GÜLCEM ALTINOĞLU

DEVELOPMENT OF NOVEL POLYMERIC PULLULAN NANOPARTICLES FOR ALZHEIMER'S DISEASE

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A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCES OF NEAR EAST UNIVERSITY

By
GÜLCEM ALTINOĞLU

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Engineering

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Approval of Director of Institute of Graduate Studies

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I hereby declare that the current PhD thesis is my own original work and I have fully cited and referenced all sources used that are not original to this work. Further, it has not been submitted to any institution for assessment purposes.

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Happiness can be found,
even in the darkest of times,
if one only remembers to turn on the light…

ABSTRACT

The current therapeutic approach for Alzheimer's disease (AD) is only symptomatic failing to provide an effective treatment with many side effects and frequent dosing. Similar to all brain targeted therapeutics, these drugs suffer from limited brain concentrations as a result of the high selectivity of the blood-brain-barrier (BBB). The limited entry to the brain has resulted in immense research in the field of nanotechnology. One such approach is the nano-encapsulation of drugs using biocompatible and biodegradable biomaterials, to enhance the transfer of therapeutics across the BBB and improve their efficacy with minimum dosing frequency. Taking these into consideration, the aim of the current dissertation is developing a novel platform for the delivery of rivastigmine (RT), clinically important reversible inhibitor of acetylcholinesterase (AChE), for AD treatment. In the current study, a non-toxic biopolymer, pullulan, has been employed for RT encapsulation due to its biocompatibility, biodegradability and ease of chemical modification. PL was further surface modified with an ACh analogue, acetylthiocholine iodide (ATCh), to develop a "smart" drug-release model, to only release the encapsulated AChE inhibitor in the presence of the AChE enzyme and prolong cholinergic neurotransmission. Synthesized particles were characterized for surface morphology, elemental composition, particle size, zeta potential and chemical structure with scanning electron microscopy, energy dispersive x-ray composition analysis, zeta sizer/potential, and Fourier transform infrared analyses. Quantity of instant drug release, *in vitro* determination of cholinesterase enzyme activity and coagulation activity were also investigated. Results demonstrated the successful synthesis of drug loaded nanoparticles in the desired nano-range (<50 nm), ideal for bypassing the BBB. Nanoparticles exhibited negative surface charge indicative of particle stability. Determination of instant drug release yielded a percentage drug release of 21%, 17% and 14% for varying amounts of incorporated RT (1.2, 1.0 and 0.8 mL respectively). Nanoparticles were also promising in terms of hemocompatibility as well as producing an apparent inhibition of the cholinesterase enzyme. These results indicate the potential of the prepared drug delivery system in AD treatment.

Keywords: Alzheimer's disease; nanotechnology; nanoparticles; pullulan; rivastigmine

ÖZET

Alzheimer hastalığı (AD) için mevcut terapötik yaklaşım pek çok yan etki ve sık dozlama ile etkili bir tedavi sağlayamamakla birlikte yalnizca semptomatiktir. Tüm beyin hedefli tedavilere benzer şekilde, merkezi sinir sistemini çevreleyen kan-beyin bariyerinin (BBB) düşük geçirgenliği nedeniyle, sınırlı beyin konsantrasyonundan muzdariptir. Beyne sınırlı erişim, nanoteknoloji alanında yoğun araştırmalara yol açmıştır. Böyle bir yaklaşım, BBB genelinde terapötiklerin beyin dağıtımını iyileştirmek ve minimum dozlama sıklığı ile etkinliklerini arttırmak için biyouyumlu ve biyolojik olarak parçalanabilir biyomalzemeler kullanarak, ilaçların nanoenkapsülasyonudur. Bunları dikkate alarak, mevcut tezin amacı, AD tedavisi için klinik olarak önemli bir geri dönüşümlü asetilkolinesteraz inhibitörü (AChE) olan Rivastigmin (RT) nanoenkapülasyonu için yeni bir platform geliştirmektir. Bu çalışmada, biyouyumluluğu, biyolojik olarak parçalanabilirliği ve kimyasal modifikasyon kolaylığı nedeniyle enkapsülasyon için toksik olmayan bir biyopolimer olan pullulan (PL) kullanılmıştır. PL, "akıllı" bir ilaç salım modeli geliştirmek için bir ACh analoğu olan asetiltiyokolin iyodür (ATCh) ile modifiye edilmiştir. Böylelikle, kolinerjik nörotransmisyonu uzatmak için, sadece AChE enziminin varlığında enkapsüle edilmis AChE inhibitor ilacini salacaktır. Sentezlenen parçacıklar yüzey morfolojisi, elemental kompozisyonu, partikül boyutu, zeta potansiyeli ve ve kimyasal yapısı için, taramalı elektron mikroskobu, enerji dağılımlı x-ray bileşim analizi, zeta sizer/potansiyeli ve Fourier dönüşümlü kızılötesi spektroskopisi kullanılarak karakterize edilmiştir. Anlık ilaç salım miktarı, *in vitro* kolinesteraz enzim aktivitesi ve koagülasyon aktivitesi tayini de araştırılmıştır. Sonuçlar, ilaç yüklü nanopartiküllerin başarılı sentezini ve BBB geçişi için ideal nano-aralıkta (<50 nm) olduğunu göstermiştir. Nanopartiküller, parçacıkların stabilitesini gösteren negatif bir yüzey yükü göstermiştir. Anlık ilaç salınımının belirlenmesi, çeşitli miktarlarda RT (sırasıyla 1.2, 1.0 ve 0.8 mL) için 21%, 17% ve 14%'lük bir ilaç salımı yüzdesi vermiştir. Nanopartiküller ayrıca hemokompatibilite açısından da umut verici sonuçlar göstermiş olup, ayni zamanda bariz bir şekilde kolinesteraz enzimini inhibe etmiştir. Bu sonuçlar, hazırlanan ilaç dağıtım sisteminin AD tedavisinde potansiyelini göstermektedir.

Anahtar Kelimeler: Alzheimer hastalığı; nanoteknoloji; nanopartiküller; pullulan; rivastigmin

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ACh: Acetylcholine

AChE: Acetylcholinesterase

AChEIs: Acetylcholinesterase inhibitors

AD: Alzheimer's Disease

AFM: Atomic force microscopy

APOE: Apolipoprotein E

APP: Amyloid precursor protein

ATCh: Acetylthiocholine iodide

Aβ: Amyloid beta

BBB: Blood-brain-barrier

BChE: Butyrylcholinesterase

BTCh: Butyrylthiocholine iodide

CDK5: Cyclin-dependent kinase-5

Ch: Choline

ChAT: Choline acetyltransferase

CNS: Central nervous system

CSF: Cerebrospinal fluid

DTNB: 5,5′-dithio-2-bis-nitrobenzoate

EDX: Energy dispersive X-Ray

ERK2: Extracellular signal-related kinase-2

FDA: The Food and Drug Administration

FTIR: Fourier transform infrared spectroscopy

GSK3β: Glycogen synthase kinase-3 beta

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hsp 70: Heat shock protein 70

MAChR: Muscarinic acetylcholine receptors

MB: Methylene blue

nAchR: Nicotinic acetylcholine receptors

NFTs: Neurofibrillary tangles

NGF: Nerve growth factor

NLCs: Nanostructured lipid carriers

NMDA: N-methyl-D-aspartate

NMDAR: N-methyl-D-aspartate receptors

NP: Nanoparticle

O/W: Oil-in-water

OS: Oxidative stress

PAMAM: Poly(amidoamine) dendrimers

PCL: Poly(ε-caprolactone)

PEG: Polyethylene Glycol

PL: Pullulan

PL/ATCh: Pullulan-Acetylthiocholine

PLA: Poly(lactic acid)

PLGA: Poly (lactic-co- glycolic acid)

PnBCA: Poly(n-butylcyano-acrylate)

PSEN1: Presenilin 1

PSEN2: Presenilin 2

ROS: Reactive oxygen species

RT: Rivastigmine

SEM: Scanning electron microscopy

SLNs: Solid lipid nanoparticles

T-80: Tween-80

TEM: Transmission electron microscopy

TPP: Sodium tripolyphosphate pentabasic

UV-Vis: Ultraviolet-visible

VIP: Vasoactive intestinal neuropeptide

W/O: Water-in-oil

CHAPTER 1
INTRODUCTION

Neurodegenerative disorders comprise a range of disorders, that are becoming a great concern due to their increasing prevalence. Among these, Alzheimer's Disease (AD), is the most prevalent type of neurodegenerative disorder, which accounts for over 80% of reported dementias worldwide (Kumar and Singh, 2015). Currently, there are around 50 million individuals living with AD, with almost 10 million new patients per year (World Health Organization, 2020), as a result of the steady growth in the aging population (Aprahamian et al., 2013). Consequently, the prevalence of AD is estimated to increase further in the coming decades, and rise over 100 million by 2050 (Brookmeyer et al., 2007). It has become the third major cause of fatality for the elderly population, following cardio/cerebrovascular diseases and malignant tumours (Du et al., 2018).

According to the Diagnostic and Statistical Manual of Mental Disorders V, the clinical diagnostic criteria for AD include the development of a new-onset memory impairment along with a gradual progression of cognitive decline in at least one of the following cognitive domains, involving executive function, complex attention, perceptual motor or social cognition (American Psychiatric Association, 2013). It is characterized by progressive and irreversible degeneration of nerve cells and their connections (Singh, 2014), that is reflected as memory impairments, along with problems regarding mental functioning, thinking and behavior. The earliest clinical manifestation of AD includes memory loss, which affects everyday life functioning of patients. Additionally, individuals may face difficulties in problem solving, performing tasks in social settings, and dealing with sudden mood changes (Alzheimer's Association, 2014). These symptoms become more pronounced as the disease progresses on to the middle stage, despite the fact that patients can still function independently. Finally, the late stage of AD is brought on by progressive decline of both cognitive and functional abilities, and losing the ability to respond to their surroundings and to control movement. These symptoms continue to worsen, leaving patients in need of constant aid, eventually leading to death.

AD etiology is multifactorial. Both genetic and environmental influences are responsible for

the formation of AD pathology (Morris and Mucke, 2006). There are three genes that are accepted as the main risk factors for AD; amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) in chromosomes 21, 14, and 1, respectively (Dai et al., 2018), where the mutations in these genes drive the formation of disease pathology. Additionally, the Apolipoprotein E (APOE) gene in chromosome 19, that has three different alleles; ε2, ε3, and ε4 alleles, is a significant risk factor for disease development (Liu, 2013). Among these, the ε4 allele is the strongest and only validated genetic risk factor for AD as a result of population studies (Bu, 2009). Therefore, individuals having at least one APOE ε4 allele possess an increased risk of AD development. It is also established that environmental factors can play a key function in disease development and progression. Advanced age is the most significant environmental factor along with other vascular and metabolic disorders including obesity, diabetes, trauma, stroke, high cholesterol, hypertension and cardiovascular disorders (Hugo and Ganguli, 2014)

To date, many aspects of the neurobiological mechanism underlying AD have been discovered. Yet, gaps of knowledge about the pivotal events in AD still continue to exist. Currently, no effective therapies are available to avoid or delay the progress of AD. Various candidate therapeutics with diverse pharmacological mechanisms have been tested in clinical trials (Becker et al., 2008). However, the course of the disorder has not been altered by therapeutic interventions, regardless of the type of treatment and/or pathological trait targeted. (Ansari et al., 2017). Since 1998, only four drugs have been approved for use by The Food and Drug Administration (FDA). It has been more than seventeen years since the FDA last approved an AD drug, as no new drugs have been approved since 2003 (Hampel et al., 2018). The approved drugs include cholinesterase inhibitors (rivastigmine, donepezil, and galantamine) and N-methyl-D-aspartate (NMDA) receptor antagonist (Memantine) (Du et al., 2018). However, they are largely symptomatic, targeting the cognitive manifestations rather than the cause of the disease (Aprahamian et al., 2013). The clinical use of cholinesterase inhibitors is based on increasing the synaptic concentration of the acetylcholine (ACh) neurotransmitter in the brain, by blocking the action of an enzyme referred to as the acetycholinesterase enzyme, that normally hydrolyzes the ACh in the synapse (Aprahamian et al., 2013) and thus prolongs its action. Acetylcholine is a key neurotransmitter used by cholinergic neurons. Almost all brain regions are innervated by

cholinergic neurons, with wide-spread activity throughout the cortex, basal ganglia, and basal forebrain (Mesulam, 2013). The brain cholinergic system has a significant role in AD as cholinergic neuron damage and/or loss is regarded as a critical pathological shift that correlates with cognitive decline in AD patients (Du et al., 2018) and memory deficits associated with age and other neurodegenerative diseases (Sultzer and David, 2018). Acetylcholinesterase inhibitors (AChEIs) are indicated to AD patients preferably in combination with the NMDA receptor antagonist, to provide a symptomatic treatment (Du et al., 2018). However, literature shows that the positive effects of AChEIs are only limited to a short period of time persisting around one to three years (Sun et al., 2008). Additionally, although they are currently the most available clinical treatment for AD therapy, the chronic use of AChEIs are not well tolerated among AD patients causing various side effects e.g. nausea, vomiting, anorexia, diarrhea, hypotension and bradycardia.

