dose-dependent growth inhibition of *Bacillus subtilis* (A, MIC: 290.0 μ M) and *Candida albicans* (B, MIC: 289.0 μ M) in the presence of G₃TAT.

Adding six arginine residues to TAT (i.e. G₃R₆TAT) significantly reduced MIC (290.0 and 289.0 vs. 75.0 μ M for *Bacillus subtilis* and *Candida albicans* respectively) (Figure 5.10).

Figure 5.10: Dose-dependent growth inhibition of *Bacillus subtilis* (A, MIC: 75.0 μ M) and *Candida albicans* (B, MIC: 75.0 μ M) in the presence of G₃R₆TAT.

The presence of TAT did not merely provide positive charges since the MIC of G₃R₁₂ against *Bacillus subtilis* and *Candida albicans* was much higher than that of G₃R₆TAT (242.0 vs. 75.0 μM) (Figure 5.11).

Figure 5.11: Dose-dependent growth inhibition of *Bacillus subtilis* (A, MIC: 242.0 μM) and *Candida albicans* (B, MIC: 242.0 μM) in the presence of G₃R₁₂.

Cell-penetrating property of TAT must play a role in inhibiting the growth of microbes (Jung & Park et al., 2006). Therefore, the introduction of TAT to G₃R₆ strongly enhanced its antimicrobial activity (MIC: 75.0 vs. > 444.4 μM) (Figure 5.12).

Figure 5.12: Dose-dependent growth inhibition of *Bacillus subtilis* (A, MIC: > 444.4 μM) and *Candida albicans* (B, MIC: > 444.4 μM) in the presence of G₃R₆.

However, the MIC of G₃R₆TAT was still much higher than that of the nanoparticles self-assembled from the amphiphilic peptide CG₃R₆TAT (10.7110.8 vs. 75.0 μ M). Clearly, the formation of core/shell nanoparticles enhanced the antimicrobial ability of the peptide, resulting in lower MICs. In addition, the nanoparticles were much more powerful in inhibiting proliferation of *Stachybotrys chartarum* than the conventional antifungal agents, fluconazole and amphotericin B (MIC: 11.0 μ M vs. > 817.0 and > 54.0 μ M respectively) (Figure 5.8C). The number of life-threatening fungal infections has increased sharply in recent years because of the growing number of immuno-compromised individuals and the emergence of increasing numbers of isolates resistant to the few effective antifungal agents available. Azole resistance in systemic isolates of *Candida albicans*, although rare, has been reported and may be increasing (Hoban & Zhanel et al., 1999). Studies on antifungal drugs have generally lagged behind those on antibiotics, mainly because fungal and mammalian cells are both eukaryotes, making differential selectivity in targets more difficult. A few native positively charged antimicrobial peptides, such as dermaseptins, pardaxin analogues, and the human-like cecropin, LL-37 (Avrahami and Shai, 2002) are both antibacterial and antifungal. They bind and permeate efficiently both zwitter-ionic and negatively charged phospholipid vesicles. Studies of their modes of action indicate that they self-associate in solution and in membranes. Self-association is driven either by a hydrophobic N- or C-terminus or by specific amino acids in the peptide sequence, resulting in the formation of α -helical bundles that could initiate strong hydrophobic binding to zwitterionic membranes.

Moreover, the nanoparticles were also superior to the conventional antibiotics, penicillin G and doxycycline in killing *Bacillus subtilis* (MIC: 11.0 vs. 1074 and 13.5 μ M respectively) (Figure 5.13).

Figure 5.13: Dose-dependent growth inhibition of *Bacillus subtilis* in the presence of penicillin G (A, MIC: 1074 μM) and doxycycline (B, MIC: 13.5 μM) (16 hours of incubation).

Next, we investigated morphological changes of *Bacillus subtilis* and *Candida albicans* before and after incubation with the nanoparticles at lethal doses for various periods of time. Untreated *Bacillus subtilis* exhibited smooth surface (Figure 5.14A1 and A2). In sharp contrast, the cell surface became extremely rough after incubation with the nanoparticles of 13.0 μM . A large number of minicells were formed, and cell debris was observed at 90 minutes (Figure 5.14A3 and A4). Treatment with the nanoparticles of 26.0 μM for 90 minutes led to more cell debris (Figure 5.14A5). The formation of minicells was also observed in *Bacillus subtilis* treated with the cationic peptide antibiotic, nisin (Hyde & Parisot et al., 2006). The nanoparticles may act with a similar mechanism as nisin does. The uptake of nanoparticles into cell wall *via* non-specific electrostatic interaction accelerated cell division, leading to the formation of minicells (Hyde & Parisot et al., 2006). The steric hindrance that the nanoparticles induced in the cell wall and hydrogen binding/electrostatic interaction between the cationic peptide and peptidoglycans of cell wall, which are made from polymers of alternating N-acetylglucosamine and N-acetylmuramic acid in β linkage, cross-linked by short peptide stems, might inhibit cell wall synthesis (Cho & Wang et al., 2007) resulting in osmotic lysis of cells. As shown in Figure 5.14B1 to B5, *Candida albicans* underwent different morphological changes. Numerous pores with size less than 50 nm were formed on the cell surfaces after treatment of nanoparticles at a concentration of 13.0 μM for 30 minutes (Figure 5.14B1 to 5.14B3). Cell wall was efficiently disrupted and protoplasts were exposed after 100 minutes because of the inhibition of cell wall synthesis (Figure 5.14B4). Thus, at 200 minutes, the majority of protoplasts broke into debris due to osmotic lysis (Figure 5.14B5). In addition to the osmotic lysis mechanism caused by the inhibition of cell-wall synthesis, the nanoparticles might permeate through the cytoplasmic membrane of both organisms due to the presence of TAT, destabilizing the membrane based on the electroporation and/or sinking raft model (Chan & Prenner et al., 2006).

Figure 5.14: Scanning electron micrographs of *Bacillus subtilis* (A) and *Candida albicans* (B) before (A1, A2, B1, B2) and after treatment of nanoparticles of 13.0 µM for 30 (B3), 90 (A3, A4), 100 (B4) and 200 minutes (B5), and 26.0 µM for 90 minutes (A5). Scale bar: 1 µm for A1, A5, B1 and B5; 100 nm for A2-A4 and B2-B4.

Figure 5.15: *Escherichia coli* growth in the presence of CG_3R_6TAT micelles.

CG_3R_6TAT micelles kill gram-positive *B. subtilis*, yeast *C. albicans* and filamentous fungi at the same concentration, yet not showing any efficacy against *E. coli* (Figure 5.15). This has been attributed principally to polyamines as constituents of the outer membranes of *E. coli* (Koski and Vaara, 1991). Cationic polyamines form a stable "roof of tiles" on the surface of the OM (Vaara, 1993) which make the gram-negative bacteria are usually much more resistant than gram-positive bacteria to a wide range of antibiotics and chemotherapeutic agents due to the reduced electrostatic interaction. Examples of agents to which most gram-negatives show such 'intrinsic' resistance include lipophilic penicillins (for example oxacillin and nafcillin), erythromycin, novobiocin, fusidic acid, some rifamycins, and the recently developed synthetic agents, oxazolidinones. In fact, a survey of the recently reported antibiotics of natural origin showed that more than 90% were active against gram-positives but lacked significant activity against *E. coli*, a representative of the gram-negative group (Nikaido, 1998).