1.1. Statement of Problem

Clinical benefits of available pharmacological therapeutic drugs for AD are also limited owing to the fact that entry of compounds into the central nervous system (CNS) is severely restricted, including the ones with good therapeutic value. This is due to the presence of a barrier, referred to as the blood-brain-barrier (BBB), which serves as a highly selective and semi-permeable border between the brain and systemic circulation. Therefore, clinical failure of therapeutics can be attributed to the insufficiencies in the method by which they are delivered rather than their potency. As a solution, the use of nanoparticles as drug delivery vehicles are proposed to address the limitations of traditional drug delivery mechanisms.

1.2 Aims of the Study

To overcome the restrictions of conventional drug delivery mechanisms, a CNS targeted nano-drug delivery system is presented in the current work. It consisted of designing and developing novel biocompatible and biodegradable polymeric NPs for the delivery of an AChEI drug, rivastigmine, across the BBB with the purpose of improving its bioavailability and therapeutic effect, as well as minimizing the current side effects of its conventional

delivery.

More specifically, the main objective of this thesis is to develop rivastigmine loaded phosphorylated pullulan nanoparticles for the treatment of AD.

The goals of the current thesis can be summarized in the following points:

(i) To develop and optimize a nanoparticle synthesis method confirmed by characterization tests

(ii) To achieve surface modified (with Acetylthiocholine iodide) phosphorylated nanoparticles as drug delivery vehicles

(iii) To evaluate the i*n vitro* efficiency of synthesized particles

(iv) To assess the drug release profile of drug loaded nanoparticles

To our knowledge, this will be the first study to develop such a system using the current materials of interest.

CHAPTER 2

ALZHEIMER'S DISEASE HYPOTHESES

The most established underlying hypotheses for AD include the amyloid beta (Aβ), tau, cholinergic and oxidative stress hypotheses (Figure 2.1), as well as other minor pathological mechanisms e.g. the inflammation and glutamate excitotoxicity hypotheses. The neuropathological features of AD include two proteins that are heavily involved in disease progression. Extracellular accumulation of Aβ peptides leading to the formation of senile plaques, and intracellular neurofibrillary tangles (NFTs) from hyper-phosphorylated tau have long been viewed as the main pathological events in AD driving neurodegeneration. However, how these proteins relate to each other is still not known and represent an active area of research.

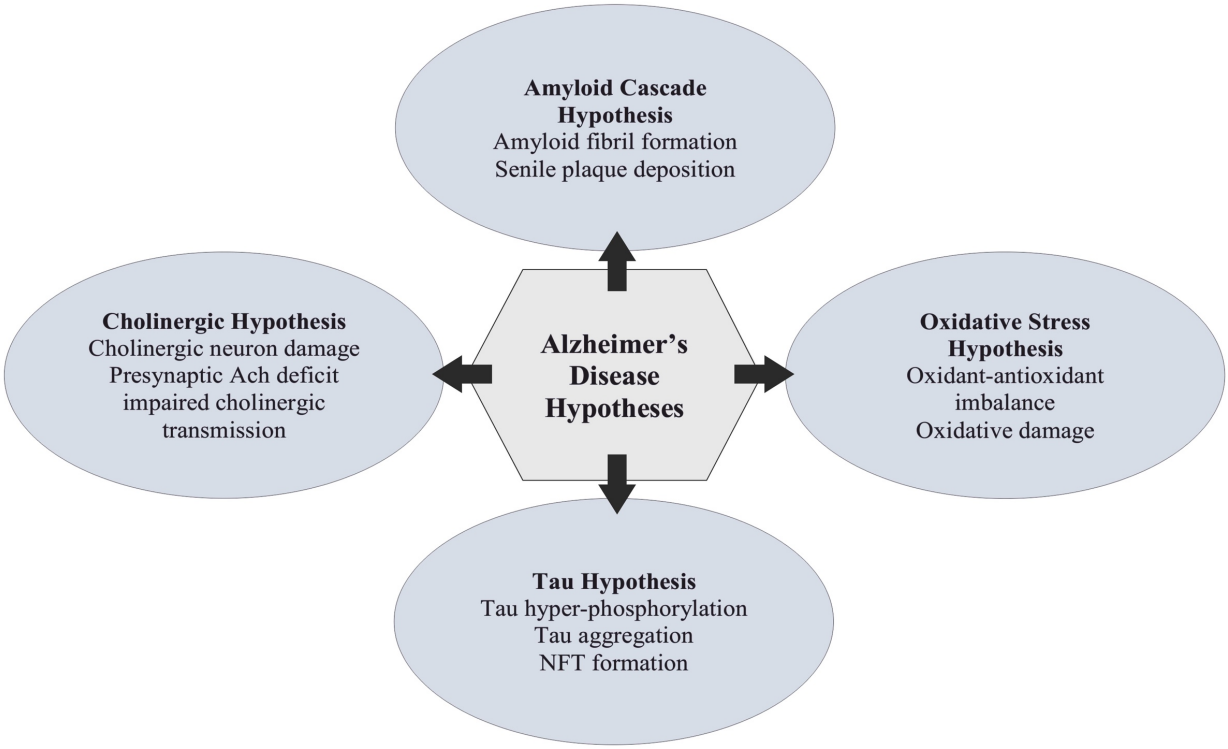


Figure 2.1: Alzheimer's disease hypotheses

Reprinted from (Altinoglu and Adali, 2020)

2.1 Amyloid Cascade Hypothesis

The amyloid cascade hypothesis is the most established underlying hypothesis of AD. It suggests that the extracellular accumulation of a peptide, referred to as the amyloid beta (Aβ) peptide, is the driving force in AD pathogenesis. Under normal conditions, the Aβ peptide is produced from the serial enzymatic cleavage of a larger transmembrane protein, amyloid precursor protein (APP), by APP cleaving enzymes; β- and γ-secretases (Chen et al., 2017) and subsequently gets degraded. APP cleavage by secretase enzymes produce several different isoforms of the Aβ peptide. In affected individuals, the normal metabolic ability to degrade Aβ is diminished, leading to its accumulation as a result of an abnormal regulation of APP secretases that leads to a disturbance in Aβ homeostasis. Aβ peptides ending at positions 40 (AB_{1-40}) and 42 (AB_{1-42}) have been found to be more prone to aggregation in AD brains, with AB_{1-42} even greater compared to AB_{1-40} (Aprahamian et al., 2013). Such spontaneous accumulation of Aβ peptides give rise to the formation of soluble oligomers that develops into insoluble fibrils with a beta-sheet structure, eventually forming neurotoxic senile plaques (Bharadwaj et al., 2009) (Figure 2.2). These plaques cause neuronal dysfunction and large scale neuronal loss, eventually leading to neurodegeneration in the brain.

Although literature still supports the validity of the amyloid cascade hypothesis and its involvement in the initiation of AD pathology, growing evidence suggests that Aβ plaques is a triggering factor in the early disease process but not sufficient in the later stages of the disease (Musiek and Holtzman, 2015). The *in vivo* observation of Aβ plaques *via* neuroimaging techniques have revealed a poor correlation between senile plaques and cognitive decline in AD patients, in contrast to the soluble oligomeric forms of the peptide (Borutaite et al., 2011). Thus, the amyloid cascade hypothesis has been reformulated and the soluble Aβ oligomers began to be taken into account. Either way, Aβ represents an early and critical event in AD pathogenesis. Yet, by what mechanism it drives AD remains unknown. Anti- Aβ treatment strategies incorporate methods with diverse mechanisms of action that inhibit the production and aggregation of Aβ peptides, and facilitate their clearance by destroying already existing Aβ plaques. Nevertheless, the failure in the development of successful Aβ targeting drugs yielded no major advancements to AD symptoms and/or progression, which shaped another rising line of evidence challenging the influence of Aβ

on AD pathogenesis, suggesting the possible role of other causes in disease progression (Terry et al., 1991) (Terry, 2006). This has shifted the attention to neurofibrillary tangles (NFTs).

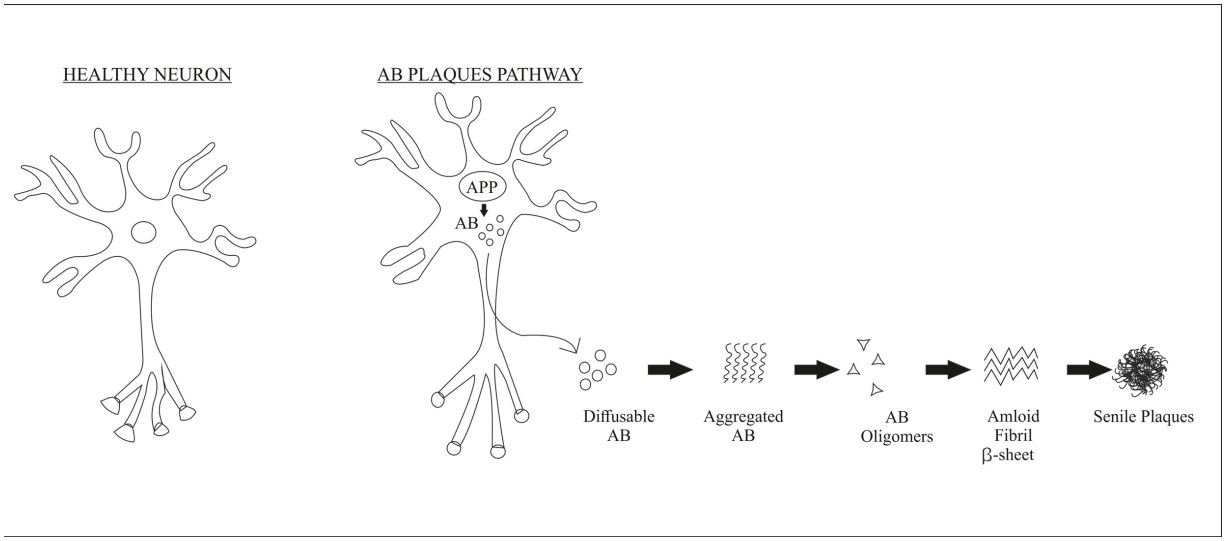


Figure 2.2: Amyloid beta (Aβ) plaque formation in Alzheimer's disease pathogenesis

Reprinted from (Altinoglu and Adali, 2020)

2.2 Tau Hypothesis

Neurofibrillary tangles (NFTs) are aggregates of tau protein. Tau is a microtubule associated scaffolding protein enriched in axons, that modulates the cytoskeletal dynamics of neurons (Pooler et al., 2013). Tau binds microtubules directly, supporting their assembly and stability (Li et al., 2018). It dynamically regulates various key functional processes including axonal transport and neurite outgrowth. Under normal conditions, tau undergoes many post-translational modifications, including phosphorylation, lysine monometyhlation, dimethylation, acetylation and ubiquitylation. The phosphorylation process is carried out by various kinases such as extracellular signal-related kinase-2 (ERK2), cyclin-dependent kinase-5 (CDK5) and glycogen synthase kinase-3 beta (GSK3β) (Takashima, 2009). Under pathological conditions, there is a state of hyper-phosphorylation. This is due to GSK3β, the

most important kinase in neurons, being overactive (Pei et al., 1999). The abnormal hyper-phosphorylation of tau leads to its dissociation from the microtubules and the collapse of the neural cytoskeleton. Dissociating tau start to pair with other threads of tau and trigger intracellular tau aggregation and oligomerization (Gong and Iqbal, 2008) to form paired helical filaments, to further originate intracellular NFTs (Meraz-Ríos et al., 2010) (Figure 2.3). NFTs impair axons of neurons, the structure of neural cytoskeleton, mitochondrial transport, and thus cause neural dysfunction and neurodegeneration. It has been shown that hyper-phosphorylated tau correlates more tightly with the cognitive impairments of AD patients and disease severity (Li et al., 2018). While the exact mechanism of tauopathy is still not fully comprehended, literature indicate the influence of damage signals including Aβ oligomers, oxygen free radicals, iron overload, and the participation of the innate immune system *via* microglial cell activation (Maccioni et al., 2010). Tau-targeted treatment strategies involve different mechanisms involving the stabilization of microtubules, modulation of tau modifications and blocking of tau aggregation (Du et al., 2018), despite their failure in clinical trials. In general, similar to amyloid cascade hypothesis, these strategies remain challenging owing to an insufficient understanding of AD and the absence of sensitive biomarkers for diagnosis.