Figure 5.16: Proposed mechanism of action of peptidic micelles based on SEM observation and *different effect* on microorganism with or without cell wall. (a) Pre-associated micelles attracted to the negative *peptidoglycan* of gram-positive bacteria or fungi; (b) Micelles settle on the bi-layer membrane and block the cell-wall synthesis; Compared with the general consensus of single *a*-helical *peptide* action in the literature (d), the heavier micelles allow deeper insertion (c) and much pronounced activity.

It has been postulated that a stabilized **amphipathic** *a*-helical conformation is an absolute requirement for **cidal** activity against Gram-positive bacteria whereas the structural requirements for activity against Gram-negative bacteria are less stringent (Giangaspero & Sandri et al., 2001; Dathe & Wieprecht et al., 1997). Above data obtained in our laboratories leads us to propose a novel mechanism (Figure 5.16). Provided that cationicity and **amphiphilicity** are maintained, **peptides** without helicity are still active. The pre-associated

cationic micelles allow attachment to cell wall first then follow a deeper insertion into the core of the lipid bilayer thereby affecting pore size and stability. Although CG₃R₆TAT micelles clearly damages target cell wall and cell membrane, given the well known micelle dynamic property and the existence of nuclear binding domain on TAT peptide, other mechanism of action such as secondary intracellular targeting after cell permeabilization and micelle dissociation, cannot be excluded.

5.5 Selectivity test

We further studied hemolysis of rat red blood cells after incubation with the nanoparticles and Amphotericin B. The nanoparticles showed low hemolytic activity at low concentrations (Figure 5.17). For example, at 16.0 μ M (i.e. 50 mg/L), a concentration higher than the MIC, less than 19% hemolysis was observed with the nanoparticles, while amphotericin B mediated more than 90% hemolysis even at concentrations lower than its MIC. Selectivity for bacterial cells compared to host cells appears to result from a variety of factors. Mammalian cell membranes, which are positively charged and rich in cholesterol, do not interact with AMPs (Zasloff, 2002). Differences in electrochemical gradients across bacterial and mammalian cell membranes also increase the selectivity.

Figure 5.17: Dose-dependent of hemolytic activity of nanoparticles in comparison with Amphotericin B. Corresponding concentration of peptide and Amphotericin B in μM : 16, 32, 80, 160 and 54, 108, 270, 540 respectively.

5.6 Transport of CG₃R₆TAT micelles into brain

To determine whether the nanoparticles were able to cross the BBB, the distribution of FITC in hippocampus brain sections of rats was observed at 2 hours after *i.v.* injection of FITC or FITC-loaded nanoparticles. FITC was first incorporated into CG3R6TAT nanoparticles. Briefly, 0.35 mg of FITC and 2.3 mg of CG3R6TAT were dissolved in 1 mL of DMSO, which was dialyzed against 500 mL of DI water for three days at 10°C using a dialysis bag with MWCO of 1,000 Da. The external water phase was replaced six times. FITC content was 5.3% in weight and the effective diameter of FITC-loaded nanoparticles was 356 nm. Naked FITC was unable to cross the BBB (Figure 5.18A). In contrast, FITC-loaded nanoparticles crossed the BBB, principally surrounding the nuclei of neurons (Figure 5.18B, white arrows).

Figure 5.18: Transport of FITC-loaded nanoparticles across the blood-brain barrier. Hippocampus brain sections of rats at 2 hours after i.v. injection. A. FITC; B. FITC-loaded nanoparticles. Scale bar: 50 and 100 μm for A and B, respectively.

5.7 Conclusion

In conclusion, we have demonstrated that the amphiphilic peptide $\text{CG}_3\text{R}_6\text{TAT}$ is able to self-assemble into cationic core/shell nanoparticles. These nanoparticles possess a broad spectrum of antimicrobial activities, which efficiently inhibit growth of bacteria (gram-positive), fungi and yeast with low MIC yet induce relatively low hemolysis. They act as an antibiotic and a carrier, excluding the necessity of other carriers. Therefore this new system addressed the low drug loading capacity of the first system. In addition, this is a macromolecular antimicrobial agent, which may overcome the multi-drug resistant problem of ciprofloxacin lactate. Furthermore, they are able to cross the BBB, providing a great potential in treating brain infections.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

6.1 Conclusion

The major problem in brain infection treatment is the presence of the blood-brain barrier separating the blood from the cerebral parenchyma and the drug resistance presented by multiple pathogens. Active targeting is a non-invasive approach, which consists in transporting antibiotics loaded nanocarriers to brain using site-specific ligands. In this study, TAT₄₇₋₅₇ peptide (YGRKKRRQRRR) was firstly conjugated with polyethylene glycol polymer terminated with cholesteryl to realize CNS penetration since it is known that the peptide can cross the BBB by adsorption-mediated macropinocytosis due to their positive charge. It was found that the ciprofloxacin lactate was well entrapped into the core of the micelles. The drug loading level could reach up to 7.3 % by optimizing the fabrication conditions. Fluorescent studies showed that these micelles were able to enter the primary human microvascular endothelial cells and astrocytes, and that these micelles had the ability to reach the cytoplasm of neurons. It was shown that polymeric nanoparticles derivatized with the TAT peptide were a useful starting material for the transportation of ciprofloxacin into the brain tissue.

TAT does not only cross the BBB but also possesses microbicidal function like other common antimicrobial peptides (AMPs). In this study, we have constructed an amphiphilic peptide having cholesterol as the hydrophobic block, six arginines and TAT as the hydrophilic block, and three glycines as the spacer in between (CG₃R₆TAT). The peptide molecules were self-assembled into cationic core/shell structured nanoparticles, which were capable of crossing the BBB in a rat model. In addition, these cationic peptide nanoparticles were highly active against *Bacillus subtilis*, *Candida albicans*, and *Stachybotrys chartarum*. Under SEM, the morphological changes of the microorganisms were observed after incubation with the nanoparticles at different time points. It was observed that numerous

pores were formed in the cell wall, leading to cell lysis. These findings suggest that far beyond secondarily conformational α -helix, β -sheet, extended or loop membrane lytic AMPs, the broad and potent antimicrobial activities of the micelles were initiated by electrostatic attraction, followed by inhibition of cell wall synthesis and ended with the burst of osmotically fragile cells. The intracellular binding to nucleoid and subsequent interfering of DNA synthesis may be involved in the process. Therefore, the relatively nonspecific and involvements of a package of mechanisms may render peptide micelles capable of overcoming multidrug resistance.