2.3 Oxidative Stress Hypothesis

Besides being a major sign of aging, significant amount of evidence has revealed that oxidative stress (OS) is a critical factor in AD pathology. OS is induced by a progressive imbalance between the oxidants and antioxidant defenses, that can result from either a decrease in antioxidant defenses or an increase in reactive oxygen species (ROS) and/or free radical production, free radicals being a species containing one or more unpaired valence electrons (Huang et al., 2016). It should be pointed out that the brain utilizes more oxygen (20% of the total oxygen consumption) than other tissues and organs, and thus is more prone to ROS exposure, making it more vulnerable to oxidative damage. Regarding free radicals, mitochondrial dysfunction is stated to be the biological foundation of their increased production that leads to oxidative damage (Huang et al., 2016). It has also been demonstrated that there is a strong correlation between oxidative damage and the concentrations of biometals e.g. iron (Fe2+), zinc (Zn2+), aluminum (Al3+) and copper (Cu2+), which may

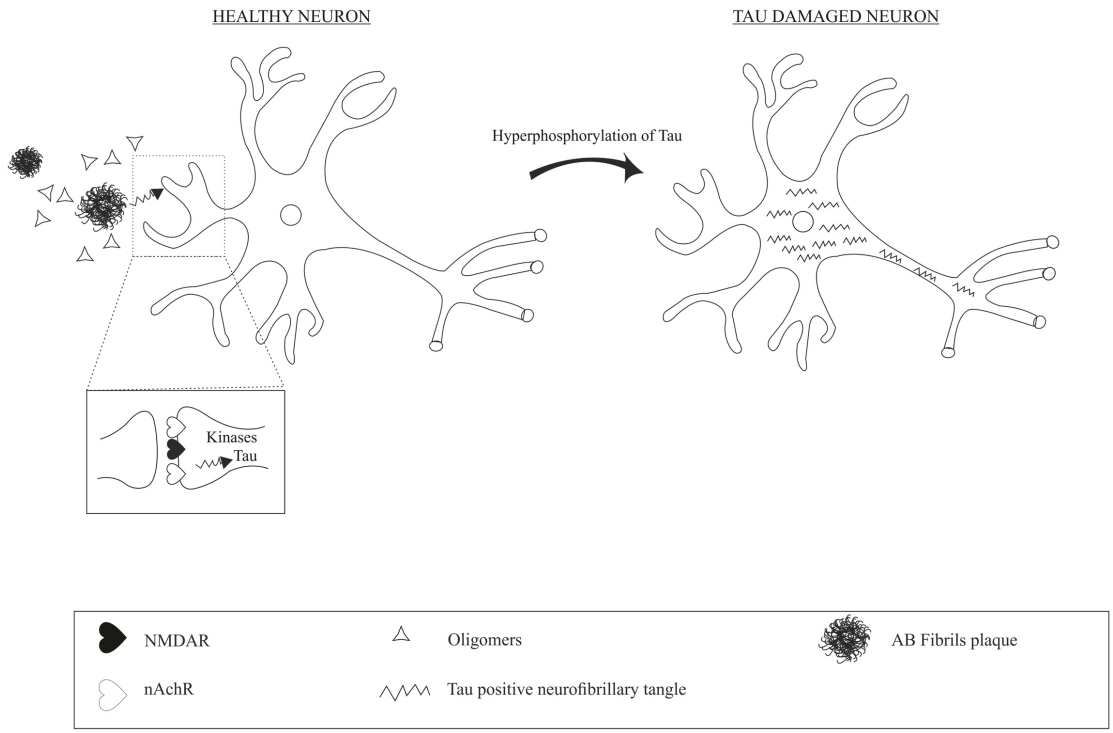


Figure 2.3: Neurofibrillary tangle (NFT) formation in Alzheimer's disease pathogenesis Reprinted from (Altinoglu and Adali, 2020)

be the source of free radicals (Zhang et al., 2014) The OS hypothesis is further supported by the fact that age is the key environmental risk factor, as the damaging effect of free radicals and ROS can accumulate over the years to trigger AD pathology (Nunomura et al., 2001). Additionally, the relationship between OS and AD is further supported by various findings, revealing lower levels of antioxidant enzymes and increased levels of DNA, protein and lipid oxidation, leading to the generation of ROS in the brains of AD patients (Barnham et al., 2004). To this end, OS have been associated with AD, and also promoting or enhancing Aβ and NFT deposition. Hence, in theory, treatment with antioxidant compounds would offer protection against OS and AD pathology. Nonetheless, OS represents a single aspect of AD, and therefore the antioxidant approach was questioned for its effectiveness over inhibiting AD progression. Instead, it is recommended as a portion of combination therapy (Persson et al., 2014) (Teixeira et al., 2013).

2.4 Cholinergic Hypothesis

Ach is a neurotransmitter used by all cholinergic neurons, that innervate almost all brain regions (Woolf and Butcher, 2011). Cholinergic neurons are widely distributed across the brain with very important roles in the peripheral and central nervous systems. Given their widespread distribution, it is hardly surprising that cholinergic neurotransmission is involved in the modulation of critical processes including learning, memory, attention and sensory information. Further studies have reported that cholinergic neurotransmission is also essential for modulation of acquisition (Blokland et al., 1992), encoding (Winters and Bussey, 2005), consolidation (Power et al., 2003), reconsolidation (Boccia et al., 2004), and retrieval of memory (Boccia et al., 2003).

ACh is synthesized from Acetyl Coenzyme A and choline in the cytosol of cholinergic neurons by choline acetyltransferase (ChAT) enzyme. Upon stimulation, ACh is released to the synaptic gap located between cholinergic neurons, and binds to receptors on postsynaptic cholinergic neurons to trigger an appropriate response. Then, cholinergic neurotransmission is terminated with the help of an enzyme, referred to as acetylcholinesterase (AChE), which is a cholinergic enzyme that breaks down or hydrolyzes ACh into acetic acid and choline.

The significance of the cholinergic system on memory, and thus AD, has come from the observation that there is a specific and severe degeneration of cholinergic neurons that strongly correlates with the intellectual and memory problems of AD patients (Whitehouse et al., 1981) (Whitehouse et al., 1982) (Figure 2.4). Furthermore, it has been illustrated that ChAT transcription is reduced in the remaining cholinergic neurons, which leads to a decrease in ChAT activity and the progression of the disease (Wilcock et al., 1982) (Bowen et al., 1976) (Strada et al., 1992). Yet, it is accepted that the observed correlation between cognitive impairment and decreased cholinergic neurotransmission alone does not establish a definitive cause of AD. Therefore, it is sensible to consider AD as a complex disease with multiple underlying causes and interactions.

Over the years, many questions have been raised on the relationship between ACh dysfunction and AD. It has been argued that ACh deficiency is a symptom of the disease rather than a primary pathological cause. However, efforts at correcting the ACh deficiency in the brain yielded the first approved medication for AD treatment, despite being only

symptomatic. Acetylcholinesterase inhibitors (AChEIs), cholinergic drugs that are currently on the market, are currently employed for AD treatment. They work by increasing the ACh levels in the synaptic gap by blocking the actions of the enzyme that breaks down ACh, referred to as AChE, and thus augmenting the ACh-mediated neurotransmission. In addition to their intention of stabilizing the cognitive decline in AD patients, AChEIs have also been reported to improve the psychological and behavioral aspects of the disease (Finkel, 2004). Furthermore, the mechanism of action of AChEIs may not be solely limited to neuron-to-neuron transmission, but also it has been hypothesized that they may offer a level of neuroprotection due to their possible anti-inflammatory action (Francis et al., 2005). However, for such an action, two important criteria must be met (i) that there is a direct and clear correlation between the cholinergic system and inflammation and (ii) that there is clear evidence demonstrating the impact of AChEIs on inflammatory mediators. This link has been established by the confirmation of the anti-inflammatory effects of the cholinergic system, linking ACh neurotransmission with the inhibition of inflammation (Shytle et al., 2004) (Zhang and Tang, 2000) (Borovikova et al., 2000). Recent evidence point to an anti-inflammatory role of AChEIs through an inhibition of free radicals and inflammatory molecules. Yet, the exact mechanism of action is still unknown. More research is required which will certainly elucidate the anti-inflammatory function of AChEIs on AD patients. Yet, it is no longer suitable to consider that enhancing cholinergic transmission is the only purpose of AChEIs in AD treatment.

2.4.1 AChEIs to treat AD- targeting the cholinergic system

As mentioned above, AChEIs increase the synaptic concentration of ACh to improve the cognitive symptoms of the disorder and thus enhance the quality of life of mild to severe AD patients. However, these drugs are able to offer a symptomatic solution, targeting the symptoms rather than the underlying causes of the disease. Additionally, reports indicate that the positive outcomes of AChEIs only persist for a short period of time, around one to three years (Sun et al., 2008). Many studies have been conducted on the efficacy of AChEIs. It has been revealed that donepezil therapy is associated with important cognitive and functional benefits over a span of twelve months in moderate to severe AD patients (Howard et al,, 2012).

In a double-blind study examining the survival of mild to moderate AD patients, long term galantamine therapy (two years) substantially reduced the mortality rate and cognitive decline, as well as enhancing their everyday life activities (Hager et al., 2014). Besides donepezil and galantamine, rivastigmine is also currently being used for AD treatment. Rivastigmine is unique in terms of its action mechanism, as it provides a dual inhibition of ACh breakdown. ACh can normally be hydrolysed by not just AChE enzyme, but also another enzyme named as butyrylcholinesterase (BChE).