In conclusion, TAT peptide conjugation on the micelles might well serve as models with which to gain better brain infection treatment. In addition, given the emergence of widespread antibiotic resistance, a better understanding of the conformation-independency of their bactericidal mechanisms could facilitate development of new peptide antibiotics. Although the killing mechanism of micelles needs to be investigated in details, the pre-association of antimicrobial peptides is an attractive strategy to enhance antimicrobial activities. These micelles may be a promising agent for treating infections caused by Gram-positive pathogens, yeast and fungi.

6.2 Future Work

6.2.1 Improvement in specific brain targeting

In the first part of TAT-PEG-b-Chol micelle antibiotic delivery study, our results suggest that ciprofloxacin could be delivered to the central nervous system. However, some aspects of this delivery system should be refined to improve the delivery efficacy. For example, biodistribution study demonstrated that TAT₄₇₋₅₇ derived micelles were accumulated in other

tissue sections, such as spleen, liver, and kidney. Therefore, brain tissue targeting should be optimized by employing other brain-specific peptides. Rabies virus glycoprotein (RVG) interacts specifically with the nicotinic acetylcholine receptor (AChR) on neuronal cells to enable viral entry into neuronal cells. Kumar and Wu et al. (2007) used a 29-amino-acid peptide from RVG to transducer siRNA amazingly specific to neuronal cells in vivo. They reasoned that this peptide might bind specifically to neuronal cells expressing the AChR. Following the 9-fluorenylmethoxycarbonyl (Fmoc) approach and using an Apex 396 peptide synthesizer, we are going to synthesize and purify (by HPLC) the 29 residue peptide (YTIWMPENPRPGTTPCDIFTNSRGKRASNG). RVG peptide will then be conjugated to PEG-b-Chol to fabricate amphiphilic RVG-PEG-b-Chol molecules. Thus, micelles with RVG peptide moieties may provide an ideal nanocarrier to enhance brain delivery.

The development of smart pH-sensitive nanocarriers capable of responding to decreased pH values in inflamed tissue (Sawant & Hurley et al., 2006; Kale & Torchilin, 2007) may increase brain specific delivery also. Ideally, TAT peptides on such constructs are shielded by long protecting PEG chains under normal pH values but lost their protective PEG shell under the action of local pathological conditions (low pH) and expose TAT for the subsequent delivery of the antibiotics. As shown in Figure 6.1, hydrophobic block-PEG5k may be made degradable by inserting the pH-sensitive hydrazone bond. TAT peptide will be coupling to PEG3k through covalent bond.

Figure 6.1: Proposed smart pH-sensitive antibiotic *delivery nanocarrier*.

6.2.2 Animal model establishment in testing brain infection treatment

Although there are several references support that the universal agreement that TAT-derived **nanoparticles** can realize **intracellular** localization in 4 seconds and can penetrate BBB within 5 minutes in **vivo**, the result of 70% drugs released **within** 2 hours may be a concern for their **clinical** application. To address this problem, we developed the second cholesteryl hybrid **peptidic** delivery system, which is able to cross the blood-brain barrier and **has** the potential to sterilize CNS pathogens. They act **as** an antibiotic and a carrier excluding the necessity of other drug carriers. Animal models of experimental **bacterial/yeast** meningitis need to be developed for the evaluation of the **pharmacokinetics** and antimicrobial effects of peptidic **micelles**. Pneumococcal meningitis is characterized by extensive meningeal **inflammation**. At **initial** stage, experimental meningitis will be established by affecting rats with *Streptococcus pneumoniae* serotype 3 (Brandt & Frimodt-Møller et al., 2006). In brief, young adult male Wistar rats (180–200 g) will be **anaesthetized** with Hypnorm®/Dormicum® and infected by **intracisternal** injection of 30 mL of the

bacterial suspension. The therapeutic index of CG₃R₆TAT system will be evaluated by using the animal model. The toxicology of CG₃R₆TAT will be studied in details.

Moreover, in order to improve specific brain binding of CG₃R₆TAT molecules, hybrid micelles may be built in mixing CRVG (cholesteryl conjugated RVG peptide) and CG₃R₆TAT.

6.2.3 Polymeric antibiotic synthesis and evaluation

In addition, there are other hurdles to overcome, including peptide liability to proteases, toxicity in certain level and the substantial expense of peptide synthesis. Further optimization of peptide composition or non-peptide backbone replacement may provide a potentially effective therapeutics. For example, quarternary ammonium salts and biguanides, both of which are positively charged at physiological pH, have been used widely as effective antibacterial agents. Design, synthesis, characterization and evaluation of novel polymeric biocides with brain targeting moieties will be carried out in the future.

Aigner, A., Wolf, S., & Gassen, H.G. (1997). Transport and detoxication: principles, approaches, and perspectives for research on the blood-brain barrier. *Angewandte Chemie International Edition*, 36, 24-41.

Aktas, Y., Yemisci, M., Andrieux, K., Gursoy, R.N., Alonso, M.J., Fernandez-Megia, E., Novoa-Carballal, R., Quinoa, E., Riguera, R., Sargon, M.F., Celik, H.H., Demir, A.S., Hincal, A.A., Dalkara, T., Capan, Y., & Couvreur, P. (2005). Development and brain delivery of chitosan-PEG nanoparticles functionalized with the monoclonal antibody OX26. *Bioconjugate Chemistry*, 16, 1503-1511.

Arda, B., Yamazhan, T., Sipahi, O.R., Islekel, S., Buke, C., & Ulusoy, S. (2005). Meningitis due to methicillin-resistant *Staphylococcus aureus* (MRSA): Review of 10 cases. *International Journal of Antimicrobial Agents*, 25, 414-418.

Available: <http://en.wikipedia.org/wiki/Antibiotic>

Azmin, M.N., Florence, A.T., Handjani-Vila, R.M., Stuart, J.F.B., Vanlerberghe, G., & Whittaker, J.S. (1985). The effect of non-ionic surfactant vesicle (niosome) entrapment on the absorption and distribution of methotrexate in mice. *Journal of Pharmacy and Pharmacology*, 37, 237-242.

Banks, W.A., Robinson, S.M., & Nath, A. (2005). Permeability of the blood-brain barrier to HIV-1 Tat. *Experimental Neurology*, 193, 218-227.

Bartfield, A.A. (2000). Bacterial meningitis. *Primary Care Update for OB/GYNS*, 7, 49-54.

Bartus, R.T., Elliott, P., Hayward, N., Dean, R., McEwen, E.L., & Fisher, S.K. (1996). Permeability of the blood–brain barrier by the bradykinin agonist, RMP-7: evidence for a sensitive, autoregulated, receptor-mediated system. *Immunopharmacol*, 33, 270–278.

Bazile, D., Prudhomme, C., Bassoullet, M., Marlard, M., Spenlehauer, G., & Veillard, M. (1995). Stealth Me.PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system. *Journal of Pharmaceutical Sciences*, 84, 493–498.

Bert, F., Ouahes, O., & Larnbert-Zechovsky, N. (1995). Brain abscess due to *Bacillus macerans* following a penetration periorbital injury. *Journal of Clinical Microbiology*, 33, 1950-1953.

Blasi, P., Giovagnoli, S., Schoubben, A., Ricci, M., & Rossi, C. (2007). Solid lipid nanoparticles for targeted brain drug delivery, *Advanced Drug Delivery Reviews*, 59, 454-477.

Brahmachary, M., Krishnan, S.P.T., Koh, J.L.Y., Khan, A.M., Seah, S.H., Tan, T.W., Brusic, V., & Bajic, V.B. (2004). ANTIMIC: a database of antimicrobial sequences. *Nucleic Acids Research*, 32, D586-D589.

Brandt, C.T., Frimodt-Møller, N., Lundgren, J.D., Pedersen, M., Skovsted, I.C., Rowland, I.J., & Østergaard, C. (2006). Evaluation of anti-pneumococcal capsular antibodies as adjunctive therapy in experimental pneumococcal meningitis, *Journal of Antimicrobial Chemotherapy*, 58, 1291-1294.

Brigati, C., Giacca, M., Noonan, D.M., & Albini, A. (2003). HIV Tat, its targets and the control of viral gene expression. *FEMS Microbiology Letters*, 220, 57-65.

Brigger, I., Morizet, J., Aubert, G., Chacun, H., Terrier-Lacombe, M. -J., Couvreur, P., & Vassal, G. (2002). Poly(ethylene glycol)-coated hexadecylcyanoacrylatenanospheres display a combined effect for brain tumor targeting. *The Journal of Pharmacology and Experimental Therapeutics*, 303, 928-936.

Brogden, K.A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature*, 3, 238-250.

BUPA's Health Information Team. (2004). Available: http://hcd2.bupa.co.uk/fact_sheets/html/meningitis.html

Calabuig, E., Pemán, J., Salavert, M., Amselem, L., Bosch, M., Viudes, A., & Gobernado, M. (2007). Meningitis caused by *Candida* spp. in a tertiary-care hospital: a 14-year review. *International Journal of Antimicrobial Agents*, 29, S348-S349

Calvo, P., Gouritin, B., Chacun, H., Desmaele, D., D'Angelo, J, Noel, J-P., Georgin, D., Fattal, E., Andreux, J.P., & Couvreur P. (2001). Long-circulating PEGylated polycyanoacrylate nanoparticles as new drug carrier for brain delivery, *Pharmaceutical Research*, 18, 1157-1166.

Cans, A., Wittenberg, N., Karlsson, R., Sombers, L., Karlsson, M., Orwar, O., & Ewing, A. (2003). Artificial cells: unique insights into exocytosis using liposomes and lipid nanotubes.

Proceedings of the National Academy of Sciences of the United States of America, 100, 400-404.

Cao, G., Pei, W., Ge, H., Liang, Q., Luo, Y., Sharp, F.R., Lu, A., Ran, R., Graham, S.H., & Chen, J. (2002). In vivo delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. *The Journal of Neuroscience*, 22, 5423-5431.

Casado, J.L., Quereda, C., Oliva, J., Navas, E., Moreno, A., Pintado, V., Cobo, J., & Corral, I. (1997). Candidal meningitis in HIV-infected patients: analysis of 14 cases. *Clinical Infectious Diseases*, 25, 673-676.

Charlton, S.T., Davis, S.S., & Illum, L. (2007). Nasal administration of an angiotensin antagonist in the rat model: Effect of bioadhesive formulations on the distribution of drugs to the systemic and central nervous systems, *International Journal of Pharmaceutics*, 338, 94-103.

Clinical and Laboratory Standards Institute/NCCLS. Performance standards for antimicrobial susceptibility testing. Fifteenth informational supplement M100-S15/. Wayne, PA: CLSI; 2005.

Coley, S.C., Jager, H.R., Szydlo, R.M., & Goldman, J.M. (1999). CT and MRI manifestations of central nervous system infection following allogeneic bone marrow transplantation. *Clinical Radiology*, 54, 390-397.

Conlon, J. M., Al-Ghaferi, N., Abraham, B., & Leprince, J. (2007). Strategies for transformation of naturally-occurring amphibian antimicrobial peptides into therapeutically valuable anti-infective agents. *Methods*, 42, 349-357.

Cordon-Cardo, C., O'Brien, J.P., Casals, D., Rittman-Grauer, L., Biedler, J.L., Melamed, M.R., & Bertino, J.R. (1989). Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood– brain barrier sites. *Proceedings of the National Academy of Sciences of the United States of America*, 86, 695–698.

Costantino, L., Gandolfi, F., Tosi, G., Rivasi, F., Vandelli, M.A., & Forni, F. (2005). Peptide-derivatized biodegradable nanoparticles able to cross the blood-brain barrier. *Journal of Controlled Release*, 108, 84-96.

Cruz-Perez, P., Buttner, M.P., & Stetzenbach, L.D. (2001). Specific detection of *Stachybotrys chartarum* in pure culture using quantitative polymerase chain reaction. *Molecular and Cellular Probes*, 15, 129-138.

da Cruz, M.T., Simoes, S., & de Lima, M.C. (2004). Improving lipoplex-mediated gene transfer into C6 glioma cells and primary neurons. *Experimental Neurology*, 187, 65-75.

Dennison, S.R., Howe, J., Morton, L.H.G., Brandenburg, K., Harris, F., & Phoenix, D.A. (2006). Interactions of an anionic antimicrobial peptide with *Staphylococcus aureus* membranes. *Biochemical and Biophysical Research Communications*, 347, 1006-1010.

Deshayes, S., Morris, M.C., Divita, G., & Heitz, F. (2005). Cell-penetrating peptides: tools for intracellular delivery of therapeutics. *Cellular and Molecular Life Sciences*, 62, 1839-1849.

Dietz, G.P., Kilic, E., & Bahr, M. (2002). Inhibition of neuronal apoptosis in vitro and in vivo using TAT-mediated protein transduction. *Molecular and Cellular Neuroscience*, 21, 29-37.

Editorial, (2003). Pneumococcal Meningitis: Antibiotics Essential but Insufficient. *Brain*, 126,1013-1014.

Edwards, K. A., & Baeumner, A. J. (2006). Liposomes in analyses. *Talanta*, 68,1421-1431.

Ewald, C., Kuhn, S., & Kalff, R. (2006). Pyogenic infection of the central nervous system secondary to dental affection-a report of six cases. *Neurosurgical Review*, 29, 163-167.

Expert reviews in Molecular Medicine, Available: <http://www.expertreviews.org>

Faustini, A., Fusco, M.A.D., & Perucci, C.A. (2007). Prognostic factors and determinants of fatal outcome due to bacterial meningitis in the Lazio region of Italy, 1996-2000. *International Journal of Infectious Disease*, 11, 137-144.

Fawell, S., Seery, J., Moore, C., Chen, L.L., Pepinsky, B., & Barsoum, J. (1994). Tat-mediated delivery of heterologous proteins into cells. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 664-668.

Fenton, M., Bone, N., & Sinclair, A. (1998). The efficient and rapid import of a peptide into primary B and T lymphocytes and a lymphoblastoid cell line. *Journal of Immunological Methods*, 212, 41-48.