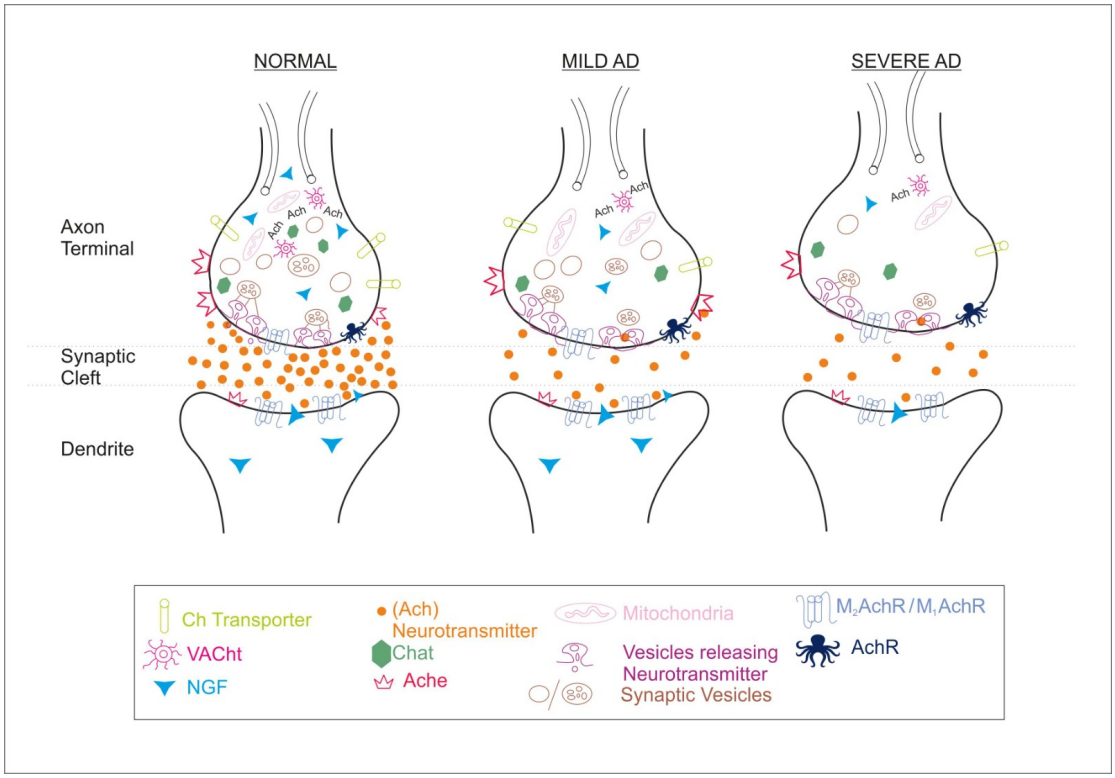


Figure 2.4: Schematic illustration of the cholinergic neuron-to-neuron transmission in different stages of Alzheimer's disease

Reprinted from (Altinoglu and Adali, 2020)

BChE is a non-specific cholinesterase enzyme that hydrolyzes a variety of choline-based compounds. BChE is very similar to AChE, where it serves as a backup to AChE by breaking

down ACh that diffuses out of the synaptic cleft. Hence, they both act to terminate the actions of ACh. In short, rivastigmine offers a dual inhibition of AChE and BChE enzymes. Literature suggests that this dual inhibition may provide additional therapeutic potential of rivastigmine. Additionally, rivastigmine is preferred due to its ease of use, as it is also offered as an oral solution and transdermal patch, and its good tolerability by patients (Dhillon, 2011). While AChEIs remain to be an important therapeutic tool against Alzheimer's disease, the chronic use of AChEIs show some adverse effects and not well tolerated among AD, including vomiting, nausea, anorexia, diarrhea, hypotension, bradycardia, increased hypermotility and hypersecretion patients (Ali et al., 2015). Moreover, despite their symptomatic benefits, none of these drugs are disease-modifying agents. By definition, disease-modifying agents are drugs that can slow down or inhibit the progression of the neurodegenerative process, and thus reduce the pathological load. To this end, such interventions may act to replenish the neural loss or even prevent it from happening. This way, they may have the potential to modify the disease rather than targeting the symptoms alone. A wide range of candidate drugs with varying mechanisms of action have been examined in recent years with *in vitro* and *in vivo* animal models. Despite their promising initial outcomes, they were unable to prove effective to humans in early clinical trials (Aprahamian et al., 2013), either due to limited efficacy or toxic effects (Hampel, 2012).

CHAPTER 3
BLOOD BRAIN BARRIER

The human brain is the most delicate organ of the human body. As a result of years of evolution, the central nervous system (CNS) has developed efficient ways to protect itself from contamination with foreign substances, including neurotoxic molecules, pathogens and circulating blood cells, that could lead to alterations in the inner and outer environments of neural cells. This, in turn, would cause impairments in neural transmission and in the control of bodily processes (Shatzmiller et al., 2016). For protection, the brain is surrounded by a series of blood-brain interfaces with varying degrees of permeability, including the blood-brain-barrier (BBB) and the blood cerebrospinal fluid barrier. The BBB is the major structure responsible for brain protection, as it is the primary physical interface between the brain and systemic circulation. It acts to maintain brain homeostasis. It serves as a highly selective and semi-permeable gateway to preserve the chemical composition of the neural milieu at homeostatic levels necessary for proper neural functioning (Brambilla et al., 2011). It regulates the influx and efflux of fluids through a dynamic combination of molecular, cellular, vascular and ionic factors (Teleanu et al., 2018). The BBB consists of microvascular endothelial cells that are connected by tight junctions, and supported by various cell types surrounding the endothelium e.g. glial cells such as astrocytes ad pericytes (Vegt et al., 2010), as well as a non-cellular component – the basement membrane (Figure 3.1).

3.1 Blood-Brain-Barrier Transport

In addition to being a "physical barrier", BBB also contains transport proteins and enzymatic barriers to functionally restrict the entrance of molecules. To this end, the BBB is also considered as a "transport barrier" and a "metabolic barrier". All of these barriers help to severely regulate the uptake and efflux of molecules between the brain and circulating blood. Regarding the transport barrier, various transcytosis mechanisms are present to mediate the transfer of molecules, while the metabolic barrier is produced by the expression of different metabolizing enzymes e.g. peptidases, monoamine oxidases and cytochrome P450 enzymes (Rooy et al., 2011).

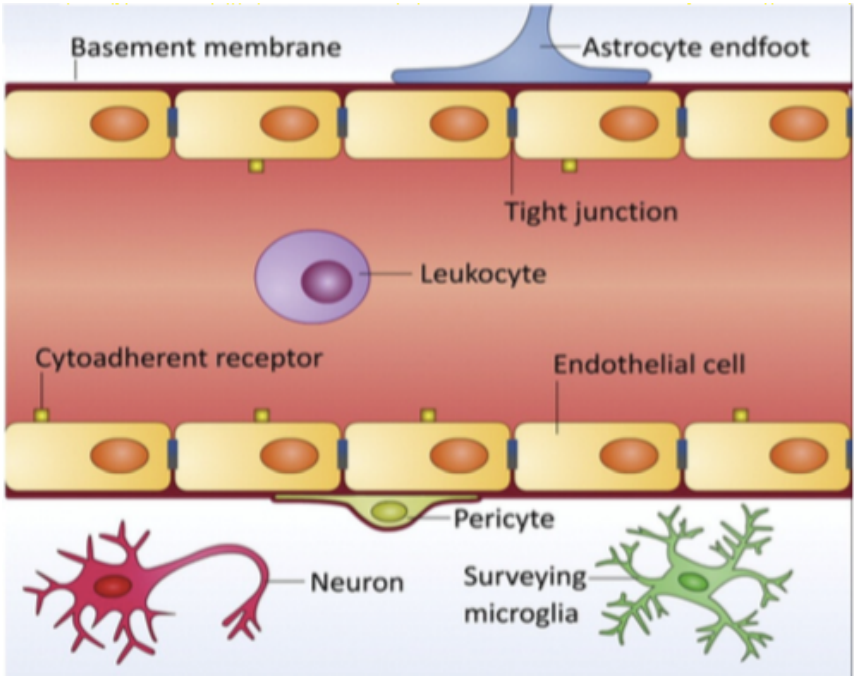


Figure 3.1: Blood-brain-barrier composition. It is a border of endothelial cells, connected by tight junctions. It is supported by a basement membrane and glial cells (astrocytes and pericytes) surrounding the endothelium
Reprinted from (Saraiva et al., 2016)

The transfer of essential molecules across the BBB is necessary for the normal physiological functioning of the brain. This transport can rely on either passive or active (energy-demanding) processes. Lipid soluble and non-polar solutes, such as carbon dioxide and oxygen, is known to passively diffuse from high concentrations to lower concentrations through the BBB, with no input of energy. For the transport of small molecules, there are three major routes that can employed depending on their characteristics (Li et al., 2019). The first one being paracellular transport, is a type of diffusion that occurs through an aqueous pathway. It is severely limited to the transfer of small hydrophilic molecules by the regulation of the transient relaxation of tight junctions connecting the endothelial cells (Barar et al., 2016). In other words, paracellular transportation does not take place to a greater degree at the BBB. In addition to paracellular transport, there is also trancellular transport, both of which are non-competetive and non-saturable. It has been revealed that small

molecules with appropriate lipophilicity and charge can enter the brain *via* transcellular diffusion. The higher the lipophilicity of a molecule, the higher is the rate of transcellular diffusion across the BBB (Pardridge, 1998). Nonetheless, given that drugs are too lipophilic, this might also give rise to their seclusion by the capillary bed and lead to a failure in reaching the BBB (Banks, 2009). Lastly, most of the remaining molecules move across the BBB through a substrate-specific process, that is driven by a transporter facilitated pathway. Endogenous substances such as glucose, amino acids and nucleosides are examples of compounds that employ a transporter mediated pathway. This type of transport is dependent on the concentration gradient of molecules across the BBB. These transporters can either be defined as receptors or carriers.

Larger molecules on the other hand e.g. peptides, proteins and antibodies, are transported across the BBB through endocytosis. In total, there are seven major specific and non-specific mechanisms of transport. Two of these mechanisms are passive pathways, that take place across the tight junctions of the BBB or through the transcellular lipophilic route. The remaining five mechanisms are active transport mechanisms including carrier-mediated transcytosis, receptor-mediated active efflux carrier transcytosis, adsorptive mediated transcytosis and through tight-junction modulation (He et al., 2018).

Despite the various crossing pathways to cross the BBB, nearly 98% of small molecules and majority of large molecules fail to bypass the BBB (Gürsoy-Özdemir et al., 2017). As a result of the highly selective nature of the BBB, the entry of solutes or drugs into the CNS is severely restricted, including the ones that may be of good therapeutic value (Leszek et al., 2017). It has been reported that many therapeutic agents that demonstrate promising results *in vitro*, are larger than 500 Da and water soluble. Thus, they are unable to penetrate the BBB in significant quantities (Mahar Doan et al., 2002). Additionally, charged molecules or compounds having rotatable bonds have a lower potential to cross the BBB. To this end, advances in the treatment of neurological conditions has been so far extremely deferred due to the fact that most potentially useful therapeutic agents fail to bypass the BBB (Pardridge, 2012) (Stamatovic et al., 2008) (Rautio et al., 2008) (Georgieva et al., 2014). Therefore, it is sensible to say that the clinical failure of pharmaceutical drugs can be attributed to the inadequacies in the method by which they are delivered, rather than the potency of the drug.

Clinical trials of therapeutic agents may have been abandoned as necessary drug concentrations in the CNS cannot be reached *via* conventional delivery mechanisms. Thus, new delivery methods are being explored to more successfully deliver pharmaceutical agents across the BBB.

3.2 Bypassing the Blood-Brain-Barrier: Nose-To-Brain

Numerous methods have been studied to overcome the limitations of the BBB and potentiate the CNS transfer of therapeutic agents. These strategies can be categorized as invasive and non-invasive techniques. Invasive approaches are neurosurgical-based, which usually includes the administration of drugs *via* intracerebral or intracerebroventricular delivery. Despite being considered an aggressive method of delivering drugs to the brain, invasive methods are extensively employed. However, they are not practical for use regarding their safety, cost and convenience. Invasive methods include BBB modulating agents, biological tissue delivery, intracerebral implants and intraventricular/interstitial drug delivery methods (Garg et al., 2015) (Lu et al., 2014). Non-invasive methods, on the other hand, utilizes the endogenous mechanisms of transport and incorporate the use of biological, chemical and colloidal drug carriers. They lack the neurosurgical component of invasive methods and thus provide ease of use and are more desirable. Non-invasive methods include the chemical modulation of BBB, prodrug approach, carrier mediated drug delivery and alternative routes of administration, which involves intranasal delivery.

Intranasal delivery is a route to deliver therapeutic compounds to the CNS through the nasal cavity. It was first established in 1989 for the CNS targeting of neurotrophic factors (Hanson and Frey, 2008). It offers a non-invasive, practical manner of bypassing the BBB. Clinically, it offers many benefits owing to its ease of administration, accessibility and better patient compliance (Gaia et al., 2011). The intranasal route allows for the passage of drugs that are unable to bypass the BBB by themselves to be transported to the CNS within minutes. Additionally, it can directly deliver drugs that are capable of crossing the BBB, without entering the systemic circulation and thus avoiding the gastrointestinal and hepatic metabolism. This way, nasal delivery improves the bioavailability of drugs, and give rise to fewer systemic side effects. Furthermore, therapeutic agents do not essentially require to be modified for nasal delivery (Hanson and Frey, 2008). The intranasal route contains the

olfactory and trigeminal neural pathways, which are the only parts of the CNS having unique connections with the external environment (Alpesh et al., 2009). Both of these pathways innervate the nasal cavity, enabling a direct CNS delivery. In other words, intranasally administered drugs are delivered to the brain *via* the olfactory and trigeminal neural pathways. Although the exact mechanisms of transport are still uncertain, it is suggested that nose-to-brain delivery take place *via* neuroepithelium and may include paracellular, trancellular and neuronal transport, where the drug is taken into neurons by endocytosis and transported *via* intracellular axonal transport (Mustafa et al., 2016). Besides these direct pathways that involve the olfactory and trigeminal nerve systems, CNS transport can take place *via* the blood vasculature and cerebrospinal fluid (CSF) present in the nasal mucosa (Kozlovskaya et al., 2014). The highly vascular nature of the nasal mucosa makes it an ideal area for the absorption of molecules into the blood. However, once again, drugs that have been absorbed into systemic circulation face with various limitations including the BBB and systemic elimination (Figure 3.2). Overall, intranasal delivery has been employed in the brain targeting of a wide variety of therapeutics and many reports have confirmed their effectiveness (Chen et al., 1998) (Hanson et al., 2004) (Banks et al., 2004) (Yu et al., 2005) (Han et al., 2006). However, it should be noted that intranasal administration of drugs possesses some limitations that must be taken into consideration during the development of nasal formulations as they are rapidly eliminated from the nasal cavity due to mucociliary clearance.

With conventional nasal solutions, low drug transfer levels have been reported (Ul Islam et al., 2020). As a solution, nanoparticulate drug delivery mechanisms have been utilized, which provide enhanced nasal penetration, stability and drug absorption (Godfrey et al., 2018). Although the exact mechanism of transfer for an increased drug absorption is not clear, nanotechnology-based nasal delivery systems have been exceptionally promising (Kumar et al., 2016). It has previously been shown that nanoemulsion particles that were 100 nm in size successfully penetrated the olfactory bulb and reached the CNS while 900 nm particles failed to do so (Ahmad et al., 2017). This suggests that there may be a cut-off point regarding the size of the particles capable of penetration. Many studies have explored the brain delivery of therapeutic agents *via* intranasal delivery comparing the effectiveness of conventional vs nanoparticulate nasal solutions.

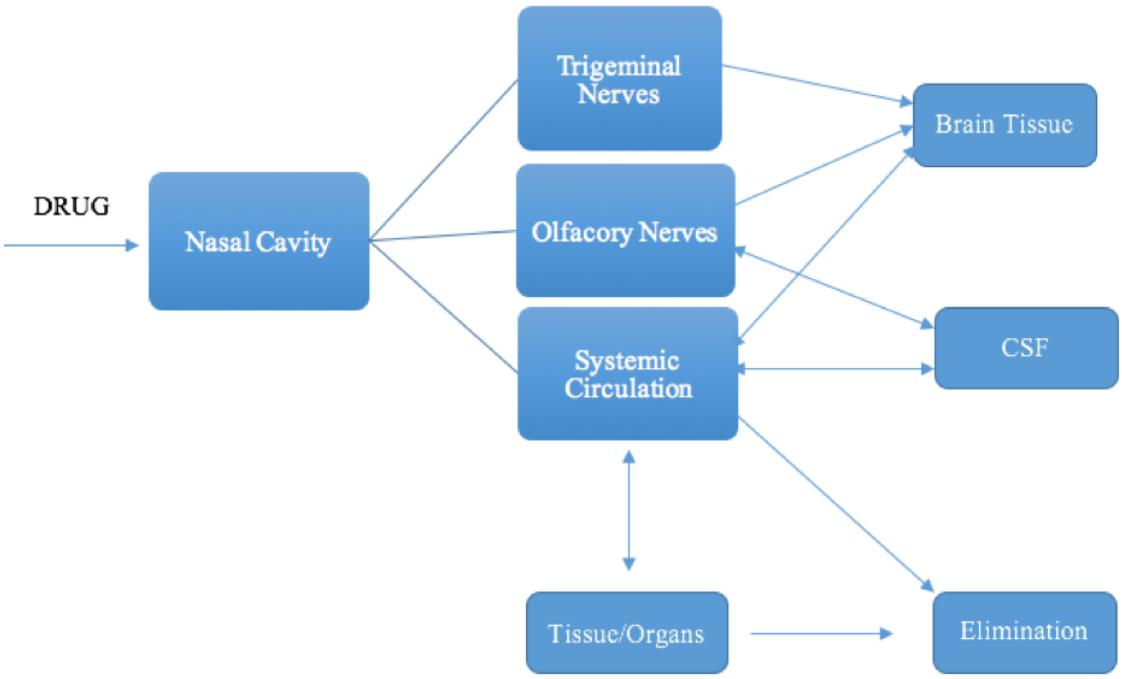


Figure 3.2: Possible fate of drugs following intranasal administration, demonstrating the 3 routes of drug transport from the nasal cavity

Adapted from (Kumar et al., 2016)

It has been demonstrated that therapeutics failed to reach the rat brains in considerable amounts when administered conventionally. However, upon their nano-based intranasal delivery, their delivery was significantly increased (Uchegbu et al., 2019) (Godfrey et al., 2018) (Shah et al., 2018), highlighting the importance of nanoparticle mediated approaches in the delivery of therapeutic agents. This approach includes the administration of drugs associated with nano-sized carriers, that are designed to (i) protect drugs from enzymatic degradation; (ii) enhance drug absorption; (iii) modify pharmacokinetic properties; and (iv) overcome biological barriers due to their reduced dimensions ranging from 1 to 1000 nm.

CHAPTER 4

NANOTECHNOLOGY

For the first time in 1959, Richard Feynman has introduced the concept of nanotechnology as a process in which individual atoms and molecules can be manipulated (Feynman, 1960). It was over a decade later when Professor Norio Taniguchi invented the term of "nanotechnology" (Nikalje, 2015). By definition, nanotechnology is a broad term combining science, engineering and technology, that is conducted at the nanoscale. Nanotechnology has been utilized in medicine for the purpose of developing novel drug delivery systems (Safari and Zarnegar, 2014) to benefit from their ability to provide targeted drug delivery, and/or controlled release of therapeutics, which leads to reduced dosing frequency and improved patient compliance. Therefore, it is suggested as an encouraging alternative for the detection and treatment of neurodegenerative diseases including AD. Although new treatment strategies are being developed, brain targeting remains very challenging and nano-carriers have proven effective in solving this problem. Drug loaded nanoparticles are considered as one of the most versatile delivery vehicles for the transport of drugs targeted at unreachable areas like the brain. To this end, various types of nanoparticle-mediated carriers have been developed for AD treatment, which will be discussed in the following sections.

Nanotechnology has thoroughly advanced in recent years, and this has allowed for novel treatment options for AD. Nanoparticle (NP) based approaches are becoming promising tools as drug carriers due to being able to manipulate their size, shape, hydrophobicity, coating, chemistry and surface charge (Birrenbach and Speiser, 1976). By definition, nanoparticles are microscopic particles that are capable of interaction within biological systems and are at least one dimension below 100nm in size. NPs consist of an internal core and a surface layer that can be functionalized with various molecules (Khan et al., 2017). Conventional treatments pose limitations regarding their passage across the BBB and transport into the CNS. NPs are able to overcome these biological barriers through the modification of different surface characteristics in order to optimize their pharmacodynamic and pharmacokinetic profiles resulting in efficient drug delivery. Advantages of nanoparticle

based drug delivery are their biocompatibility, biodegradability, stability, controlled/sustained drug release, low toxicity and immunogenicity response in addition to their ability to provide a lower quantity and frequency of dosing which in turn minimizes any resulting side effects (Wen et al., 2016) (Table 4.1).

Table 4.1: Advantages and disadvantages of nano-mediated CNS drug delivery (Altinoglu and Adali, 2020)

Advantages	Disadvantages
1. Facilitates BBB transport for CNS delivery	1. BBB transport is not indicative of biological activity
2. Good stability, biocompatibility and biodegradability	2. May influence the physiology of any given cell in the body
3. Drug protection against enzymatic degradation	3. Limited information on the metabolic fate and pathway
4. Improved half-life and circulation time	4. Possible aggregation
5. Improved bioavailability	5. May cause toxicity
6. Controlled and sustained drug release	6. Possibility to further disease progression
7. Minimizes the quantity and frequency of dosage	

4.1 Nanoparticle Characterization Techniques

NP are generally characterized for surface morphology, average particle size, size distribution, zeta potential, and structural analysis.

4.1.1 Imaging techniques/microscopy for nanoparticle morphology

For analyzing particle morphology, imaging methods like scanning electron microscopy (SEM) and transmission electron microscopy (TEM) that utilizes a beam of electrons can be employed. This is due to the fact that conventional optical microscopy is not feasible due to the extremely small dimension of the particles. To this end, more advanced techniques like SEM and TEM are developed to enable the visualization of such small particles. In addition to these methods, atomic force microscopy (AFM) is considered as an alternative technique for characterizing sample morphology. Besides morphology, with these techniques, the size

of NPs can also be examined by measuring the image height of the NPs, given that the NPs are placed on a flat substrate (Scalf and West, 2006) (Hubenthal et al., 2012).

1. Scanning Electron Microscopy: First developed in 1948, scanning electron microscopy utilizes a high energy electron beam that is scanned over the surface of samples, where the back scattering of the electron beam is measured (Rochow and Tucker, 2013) (Bogner et al., 2007). SEM provides a high-resolution imaging of samples with sizes below 10 nm (Hodoroaba et al., 2016) and hence preferred in the characterization of nano-scale systems. Suitable sample preparation is essential in obtaining high quality SEM images. Prior to introduction of a sample to the microscope, the sample must initially be coated to provide electrical conductance to its surface, typically by sputter coating of non-conductive material (unless they are already conductive e.g. metal samples). Once prepared, the sample is placed and fixated on a stub, under high vacuum chamber (Muscariello et al., 2005). It is critical to generate a uniform distribution of particles across the entire measurement substrate. SEM imaging without the coating process can also be achieved, for the option of acquiring SEM micrographs of samples that are wet and/or uncoated, by using a low pressure gaseous environment in the chamber, referred to as environmental SEM (Donald et al., 1998).

Energy dispersive X-Ray (EDX) composition analysis is an X-ray technique, used together with SEM, to provide elemental identification. It can be utilized for both qualitative and quantitative analysis, allowing for the identification of the type of elements that are present, and the percentage of each element's concentration. The data generated consist of spectra with peaks that correspond to the elements making up the chemical composition of samples. It is dependent on the atomic mass of the elements being detected.

2. Transmission Electron Microscopy: First developed in 1930s, transmission electron microscopy has been used as a visualization technique in which a beam of electrons is transmitted through a sample (Guston, 2010). The interaction of electrons with the sample as the beam is transmitted leads to the formation of an image. The sample is most often a suspension or an ultrathin section that is placed in a high vacuum chamber, similar to SEM. Environmental TEM is also available for by using low pressure gas chamber. When compared to SEM, TEM utilizes stronger electron beams and thus offers an even higher

image resolution with better quality. TEM is considered to be the most popular imaging technique in analyzing the morphology of nanomaterials. However, both TEM and SEM are able to demonstrate the size, dispersion, heterogeneity and degree of aggregation of nanoparticles.