Forster, S., Zisenis, M., Wenz, E., & Antonietti, M. (1996). Micellization of strongly segregated block copolymers. *Journal of Chemical Physics*, 104, 9956-9970.

Foster, D.R., & Rhoney, D.H. (2005). Enterobacter meningitis: organism susceptibilities, antimicrobial therapy and related outcomes, *Surgical Neurology*, 63, 533-537.

Fretz, M.M., Mastrobattista, E., Koning, G.A., Jiskoot, W., & Storm, G. (2005). Strategies for cytosolic delivery of liposomal macromolecules. *International Journal of Pharmaceutics*, 298, 305-309.

Friden, P.M. (1994). Receptor-mediated Transport of Therapeutics across the Blood-Brain Barrier. *Neurosurgery*, 35, 294-298.

Fukui, H., Koike, T., Saheki, A., Sonoke, S., Tomii, Y., & Seki, J. (2003). Evaluation of the efficacy and toxicity of Amphotericin B incorporated in lipid nano-sphere (LNS). *International Journal of Pharmaceutics*, 263, 51-60.

Ganz, T. (2002). Versatile defensins. *Science*, 298, 977-979.

Garcia-Garcia, E., Andrieux, K., Gil, S., & Couvreur, P., (2005). Colloidal carriers and blood-brain barrier (BBB) translocation: A way to deliver drugs to the brain? *International Journal of Pharmaceutics*, 298,274-292.

Goldberg, A.N., Oroszlam, G., & Anderson, T. (2001). Complications of frontal sinusitis and their management. *Otolaryngologic Clinics of North America*, 34, 211-225.

Goumon, Y., Lugardon, K., Kieffer, B., Lefevre, J.F., Van Dorsselaer, A., Aunis, D., Metz-Boutigue, M.H. (1998). Characterization of antibacterial COOH-terminal proenkephalin-A-derived peptides (PEAP) in infectious fluids. Importance of enkelytin, the antibacterial PEAP209-237 secreted by stimulated chromaffin cells. *The Journal of Biological Chemistry*, 273, 29847–29856.

Gref, R., Minamitake, Y., Peracchia, V., Trubetskoy, V., Torchillin, V., & Langer, R. (1994). Biodegradable long-circulatingpolymeric microspheres.*Science*, 263, 1600-1603.

Greig, N.H., Daly, E.M., Sweeney, D.J., & Rapoport, S.I. (1990). Pharmacokinetics of chlorambucil-tertiary butyl ester, a lipophilic chlorambucil derivative that achieves and maintains high concentrations in brain. *Cancer Chemotherapy and Pharmacology*, 25, 320–325.

Hancock, R.E.W., & Sahl, H. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology*, 24, 1551-1557.

Hardebo, J.E., & Kahrstrom, J. (1985). Endothelial negative surface charge areas, and blood–brain barrier function. *ACTA Physiologica Scandinavica*, 125,495-499.

Hinson, V.K., & Tyor, W.R. (2001). Update on viral encephalitis. *Current Opinion Neurology*, 14,369-374.

[http://www. ninds.nih.gov](http://www.ninds.nih.gov)

Hu, F.Q., Ren, G.F., Yuan, H., Du, Y.Z., Zeng, S. (2006). Shell cross-linked stearic acid grafted chitosan oligosaccharide self-aggregated micelles for controlled release of paclitaxel. *Colloids and Surfaces B: Biointerfaces*, 50, 97-103.

Hughes, G.A., (2005). Nanostructure-mediated drug delivery, *Nanomedicine: Nanotechnology, Biology, and Medicine*, 1, 22-30.

Hunter, H.N., Fulton, D. B., Ganz, T., & Vogel, H.J. (2002). The solution structure of human Hecidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis. *The Journal of Biological Chemistry*, 277,37597–37603.

Illum, S.L., & Davis, S.S. (1983). Effect of the nonionic surfactant poloxamer 338 on the fate and deposition of polystyrene microspheres following intravenous administration, *Journal of Pharmaceutical Sciences*, 72, 1086-1089.

Illum, S.L., Davis, S.S., Müller, R.H., Mak, E., & West, P. (1987). The organ distribution and circulation time of intravenously injected colloidal carriers sterically stabilized with a

block copolymer--poloxamine 908. *Life Sciences*, 40, 367-374.

Islam, Z., Harkema, J.R., & Pestka, J.J. (2006). Satratoxin G from the black mold *Stachybotrys chartarum* evokes olfactory sensory neurons loss and inflammation in the murine nose and brain. *Environmental Health Perspectives*, 114, 1099-1107.

Jafari, H.S., Saez-Llorens, X., Severien, C., Parras, F., Friedland, I., Rinderknecht, S., Ehrett, S., Olsen, K.D., Abramowsky, C., & McCracken, G.H. (1994). Effects of antifungal therapy on inflammation, sterilization, and histology in experimental *Candida albicans* meningitis. *Antimicrobial Agents Chemotherapy*, 38, 83-89.

Jenssen, H., Hamill, P., & Hancock, R.E.W. (2006). Peptide antimicrobial agents, *Clinical Microbiology Reviews*, 19, 491-511.

Jung, H.J., Park, Y., Hahm, K-S., & Lee, D.G. (2006). Biological activity of Tat (47-58) peptide on human pathogenic fungi. *Biochemical and Biophysical Research Communication*, 345, 222-228.

Kale, A.A., & Torchilin, V.P. (2007). Design, synthesis, and characterization of pH-sensitive PEG-PE conjugates for stimuli-sensitive pharmaceutical nanocarriers: the effect of substitutes at the hydrazone linkage on the pH stability of PEG-PE conjugates. *Bioconjugate Chemistry*, 18, 363-370.

Kastrup, O., Wanke, I., & Maschke, M. (2005). Neuroimaging of infections. *NeuroRx*, 2, 324-332.

Kataoka, K., Harada, A., & Nagasaki, Y. (2001). Block copolymer micelles for drug delivery: design, characterization and biological significance. *Advanced Drug Delivery Reviews*, 47, 113-131.

Kilic, E., Dietz, G.P.H., Hermann, D.M., & Bahr, M. (2002). Intravenous Tat-Bcl-XL is protective after middle cerebral artery occlusion in mice. *Annals of Neurology*, 52, 617-622.

Kim, B.Y., Kang, J., Kim, K.S. (2005). Invasion processes of pathogenic *Escherichia coli*. *International Journal of Medical Microbiology*, 295, 463-470.

Kim, K.S. (2006). Microbial translocation of the blood-brain barrier. *International Journal for Parasitology*, 36, 607-614.

Koo, Y.L., Reddy, G.R., Bhojani, M., Schneider, R., Philbert, M.A., Rehemtulla, A., Ross, B. D., & Kopelman, R. (2006). Brain cancer diagnosis and therapy with nanoplatfomns. *Advanced Drug Delivery Reviews*, 58, 1556-1577.

Koski, P., & Vaara, M. (1991). Polyamines as constituents of the outer membranes of *Escherichia coli* and *Salmonella typhimurium*. *Journal of Bacteriology*, 173, 3695-3699.