4.1.2 Zeta potential

Zeta potential, also referred to as the electrokinetic potential, is a principal indicator of the stability of colloidal dispersions against aggregation (Deryabin et al., 2015). It is the charge that develops at the interface between a solid surface and its liquid medium. Its main focus is obtaining information about the surface charge of a material. The surface of particles is surrounded by two layers; an inner layer, also known as the Stern layer, that includes ions bound relatively tightly; and an outer layer, or the diffuse layer, where ions are loosely bound to the surface (Shnoudeh et al., 2019) (Figure 4.1). When the particles are in motion within a liquid environment, the ions in the Stern layer will move together due to being firmly bound, whereas ions in the diffuse layer will not move together. The electrical potential formed at the diffuse layer is the measured zeta potential value. It is measured by the addition of a solution to a cell containing two gold electrodes. Upon the application of a voltage to the electrode, particles move toward the electrode with the opposite charge, as like chargers repel and opposite charges attract. Then, particle velocity is measured as a function of voltage, at multiple voltages to calculate the zeta potential.

The magnitude of the zeta potential, which is measured in millivolts (mV), demonstrates the degree of electrostatic repulsion between similarly charged adjacent particles in a colloidal dispersion. ZP values are usually between +100 and −100 mVs. It is suggested that NPs with zeta potential values of > +25 mV or < −25 mV normally have higher degree of stability. Lower zeta potential values are also known to give rise to aggregation and coagulation of particles due to van der Waals interparticle attractions (Sapsford et al., 2011).

4.1.3 Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a characterization method used for the identification or quantification of samples by producing a unique molecular fingerprint for many different components. It identifies the chemical bonds in a molecule by creating an infrared absorption or emission spectrum. FTIR spectroscopy is possible owing to the fact that each molecule creates a distinctive fingerprint that can be used for chemical identification. It is carried out by sending an infrared radiation through a sample, with some radiation absorbed and some passing through, in other words, transmitted. The resulting signal at the detector is converted to an interpretable spectrum, representing the molecular fingerprint of the sample, that can be used to detect functional groups and thus covalent bonding information in materials.

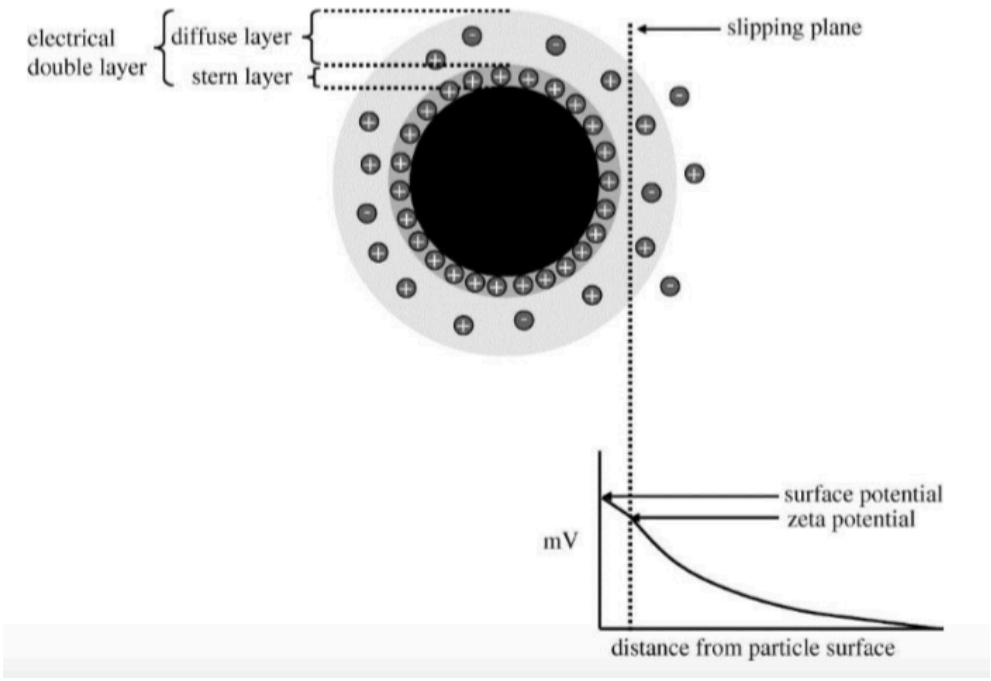


Figure 4.1: Representation of the Stern layer and diffuse layer on a particle's surface Reprinted from (Kaszuba et al., 2010))

4.2. Important NP Characteristics for Brain Drug Delivery

There are several factors that need to be considered when designing NPs. Along with their need to be biocompatible, biodegradable, non-toxic and non- reactive, their size, zeta potential, molecular weight, structural conformation, lipophilicity and the affinity for cellular proteins are all among the important characteristics that need to be taken in to consideration when designing brain targeted NPs (Shadab et al., 2012). All of these features allows for optimal BBB passage, stability, ability to escape from rapid circulation clearance, and drug release (Goldsmith et al., 2014).

BBB transfer and thus the brain delivery of NPs is influenced by several factors. Many studies have revealed a negative correlation between the ability to overcome the BBB and particle size (Sonavane et al., 2008) (Etame et al., 2011) (Hanada et al., 2014). NPs with diameters between 50 – 100 nm have been favored in order to achieve successful transport in animal models (Saraiva et al., 2016). Smaller sized particles have also been linked to a more uniform distribution within the body (Decuzzi et al., 2010) as well as more sustained duration of action (Banerjee et al., 2002). Accompanying this, smaller sized particles also influence drug loading and release. However, larger NPs with larger internal cores provide a more efficient drug encapsulation in terms of quantity, where larger cores can store higher amounts of drug per particle. Larger NPs also result in slower drug release when compared to smaller NPs which release their contents rapidly. This is due the larger surface area to volume ratio of smaller NPs, whereby the drug encapsulated is nearer the NP surface (Redhead et al., 2001). However, drug loading and drug release is not only limited to drug particle size but also an array of other various factors depending on the biomaterial of choice and drug of interest.

In terms of surface characteristics, nanoparticles differ in shape ranging from spherical to rod shaped, cubic and cylindrical NPs. NP shape impacts not only BBB uptake but also their clearance and distribution (Decuzzi et al., 2010). They also differ in terms of surface charge (positive, zwitterionic, and negative), which is another factor influencing their tendency to bypass the BBB. As mentioned previously, zeta potential represents the surface charge on NPs and functions as an indication of particle stability (Pan et al., 2012). Findings have revealed that majority of CNS targeted NPs have moderate (-1 to -15 mV) or highly negative

(-15 to -45 mV) zeta potentials (Bramini et al., 2014) (Wiley et al., 2013). Highly positive zeta potentials have been correlated with the induction of BBB toxicity, however, some NPs with zeta potentials of 15 mV or above have been successfully used in drug delivery (Jallouli et al., 2007). NPs that are neutral and zwitterionic have also been found to provide longer circulation time and increased clearance in contrast to positively and negatively charged NPs (Arvizo et al., 2011). The exact mechanism of transport across the BBB is yet to be elucidated. However, studies suggest their transport occurs through diffusion, endocytosis and/or transcytosis (Doggui et al., 2012), which are dependent on the physiochemical characteristics of NPs.

There are various types of NPs that can be classified into different categories, main ones being polymeric NPs, lipid-based NPs, liposomes and nano-emulsions.

4.3. Types of Nanoparticles

4.3.1 Polymeric nanoparticles

Polymeric NPs are solid colloidal particles capable of transfer of therapeutic compounds through their encapsulation (Cupaioli et al., 2014). Due to their biocompatible, biodegradable and stable nature in addition to their low toxicity and immunogenicity properties, these particles have been extensively studied as suitable nano-carriers (Naahidi et al., 2013). They can be prepared from both natural polymers (e.g. polysaccharides, amino acids, or proteins) (Karatas et al., 2009), and synthetic polymers (e.g. poly (lactic-co-glycolic acid) (PLGA), poly (n-butylcyano-acrylate) (PBCA), poly(ε-caprolactone) (PCL), and poly (lactic acid) (PLA)), or from inorganic materials, such as silicon dioxide, gold and alumina (Saraiva et al., 2016). NPs acquired from natural polymers has the benefit of delivering biological signals for specific receptors and transporters, however are accompanied by some intrinsic limitations due to restrictions posed by their extent to be modified. Synthetic polymers, on the other hand, can be modified to acquire a diverse range of characteristics (Cupaioli et al., 2014). Polymeric NPs are prepared through a range of methods e.g. polymer polymerization, ionic gelation, spray drying, nano-precipitation and emulsion solvent evaporation (Cheng et al., 2015). The technique used differs based on the characteristics of the particular polymer used and the encapsulated material in order to

produce the desired NP.

4.3.2 Lipid-based nanoparticles

Lipid based NPs are nano-particulate systems that have efficient loading capabilities, drug degradation protection, prolonged and controlled drug release as well as low toxicity. They are the first generation of nanoparticle-mediated delivery systems with the capability of crossing the BBB *via* endocytosis due to being lipophilic in nature. Moreover, their lipophilic nature aids in the ease of loading of therapeutics and also in surface functionalization (Gastaldi et al., 2014). In comparison to polymeric NPs, lipid carriers have increased drug loading capacities, that enables greater control over drug release (Mishra et al.., 2010). They do, however, possess some limitations in the form of poor loading of hydrophilic drugs and poor *in vivo* stability (Md et al., 2018).

Lipid nanoparticles can be classified into two categories; solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs). SLNs consist of a solid lipid core matrix where drugs can be dispersed or dissolved in, and they range in sizes starting from as small as 40 nm to 200 nm (Kaur et al., 2008). Their nano size allows them to evade the clearance systems and pass through the BBB endothelial cells (Neves et al., 2015). Various preparation techniques can be used to produce SLNs including high pressure homogenization, spray drying, ultra-sonication/high-shear technique, solvent emulsification – diffusion, evaporation, and double emulsion techniques where they can be synthesized from lipids, emulsifying agents and water/solvent that are biocompatible for humans (Robinson et al., 2015). NLCs however consist of both solid and liquid lipid cores. These NPs were produced in order to overcome the limitations of SLNs that include poor drug loading capacity and expulsion during storage (Fang et al., 2013).

4.3.3 Liposomes

Liposomes are multiple layered phospholipid bilayers that form spherical vesicles by self-assembling around an aqueous core. They are classed either as uni- lamellar or multi-lamellar (Hadavi and Poot, 2016). They range in sizes from 50 to 100 μm. Liposomes are not only used as non-toxic and biocompatible transporters of lipophilic or hydrophilic drugs,

but can also entrap amphiphilic and hydrophobic agents within the aqueous core owing to their phospholipid nature. Therefore, they are capable of transporting a wide range of therapeutic agents that occur either *via* lipid mediated diffusion or endocytosis. Liposome preparation consists of the thin lipid film followed by agitation, sonication, high-pressure homogenization, reverse-phase evaporation, and extrusion (Fonseca-Santos et al., 2015).

4.3.4 Nano-emulsions

Nano-emulsions are nanometric-scale water-in-oil (W/O) or oil-in-water (O/W) emulsions that vary in droplet sizes ranging between 20 to 200 nm. (Fernandes et al., 2010). These kinetically stable and thermodynamically metastable NPs are heterogeneous mixtures that consist of oil droplets, surfactant(s)/stabilizers and a suitable aqueous medium e.g. water (Wen et al., 2016). Nano emulsion preparation includes the use of high energy input methods e.g. high-pressure homogenizers, ultrasound generators and phase inversion temperature techniques (Fernandes et al., 2010). The oil of choice used in the preparation of these emulsions dictates their ability to be uptaken into the CNS. Their poor stability during storage which gives rise to subsequent burst–release effect is among the limitations for this system (Silva et al., 2013). Despite this disadvantage, many studies conducted have revealed that nano-emulsion mediated systems enhance drug delivery to the brain.

CHAPTER 5

NANO-ENABLED ANTI-AD APPROACHES

5.1. Nano-enabled Anti-Aβ Strategies

Neuroprotective peptides have recently indicated promise in terms of a therapy strategy for AD (Saraiva et al., 2016). With Aβ aggregation being the most commonly conveyed pathological hallmark of AD, these agents function through the breakdown of Aβ plaque formation by degrading the AB peptide as well as modulating Aβ cleaving enzymes (Salomone et al., 2012). Neuro-regenerative approaches also aim to seek to reconstruct damaged tissue and reverse disease aetiology (Cummings, 2009). On this basis, numerous nano-carrier systems have been evaluated.