Krama, S.D., & Wunderli-Allenspach, H. (2003). No entry for TAT (44-57) into liposomes and intact MDCK cells: novel approach to study membrane permeation of cell-penetrating peptides. *Biochimica et Biophysica Acta*, 1609, 161-169.

Kreméry, V., Paradisi, F., & Pediatric Nosocomial Meningitis Study Group. (2000). Nosocomial bacterial and fungal meningitis in children; an eight year national survey reporting 101 cases. *International Journal of Antimicrobial Agents*, 15, 143-147.

Kumar, P., Wu, H., McBride, J.L., Jung, K.E., Kim, M.H., Davidson, B.L., Lee, S.K., Shankar, P., & Manjunath, N. (2007). Transvascular delivery of small interfering RNA to the central nervous system. *Nature*, 448, 39-43.

Kuroda, K., Fujimoto, K., Sunamoto, J., & Akiyoshi, K. (2002). Hierarchical self-assembly of hydrophobically modified pullulan in water: Gelation by networks of nanoparticles. *Langmuir*, 18, 3780–3786.

Kusuhara H., Suzuki H., Naito M., Tsuruo T., & Sugiyama Y. (1998). Characterization of efflux transport of organic anions in a mouse brain capillary endothelial cell line. *The Journal of Pharmacology and Experimental Therapeutics*, 285, 1260–1265.

Laban, J.T., & O'Neill, K. (2007). *CNS infection. Surgery (Oxford)*, 25, 517-521.

Li, Y., Pei, Y., Zhang, X., Gu, Z., Zhou, Z., Yuan, W., Zhou, J., Zhu, J., & Gao, X. (2001). PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation, and biodistribution in rats. *Journal of Controlled Release*, 71, 203-211.

Lindsay, K.W., Bone I. *Neurology and neurosurgery illustrated*, 3rd edn. (1997). Edinburgh: Churchill Livingstone.

Lipowsky, R., & Sackmann, E. (Eds.). (1995). Structure and dynamics of membranes-from cells to vesicles. Amsterdam: Elsevier Science

Liu, X.M., Pramoda, K.P., Yang, Y. ., Chow, S., & He, C. (2004). Cholesteryl-grafted functional amphiphilic poly(N-isopropylacrylamide-co-N-hydroxylacrylamide): synthesis, temperature-sensitivity, self-assembly and encapsulation of a hydrophobic agent. *Biomaterials*, 25,2619–2628.

Loftus, L.T., Li, H.F., Gray, A.J., Hirata-Fukae, C., Stoica, B.A., Futami, J., Yamada, H., Aisen, P.S., & Matsuoka, Y. (2006). In vivo protein transduction to the CNS, *Neuroscience*, 139,1061–1067.

Lorin, A., Thomasa, A., Stroobant, V., Brasseur, R., & Lins, L. (2006). Lipid-destabilising properties of a peptide with structural plasticity. *Chemistry and Physics of Lipids*, 141, 185-196.

Lu, W., Tan, Y.Z., Hu, K.L., & Jiang, X.G. (2005). Cationic albumin conjugated pegylated nanoparticle with its transcytosis ability and little toxicity against blood-brain barrier. *International Journal of Pharmaceutics*, 295,247-260.

Luer, M.S., & Hatton, J. (1993). Vancomycin administration into the cerebrospinal fluid: A review. *The Annals of Pharmacotherapy*, 27, 912-921.

Matsuzaki, K. (1999). Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochimica et Biophysica Acta*, 1462, 1-10.

Minchin, R. (2008) Sizing up targets with nanoparticles. *Nature Nanotechnology*, 3, 12-13.

Nagahara, H., Vocero-Akbani, A.M., Snyder, E.L., Ho, A., Latham, D.G., Lissy, N. A., Becker-Hapak, M., Ezhevsky, S.A., & Dowdy, S.F. (1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nature Medicine*, 4, 1449-1452.

Neuwelt, E. (2004). Mechanism of disease: the blood–brain barrier. *Neurosurgery*, 54, 131–141.

Nikaido, H. (1998). The role of outer membrane and efflux pumps in the resistance of gram-negative bacteria. Can we improve drug access? *Drug Resistance Updates*, 1, 93-98.

Obernaier, B., Klein, M., Koedel, U., & Pfister, H.W. (2006). Disease models of acute bacterial meningitis. *Drug Discovery Today: Disease Models*, 3, 105-112.

Pardridge W.M. (1999). Vector-mediated drug delivery to the brain. *Advanced Drug Delivery Reviews*, 36,299–321.

Oliver, J. (2005). Drug transport to brain with targeted nanoparticles. *NeuroRx*[®], 2, 108-119.

Pardridge, W.M. (2005). The blood-brain barrier: bottleneck in brain drug development development. *NeuroRx*[®], 2, 3-14.

Peracchia, M.T., Gref, R., Minamitake, Y., Domb, A., Lotan, N., & Langer, R. (1997). PEG-coated nanospheres from amphiphilic diblock and multiblock copolymers: investigation of their drug encapsulation and release characteristics. *Journal of Controlled Release*, 46, 223-231.

Peracchia, M.T., Fattal, E., Desmaele, D., Besnard, M., Noel, J.P., Gomis, J.M., Appel, M., d'Angelo, J., & Couvreur, P. (1999). Stealth® PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. *Journal of Controlled Release*, 60, 121-128.

Peracchia, M.T., Vautier, C., Pusieux, F., & Couvreur, P. (1997). Development of sterically stabilized poly(isobutyl-2-cyanoacrylate) nanoparticles by chemical coupling of poly(ethylene glycol). *Journal of Biomedical Materials Research*, 34, 317-326.

Pien, F.D., & Pien, B.C. (1999). *Angiostrongylus cantonensis* eosinophilic meningitis. *International Journal of Infectious Diseases*, 3, 161-163.

Regoes, R.R., Wiuff, C., Zappala, R.M., Gamer, K.N., Baquero, F., & Levin, B.R. (2004). Pharmacodynamic functions: a multiparameter approach to the design of antibiotic treatment regimens. *Antimicrobial Agents and Chemotherapy*, 48, 3670-3676.

Rosenstein, N.E., Perkins, B.A., Stephens, D.S., Popovic, T., & Hughes, J.M. (2001). Meningococcal disease. *The New England Journal of Medicine*, 344, 1378-1388.

Rousselle, C., Clair, P., Smirnova, M., Kolesnikov, Y., Pasternak, G.W., Gac-Breton, S., Rees, A.R., Scherrmann, J-M., & Temsarnani, J. (2003). Improved brain uptake and pharmacological activity of dalargin using a peptide-vector-mediated strategy. *Journal of Pharmacology and Experimental Therapeutics*, 306, 371-376.

Sabatier, J.M., Vives, E., Mabrouk, K., Benjouad, A., Rochat, H., Duval, A., Hue, B., & Bahraoui, E. (1991). Evidence for neurotoxic activity of tat from human immunodeficiency virus type 1. *Journal of Virology*, 65, 961-967.

Sanchez-Portocarrero, J., Perez-Cecilia, E., Corral, O., Romero-Vivas, J., & Picazo, J.J. (2000). The central nervous system and infection by *Candida* species. *Diagnostic Microbiology and Infectious Disease*, 37, 169–179.

Santra, S., Yang, H., Stanley, J.T., Holloway, P.H., Moudgil, B.M., Walter, G., & Mericle, R.A. (2005). Rapid and effective labeling of brain tissue using TAT-conjugated CdS:Mn/ZnS quantum dots. *Chemical Communications*, 25, 3144-3146.

Sato, H., & Feix, J.B. (2006). Peptide-membrane interactions and mechanisms of membrane destruction by amphipathic α -helical antimicrobial peptides. *Biochimica et Biophysica Acta*, 1758, 1245-1256.

Sawant, R.M., Hurley, J.P., Salmaso, S., Kale, A., Tolcheva, E., Levchenko, T.S., & Torchilin, V.P. (2006). "SMART" drug delivery systems: Double-targeted pH-responsive Pharmaceutical Nanocarriers. *Bioconjugate Chemistry*, 17, 943-949.

Schmutzhard, E. (2001). Viral infections of the CNS with special emphasis on herpes simplex infections. *Journal of Neurology*, 248, 469-477.

Schröder, U., & Sabel, B.A. (1996). Nanoparticles, a drug carrier system to pass the blood-brain-barrier, permit central analgesic effects of intravenous dalargin injections, *Brain Research*, 710, 121-124.

Schwarze, S.R., Ho, A., Vocero-Akbani, A., & Dowdy, S.F. (1999). In vivo protein transduction: delivery of a biologically active protein into the mouse, *Science*, 285, 1569-1572.

Scotti, G., & Gerevini, S. (2001). Diagnosis and differential diagnosis of acute transverse myelopathy. The role of neuroradiological investigations and review of the literature. *Neurological Sciences*, 22, (suppl 2) S 69-73.

Seki, J., Sonoke, S., Saheki, A., Fukui, H., Sasaki, H., & Mayumi, T. (2004). A nanometer lipid emulsion, lipid nano-sphere (LNS), as a parenteral drug carrier for passive drug targeting, *International Journal of Pharmaceutics*, 273, 75-83.

Seki, J., Sonoke, S., Saheki, A., Koike, T., Fukui, H., Doi, M., & Mayumi, T. (2004) Lipid transfer protein transports compounds from lipid nanoparticles to plasma lipoproteins. *International Journal of Pharmaceutics*, 275, 239-248.

Shai, Y. (1999). Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α -helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochimica et Biophysica Acta*, 1462, 55-70.

Shinoda, K., Carlsson, A., & Lindman B. (1996). On the importance of hydroxyl groups in the polar head-group of nonionic surfactants and membrane lipids. *Advances in Colloid and Interface Science*, 64, 253-271.

Sims, L., Lim, M., & Harsh, G.R. (2005). Review of brain abscesses. *Operative Techniques in Neurosurgery*, 7, 176-181.

Straus, S.K., & Hancock, R.E.W. (2006). Mode of action of the new antibiotic for gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides. *Biochimica et Biophysica Acta*, 1758, 1215-1223.

Sáez-Llorens, X., & McCracken Jr, G.H. (2003). Bacterial meningitis in children. *Lancet*, 361, 2139-2148.

Tan, Y., Tillett, D. J. & McKay, I.A. (2000). Molecular strategies for overcoming antibiotic resistance in bacteria. *Molecular Medicine Today*, 6, 309-314.

Thorne, R.G., Emory, C.R., Ala, T.A., & Frey, W.H. (1995). Quantitative analysis of the olfactory pathway for drug delivery to brain. *Bruin Research*, 692, 278-282.

Torchilin, V.P., Rammohan, R., Weissig, V., & Leuchenko, T.S. (2001). TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors, *Proceedings of the National Academy of Sciences of the United States of America*, 98, 8786-8791.

Torriero, A.A.J., Ruiz-Díaz, J.J.J., Salinas, E., Marchevsky, E.J., Sanz, M.I., & Raba, J. (2006). Enzymatic rotating biosensor for ciprofloxacin determination. *Talanta*, 69, 691-699.

Tossi, A., Sandri, L., & Giangaspero, A. (2000). Amphipathic α -helical antimicrobial peptides. *Biopolymers*, 55, 4-30.

Tsuji, A., & Tamai, H. (1999). Carrier-mediated or specialized transport of drugs across the blood-brain barrier, *Advanced drug delivery reviews*, 36, 277-290.

Tyagi, M., Rusnati, M., Presta, M., & Giacca, M. (2001). Internalization of HIV-1 Tat requires cell surface heparan sulfate proteoglycans. *The Journal of Biological Chemistry*, 276, 3254-3261.

Tzankaki, G., Markou, F., Kesanopoulos, K., Levidiotou, S., Pangalis, A., Tsolia, M., Liakou, V., Papavasiliou, E., Voyiatzi, A., Kansouzidou, A., Blackwell, M.C., & Kremastinou, J., (2006). Phenotypic assessment of *Neisseria meningitidis* isolates obtained from patients with invasive meningococcal disease in Greece, 1993-2003: Implications for serogroup B vaccines based on PorA serosubtype antigens. *Vaccine*, 24, 819-825

Uchegbu, I. F. (Ed.). (2000). Review of synthetic surfactant vesicles. Amsterdam: Harwood Academic Publishers.

United States Patent 20040157260, abstract available at:
<http://www.fi-epatentsonline.com/20040157260.html>

Vaara, M. (1993). Antibiotic-supersusceptible mutants of *Escherichia coli* and *Salmonella typhimurium*. *Antimicrobial Agents and Chemotherapy*, 37, 2255-2260.

Vives, E., Brodin, P., & Lebleu, B. (1997). A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *The Journal of Biological Chemistry*, 272, 16010-16017.

Vordbrodt, A.W. (1988). Ultrastructural cytochemistry of blood-brain barrier endothelia. *Progress in Histochemistry and Cytochemistry*, 18, 1-99.

Wade, L.A., & Katzman, R. (1975). Synthetic amino acids and the nature of L-DOPA transport at the blood-brain barrier, *Journal of Neurochemistry*, 25, 837-842.

Wadia, J.S., Stan, R.V., & Dowdy, S.F. (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nature Medicine*, 10, 310-315.

Wang, Y., Gao, S., Ye, W., Yoon, H.S., & Yang, Y. (2006). Co-delivery of drugs and DNA from cationic core-shell nanoparticles self-assembled from a biodegradable copolymer, *Nature Materials*, 5, 791-796.

Weaver, M., & Laske, D.W. (2003). Transferrin receptor ligand-targeted toxin conjugate (Tf-CRM107) for therapy of malignant gliomas. *Journal of Neuro-Oncology*, 65, 3-13.

Yang, L., Weiss, T. M., Lehrer, R. I. & Huang, H. W. (2000). Crystallization of antimicrobial pores in membranes: magainin and protegrin. *Biophysical Journal*, 79, 2002-2009.