Aβ sub-fragments have been suggested to protect nerve cells from AD pathology, however, due to having low permeability through the BBB, *in vivo* applications have been restricted. Songjiang and Lixiang (2009) synthesized a NP system, using the widely used biocompatible and biodegradable natural polymer, chitosan, and loaded with Aβ sub-fragments. Results revealed that this nano-mediated delivery system was able to carry out Aβ delivery to the brain with a marked immunogenic response. These results solidify the importance of peptide vaccine research for AD. Another promising strategy is the employment of iAβ5 peptide. iAβ5 is a peptide that is a shorter anti-β-sheet peptide with key advances in its capability to hinder Aβ fibrillogenesis. However, this peptide is not stable and easily degraded by proteases, therefore, unsuitable for use in *in vivo* applications (Permanne et al., 2002). Taking this in consideration, loading NPs with this peptide poses an attractive approach for targeting AB fibrillogenesis. PLGA NPs are another type of widely used polymeric carriers owing to their biocompatibility, enhanced drug loading capacity and ease of surface functionalization, with low toxicity issues (Bala et al., 2004). A study conducted, employed iAβ5 peptide loaded PLGA NPs and surface functionalized with two antibodies; an anti-transferrin receptor monoclonal antibody OX26 (surface receptor on the brain capillary endothelium) and anti-Aβ DE2B4 (Aβ targeting peptide). Results demonstrated that CNS delivery for functionalized NPs was considerably improved when

compared with NPs with no functionalization, with the intention of enhancing their brain concentration and weakening the senile plaques.

Another major neuroprotective peptide established in the central and peripheral nervous system is vasoactive intestinal neuropeptide (VIP). Along with its anti-inflammatory effects, VIP has been shown to prevent excitotoxicity mediated neural death and Aβ induced toxicity in both *in vivo* and *in vitro* models (Brenneman et al., 1998) (Gozes et al., 2003). The limited bioavailability and instability of VIP hindered the process of developing a successful VIP based drug delivery system to treat AD. Therefore, the encapsulation of this peptide within NPs for successful brain delivery was sought out by various research groups. One group incorporated I^{25} vasoactive intestinal peptide (I^{25} VIP) into surface modified PEG-PLA NPs. They stated a 2-fold enhanced uptake of surface functionalized NPs in comparison to uncoated NPs. Another study from Zhang et al. (2014) established a dual-functional Aβ-targeting system, with PEGylated PLA NPs, surface modified with two targeting peptides; TGN and QSH. TGN was intended to target BBB ligands for BBB transfer, while QSH was used for specific targeting of Aβ plaques owing to its high affinity for Aβ1-42 peptides. They reported an increased and accurate delivery in AD mice models providing a successful targeting system. In conclusion, surface modified polymeric NPs are a positive avenue of research for the efficient and successful delivery of anti-amyloid agents.

Another attractive therapeutic approach is the encapsulation of hormones into NPs. Preclinical studies have revealed that gonadal steroids (e.g. estrogens and androgens) play a key part in the functioning of the brain, whereby they can influence a diverse range of brain processes (Rubinow and Schmidt, 2002). Estrogen has also been implicated to play a role in the reduction of cerebral amyloid aggregates and positively influence the growth and survival of cholinergic neurons in the brain (Amtul et al., 2010). Mittal et al. (2007) loaded estradiol in PLGA NPs with varying molecular weight and polymer composition to investigate the effect of drug release. Results revealed that tweaking of these two variables produced a 10-fold increase in drug bioavailability in comparison to free estradiol. Following this, they have coated the surface of NPs with tween-80. Surface coated PLGA NPs produced a marked increase in brain estradiol levels in comparison to non-coated PLGA NPs 24 h after oral administration. The comparison regarding the preventative effect of both

NPs also unveiled the successful inhibition of Aβ42 immunoreactivity expression for coated NPs compared to non-coated NPs, signifying the impact of surface coating for drug delivery (Mittal et al., 2011).

5.2. Nano-enabled Anti-Tau Strategies

The clinical manifestation of AD symptoms is closely linked with Tau aggregation (Chen et al., 2018). However, encapsulation of tau inhibitors is an avenue that has not been extensively studied. Some approaches have been undertaken to encapsulate anti-tau agents in order to examine their therapeutic relevance. Among them, is a tau aggregation inhibitor methylene blue (MB) also known as methylthioninium chloride. This drug has been observed to slow down the onset of AD progression along with other taupathies. It is, however, restricted by its highly hydrophilic nature which in turn limits its bioavailability and effectiveness in the brain (Karthivashan et al., 2018). Following this, glutathione coated PLGA NPs have been synthesized in order to increase its availability in the brain and were reported as appropriate for BBB penetration. Studies on Transwell-based BBB models demonstrated an enhanced transfer of MB loaded NPs in comparison to the free drug solution alone. MB NP administration was therapeutically examined on two different *in vitro* AD models expressing the tau protein. They reported a significant decline in endogenous and over-expressed tau protein levels upon MB loaded NP administration (Jinwall et al., 2014). Another study conducted by Chen et al. (2018) encapsulated MB intended to target hyper-phosphorylated tau *in vivo*. Results revealed a decline in tau hyper-phosphorylation, mitochondrial oxidative stress and neuronal loss. Along with this, AD rats exhibited a substantial memory reversal in mice, signifying its potential as a tau-targeting nano-system (Chen et al.,2018). Taking into account the role that protein misfolding plays in protein aggregation, molecular chaperones may serve as an attractive target for therapeutic intervention. Molecular chaperone and protein folding catalyst heat shock protein 70 (HSP70) is involved in the protein misfolding process and has received great interest as a possible therapeutic target in tackling AD disease progression (Lu et al., 2014). The drug MKT-077, a highly water soluble rhodacyanine dye with suggested anti-cancerous properties, has exhibited encouraging results through targeting the tau mediated HSP70 pathway (Koya et al., 1996). Its potential use in the treatment of AD, however, is restricted

by its limited BBB transport along with significant toxicity issues. To address these problems, PEG-PLGA NPs coated in 2% glutathione were synthesized and loaded with MKT-077, and provided a successful penetration across an *in vitro* BBB model, with a considerably highly permeation that standalone MKT-077 solution. In terms of therapeutic action, MKT-077 NPs also displayed a decrease in tau aggregates in *in vitro* experimental models (Jinwal et al., 2014), holding promise for AD and other tauopathies.

Nicotinamide, the amide form of vitamin B3, has not only been related with neuronal development and survival but also appears to possess neuroprotective effects (Fricker et al., 2018). Preclinical studies have revealed cognitive enhancement upon nicotinamide treatment, hence solidifying the efficacy of nicotinamide therapy on AD progression (Fillit et al., 2017). Although this treatment is established and can readily cross the BBB, nicotinamide suffers from a sink condition and is only available at relatively low concentrations, making it necessary to take multiple doses per day (Vakilinezhad et al., 2018). To solve this issue, a nano- based delivery system was developed. Vakilinezhad et al. (2018) prepared nicotinamide loaded SLNs that were functionalized with one of the three different coatings; polysorbate 80, phosphatidylserine or phosphatidic acid. Results unveiled the benefits of functionalization in improving brain bio-distribution. These particles were also deemed safe in the *in vitro* cytotoxicity tests except for polysorbate 80 coated NPs. A decline in tau hyper- phosphorylation and neuronal cell preservation in AD rats were observed for phosphatidylserine coated Nicotinamide-NPs in parallel with the results of spatial and memory test. Following these results, it can be concluded that these NPs can be successfully employed as a delivery system in order to overcome the setbacks of conventional nicotinamide administration therapies in order to target AD more efficiently.

5.3. Nano-enabled Cholinergic Strategies

The dysfunction of the cholinergic system is known to have a major effect on the learning and memory of patients with AD (Francis, 2005). Currently, the most established therapeutic method for AD patients is the inhibition of AChE in order to enhance cholinergic transmission (Karthivashan et al., 2018). The non-competitive and reversible inhibitor of AChE enzyme, rivastigmine, is commonly used for the treatment of AD patients. Yet, it's

efficacy is relatively restricted due to its poor brain uptake that ultimately results in adverse cholinergic effects of peripheral organs (Ahmad et al., 2017). For this reason, many studies have attempted to encapsulate rivatigmine in NPs to increase the chances of successful delivery to brain and decrease potential side effects. A study conducted Wilson et al. (2008) involved the preparation of polysorbate 80-coated PBCA NPs in order to increase the brain bioavailability of rivastigmine in rats *via* intravenous injection. Results displayed a 3.82-fold increase in the uptake of the drug in comparison to its free administration (Wilson et al., 2008). Another study conducted by Joshi et al. (2010) encapsulated rivastigmine not only with PBCA NPs but also with PLGA NPs. They demonstrated an enhanced brain uptake of rivastigmine along with an improved therapeutic effect with increased memory regain on scopolamine-induced amnesic mice. In addition to this, numerous studies have loaded rivastigmine in chitosan NPs for AD therapy purely for its ease of manufacture and cost effectiveness in comparison to other biodegradable polymers (Hanafy et al., 2015), where they all achieved increased brain concentrations of the drug *via* the intranasal route (Wang et al., 2008) (Bhavna et al., 2014) (Alam et al., 2012), supporting the notion to use AChEI-loaded NPs in AD treatment.

Other AChE inhibitors have also been loaded in NPs to target AD. One includes the drug Tacrine, which was the first drug to be approved for the use in AD treatment in 1993 (Tumiatti et al., 2010). It was, however, discontinued due to its poor tolerability. Nonetheless, tacrine loading into NPs has been investigated. A study conducted, synthesized tacrine loaded PBCA NPs with altered drug polymer ratios. The results revealed that the brain concentrations of intravenously injected 1% polysorbate-80 coated tacrine NPs were improved up to 4.07 fold compared to the free drug (Wilson et al., 2008). Wilson et al. (2010) conducted a similar study by using tacrine loaded chitosan NPs to test the bio- distribution on rats after intravenous injection. Their findings showed that NPs coated in 1% polysorbate 80 enhanced brain drug concentrations, with minimal reticuloendothelial system uptake and longer half-lives, further solidifying the role this coating plays in effective brain drug delivery. Another AChE drug, Galantamine was also encapsulated in order to overcome the limitation of the BBB and improve its brain bioavailability. This drug was effectively delivered to the brain upon intranasal administration in hydro-bromide incorporated chitosan NPs with higher transport efficiency in comparison to its oral administration, with no toxicity

issues (Hanafy et al., 2015). A more recent study used galantamine loaded thiolated chitosan NPs to successfully deliver galantamine to the CNS and established a significant recovery in amnesia induced mice (Sunena et al., 2019). Along with AChEIs, the direct effects of ACh brain delivery have also been tested. Fan et al. (2018) were the first to establish that ACh-loaded human serum albumin NPs may result in a better therapeutic outcome with enhanced spatial learning and memory, while reducing OS in mice. The above formulations illustrate that the delivery of AChEIs and ACh itself *via* nano-mediated approaches holds significant potential to be developed as a treatment option with enhanced brain targeting in comparison to their conventional delivery.

5.4. Nano-enabled Antioxidant Strategies

An additional method for the treatment of AD focuses on the delivery of antioxidants to the brain, owing to their capability of neutralizing oxidative stress mediated free radicals and their association in the prevention of neurodegenerative diseases (Leszek et al., 2017). Most antioxidants, however, are quickly metabolized and eradicated from the body meaning they have very limited bioavailability. As a potential solution, use of antioxidant loaded NPs have been proposed. The yellow curry spice, curcumin, acquired from rhizome of the curcuma species has medical properties as well as playing a neuroprotective, anti-oxidant and anti-inflammatory role in the body (Brambilla et al., 2011). *In vitro* and *in vivo* studies have also revealed the positive effects of curcumin in AD pathology. It acts by decreasing the formation of Aβ from APP protein, inhibits metal induced Aβ formation, Aβ aggregation, as well as destabilizing preformed Aβ fibrils (Huang et al., 2012) (Zhao et al., 2012) (Baum and Ng, 2004). In this regard, Sood et al. (2013) synthesized curcumin/donepezil loaded SLNs. Results revealed enhanced brain concentrations of the drug following intranasal administration in addition to improved learning and memory behaviors in rats. Kakkar et al. (2013) also evaluated brain targeting through oral administration in rats by loading curcumin into SLNs. Results showed a 2-fold increase in the concentration of curcumin in the brain along with improved AChE activity when compared to the free drug. Polymeric NPs such as PBCA NPs have also been used to encapsulate curcumin. Although the biological activity of curcumin in the body has not yet been evaluated for the process of encapsulation itself, it has significantly improved the circulation time and concentration in the brain when

compared to free curcumin (Sun et al., 2010) (Mulik et al., 2009).