Yih, T.C., & Al-Fandi, M., (2006). Engineered nanoparticles as precise drug delivery systems. *Journal of Cellular Biochemistry*, 97, 161-171.

Young, J.L., Fritz, A., Liu, G., Thoburn, K., Kres, J., & Roffers S. (2000). Available: http://training.seer.cancer.gov/module_anatomy/unit5_3_nerve_org1_cns.html

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, 415, 389-395.

Zhang, X., Xie, J., Li, S., Wang, X., & Hou, X. (2003). The Study on Brain Targeting of the Amphotericin B Liposomes. *Journal of Drug Targeting*, 11, 117-122.

Zhao, M., Kircher, M.F., Josephson, L., & Weissleder, R. (2002). Differential conjugation of tat peptide to superparamagnetic nanoparticles and its effect on cellular uptake, *Bioconjugate Chemistry*, 13, 840-844.

Zheng, Y.G., Gao, S.J., & Ying, J.Y. (2007). Synthesis and cell-imaging applications of glutathione-capped CdTe quantum dots. *Advanced Materials*, 19, 376-380.

Ziegler, A., Li, X.B., Seelig, A., & Seelig, J. (2003). Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis. *Biochemistry*, 42, 9185-9194.

Ziegler, A., Nervi, P., Diirrenberger, M., & Seelig, J. (2005). The cationic cell-penetrating peptide CPPTAT derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical, and metabolic evidence. *Biochemistry*, 44, 138-148.

APPENDICES

LIST OF PAPERS FINISHED

DURING PHD STUDY

Patent

Cationic Core/Shell Nanoparticles Self-Assembled from Amphiphilic Cell-penetrating Peptide as Antimicrobial Agent Capable of Crossing the Blood-Brain Barrier

International referred journals

1. S. M. Liu, W. D. Zhang, Z. L. Liu, and L. Liu*, "Chemical fabrication of Al₂O₃ nano-trilobes", Applied Catalysis A, General, 287 (2005) 108-115.
2. C. Y. Wang, S. M. Liu, X. Bai and L. Liu*, "Synthesis of cobalt–alurninate spinels via glycine chelated precursors", Materials Chemistry and Physics, 96 (2006) 361-370.
3. L. Liu, X. Y. Tan and S. M. Liu, "Ytria Stabilized Zirconia Hollow Fiber Membranes", Journal of the American Ceramic Society, 89 (2006), 1156-1159.
4. L. Liu, Y. Yu, S. Gao, S. M. Liu*, Development of bio-ceramic hollow fiber membranes for bone tissue engineering and immunoisolation devices, Journal of Membrane Science, 280 (2006) 375-382.
5. K. S. Soppimath, L. Liu, W. Y. Seow, S. Q. Liu, R. Powell, P. Chan and Y. Y. Yang*, "Multi-Functional Polymer Core-Shell Nanoparticles Self-Assembled from pH-Induced Thermally Responsive Polymer for Targeted Anticancer Drug Delivery," Advanced Functional Materials, 17 (2007) 355-362.

6. S. M. Liu, L. Liu, Z. P. Xu, A. Yu, G. Q. Lu* and J. C. D. da Costa, "Bioceramic Macrocapsules for cell immunoisolation," *Angewandte Chemie International Edition*, 46 (2007) 3062-3065.
7. S. M. Liu, Z. P. Xu, H. B. Su, L. Liu*, "From transparent chips to nano-optical tag," *Smart Materials and Structures*, 16 (2007) 2214-2221.
8. H. Chen, L. Liu, H. W. Yu, L. S. Wang, C. B. Ching and Y. Y. Yang*, "Thermally Responsive Reversed Micelles for Immobilization of Enzymes," *Advanced Functional Materials*, 18, (2008) 95-102.
9. L. Liu, K. Guo, J. Lu, S. S. Venkatraman, D. Luo, Y. Zheng, K. C. Ng, E. Ling, S. Mookhala and Y. Yang*, "Biologically Active Core/Shell Nanoparticles Self-Assembled from Cholesterol-Terminated PEG-TAT for Drug Delivery across the Blood-Brain Barrier," *Biomaterials*, 29, (2008) 1509-1517.
10. L. Liu, S. S. Venkatraman, Y. Yang*, K. Guo, J. Lu, B. He and L. Kan, "Delivery of Ciprofloxacin Lactate Targeted to Blood-Brain Barrier by Polymeric Micelle Carrier Anchored with TAT Peptide", *Peptide Science*, early view.
11. L. Liu, K. Guo, J. Lu, S. S. Venkatraman, Y. Yang*, "Self-assembled Cationic Peptide Nanoparticles as an Efficient Antimicrobial Agent Capable of Crossing the Blood-brain Barrier", submitted to *Nature Nanotechnology*, in revision.

International conference

1. L. Liu and Y. Y. Yang, "Targeted Delivery of Anticancer Drugs Using pH- and Temperature-Sensitive Core-Shell Nanoparticles Conjugated with Folate", 3rd International Conference on Materials for Advanced Technology, Singapore, July 2005, talk.
2. Y.Y. Yang, Y. Wang, L. Liu, R. Powell, P. Chan, K. Soppimath, X. Jiang and S. Gao, "Smart Polymer Nanostructures for Targeted Drug Delivery," International Congress of Nanotechnology (ICNT), San Francisco, October 31- November 4, 2005, talk.
3. L. Liu and Y. Y. Yang, "Smart Core-shell Nanoparticles for Targeted Anticancer Drug Delivery", 2nd Material Research Society- Singapore Conference on Advanced Materials, Singapore, January, 2006, poster.
4. L. Liu, "Wet Etching Fabrication of Transparent Chips and Their Potential Application in Nano-Optical Tagging Biosensor", 3rd Asia-Pacific Conference of Transducers and Micro-Nano Technology (APCOT), Singapore, June 2006, poster.
5. L. Liu, K. Guo, J. Lu, and Y. Y. Yang, "Delivery of Ciprofloxacin Targeted to Blood-Brain Barrier by Cholesterol-Terminated PEG-TAT Micelle Carrier", 4th International Conference on Materials for Advanced Technology (ICMAT), Singapore, July 2007, talk.
6. L. Liu, Y. Wang and Y. Y. Yang, "Antimicrobial activities of cationic micelles formed from a biodegradable amphiphilic copolymer", ICBN, Singapore, August 2007, poster.

7. L. Liu and C. Yong, "Strontium related discriminating mechanical strength between ovine and porcine cortical xenografts", Colloquium Spectroscopicum International XXXV, Xiamen, China, September 2007, talk.
8. L. Liu, S. S. Venkatraman, and Y. Y. Yang, "Cholesterol-Terminated PEG-TAT Micelle as Novel Carrier for Ciprofloxacin Delivery Targeted to Blood-Brain Barrier", 7th Australian Peptide Conference and the 2nd Asia-Pacific International Peptide Symposium, October 2007, poster.
9. L. Liu, "Silica contribution to biomineralization inside bilayer phospholipid matrix vesicle", 2nd Singapore Lipid Symposium, March 2008, poster.