Another agent that has received great attention in the treatment of AD owing to its neuroprotective effects against Aβ toxicity (Jang and Surh, 2003), antioxidant and anti-inflammatory properties (Soleas et al., 1997) is resveratrol. Resveratrol is a natural polyphenol found in the seed and skin of grapes and red wine (Markus and Morris, 2008). This drug, however, similar to curcumin, is rapidly metabolized and does not reach target organs. It is therefore crucial to stabilize resveratrol in order to conserve its biological activity and improve its bioavailability. Frozza et al. (2010) synthesized resveratrol loaded SLNs and administered them into mice to reveal a 3 to 6-fold increase in concentration of resveratrol in the brain, liver and kidneys accompanied by improvements in memory through the nano-encapsulation technique.

Another potential agent in the treatment of AD is the antioxidant ferulic acid. This antioxidant is a naturally occurring and abundant phenolic found in the cell wall of plants. To this end, Bondi et al. (2009) synthesized ferulic acid loaded SLNs and showed that these SLNs were capable of significantly reducing radical oxygen species when compared to free ferulic acid alone. Although the neuroprotective effects of antioxidant compounds are clear, it is important to point out that no human clinical trials have been undertaken concerning their role as a therapeutic in the treatment of AD. All of the mentioned nano-enabled AD therapies in Chapter 5 and more are illustrated in Table 5.1

Table 5.1: Nano-enabled drug delivery systems for AD therapy (Altinoglu and Adali, 2020)

Nano-delivery Systems	Therapeutic agent	Category	Proposed Target Hypothesis	Carrier Material	References
Polymeric NPs	Rivastigmine	AChEI	Cholinergic system	PnBCA	(Wilson et al., 2008) (Joshi et al., 2010) (Fazil et al., 2012)
				PLGA	
				Chitosan	
	Galantamine-hydrobromide	AChEI	Cholinergic system	Chitosan	(Hanafy et al., 2015) (Fornaguera et al., 2015)
	Donepezil	AChEI	Cholinergic system	PLGA	
				Chitosan	(Bhavna Shadab et al., 2014) (Md et al., 2014) (Baysal et al., 2017)
				PLGA	
				(PEG)PLGA	
	Galantamine	AChEI	Cholinergic system	Chitosan	(Sunena et al., 2019)
	Tacrine	AChEI	Cholinergic system	Chitosan	(Wilson et al., 2008) (Wilson et al., 2010)
				PnBCA	
	Acetylcholine	Proteins and peptides	Cholinergic system	Human serum albumin	(Fan et al., 2018)
	Aβ sub-fragments	Anti-amyloid	Amyloid cascade	Chitosan	(Songjiang et al., 2009) (Agyare et al., 2008)
	Coenzyme Q10	Anti-amyloid and	Amyloid cascade	PLGA	(Wang et al., 2010)
		antioxidant	and oxidative damage		
	PPAR- γ agonist	Anti-amyloid	Amyloid cascade	PEG-PLGA	(Silva-Abreu et al., 2018)
	Methylene blue	Tau aggregation inhibitor	Tau cascade	PEG-PLGA	(Jinwal et al., 2014)
	MKT-077	Tau aggregation inhibitor	Tau cascade	PEG-PLGA	(Jinwal et al., 2014)
		Proteins and peptides	Neuroprotective	PEG-PLGA	(Zhang et al., 2014)

Table 5.1 continued					
	Nerve growth factor	Proteins and peptides	Cholinergic system	PnBCA	(Kurakhmaeva et al., 2009)
	VIP	Proteins and peptides	Anti-amyloid and antioxidant	PEG-PLA	(Gao et al., 2007) (Kanwar et al., 2009) (Gozes et al., 2003)
	iAβ5- β-sheet breaker peptide	Proteins and peptides	Amyloid cascade	PLGA	(Loureiro et al., 2016)
	TGN and QSH	Proteins and peptides	Targeted delivery to amyloid cascade	PEG-PLA	(Zhang et al., 2014)
	NAPVSIPQ (NAP)	Proteins and peptides	Neuroprotective peptide	PEG-PLA	(Liu et al., 2013)
	Estradiol	Hormones	Amyloid cascade and Cholinergic system	Chitosan, PLGA	(Wang et al., 2008) (Mittal et al., 2007) (Mittal et al., 2011)
	Melatonin	Hormones	Oxidative stress	Eudragit S100®	(Schaffazick et al., 2005)
	Mifepristone	Hormones	Amyloid cascade	PLGA	(He et al., 2007)
	Curcumin	Polyphenols (Antioxidant)	Oxidative stress	PnBCA PLGA	(Sun et al., 2010) (Mulik et al., 2009)
	Resveratrol	Polyhphenols (Antioxidant)	Oxidative stress	PEG-PCL	(Lu et al., 2009)
	ECGC	Polyphenols (Antioxidant)	Oxidative stress	Chitosan	(Dube et al., 2010)
	Piperine	Polyphenols (Antioxidant)	Oxidative stress	Chitosan	(Elnaggar et al., 2015)
	Nano-N2PY	Chelating agent (Iron)	Metal induced oxidative stress	Polystyrene	(Liu et al., 2009)
	D-penicillamine	Chelating agent (Copper)	Metal induced oxidative stress	MPB-PE	(Cui et al., 2005)
Solid Lipid NPs	Galantamine hydrobromide	AChEI	Cholinergic system	Glycerylbehnate (compritol)	(Misra et al., 2016)
	Rivastigmine	AChEI	Cholinergic system	Compritol 888 ATO	(Shah et al., 2015)
	Curcumin	Polyphenols (Antioxidant)	Cholinergic system		(Kakkar et al., 2013)

Table 5.1 continued					
	Resveratrol	Polyphenols (Antioxidant)	Oxidative stress		(Frozza et al., 2010) (Loureiro et al., 2017)
	Ferulic acid	Polyphenols (Antioxidant)	Oxidative stress	Compritol 888 ATO	(Bondi et al., 2009) (Picone et al., 2009)
	epigallocatechin-3-gallate (ECGC)	Polyphenols (Antioxidant)	Oxidative stress and amyloid cascade	monophasic liquid preparations	(Smith et al., 2010)
	D-penicillamine	Chelating agent (Copper)	Metal induced oxidative stress	hexadecanol and 1,2-dioleoyl-sn-glycero-3-phospho- ethanolamine-N-[3-(2-pyridyldithio)-propionate]	(Cui et al., 2005)
	Nerve growth factor	Proteins and peptides	Cholinergic system	(Heparin-conjugated stearic acid; stearylamine-cationic lipid; esterquat	(Kuo and Rajesh., 2017)
	Nicotinamide	Neuroprotective agent	Tau cascade	Polysorbate 80, phosphatidylserine or phosphatidic acid coating	(Vakilinezhad et al., 2018)
Nanostructured lipid carriers	Curcumin-donepezil	Antioxidant-AChEI	Oxidative stress and cholinergic system		(Sood et al., 2013)
Liposomes	Galantamine	AChEI	Cholinergic system	soya phosphatidylcholine, cholesterol, and propylene glycol	(Li et al., 2012)
	Rivastigmine	AChEI	Cholinergic system	EPC, cholesterol, DSPE-PEG-CPP phosphatidylcholine/cholesterol soya lecithin/cholesterol	(Yang et al., 2013) (Mutlu et al., 2011) (Arumugam et al., 2008)
	Donepezil	AChEI	Cholinergic system	1,2-distearyl-sn-glycero-3-phosphocholine (DSPC),	(Al Asmari et al., 2016)

Table 5.1 continued					
	Brain derived neurotrophic factor	Proteins and peptides	Neuroprotective peptide		(Spuch and Navarro, 2011)
	Curcumin	Polyphenols	Oxidative stress and amyloid		(Mourtas et al., 2011)
		(Antioxidant)	cascade		
Nano-emulsions	Huperzine A	Natural AChEI	Cholinergic system		(Patel et al., 2013)
	Ginkgo biloba	Antioxidant	Oxidative stress		(Jin et al., 2013)
	Tabernaemontana	Natural AChEI	Oxidative stress		(Chaiyana et al., 2013)
	divaricate				
	Beta-asarone	Neuroprotective agent	Oxidative stress		(Zhang et al., 2014)
Nanocomposites	Methylene blue	Tau aggregation inhibitor	Tau cascade	CeNC/IONC/MSN-T807	(Chen et al., 2018)

CHAPTER 6

CURRENT NANO-MEDIATED DRUG DELIVERY SYSTEM

In this study, rivastigmine (RT) (Figure 6.1), clinically important reversible inhibitor of AChE enzyme, which has 4-17 times higher specificity for AChE inhibition in the brain as compared to the heart and blood, has been encapsulated (Fazil et al., 2012). RT has been shown to enhance or maintain the cognitive function and behavior of AD patients. Yet, its brain delivery is restricted due to its poor dissolution, absorption and hydrophilicity, which necessitates frequent dosing that results in severe cholinergic side effects (Joshi et al., 2010). To this end, its clinical application is limited, making it an ideal candidate for nano-encapsulation.

As mentioned in Chapter 1, RT is unique in terms of its mechanism of action, as it provides a dual inhibition of ACh breakdown, that may provide an additional therapeutic potential. Additionally, it is suggested that the therapeutic potential of AChEIs may not only be limited to increasing the synaptic availability of ACh, but also provide an anti-inflammatory effect by protecting neurons from inflammation and free radical toxicity. Based on these reasons, RT has been chosen as the drug of interest in the current work.

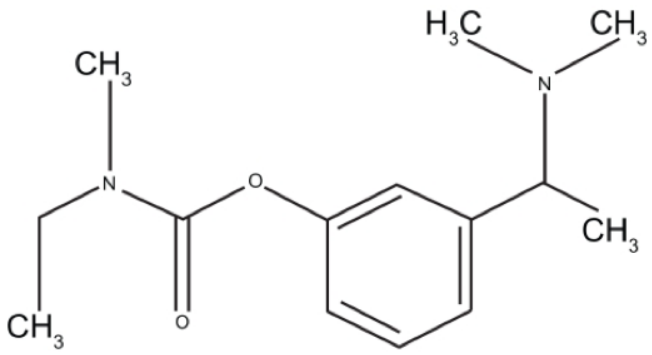


Figure 6.1: Chemical structure of rivastigmine
Reprinted from (Cummings and Winblad, 2007)

The present study aimed at nano-encapsulating RT with a biopolymer, pullulan (PL), that can potentially improve its brain bioavailability, dosing frequency and possible side effects. Polymeric NPs have received increased attention due to their stable, biocompatible and biodegradable nature, with an efficient drug accumulation at the target sites (Joshi et al., 2010). PL is a neutral, water soluble polysaccharide produced from starch by the fungus *Aureobasidium pullulans* that is commonly found in phyllosphere of crop plants and on many tropical fruits (Singh et al., 2015). It is a polysaccharide comprising of maltotriose units connected by 1,6 glycosidic bonds (Rekha and Sharma, 2007). It is non-toxic, non-immunogenic and non-mutagenic in nature and thus it has been employed in various biomedical and pharmaceutical applications (Mishra et al., 2011), including gene delivery, tissue engineering, wound healing and brain drug delivery (Thomsen et al., 2011). PL has a large amount of hydroxyl groups, and thus makes it possible to graft small molecules for surface modification (Figure 6.2). Therefore, based on its ease of chemical modification and potential in nanoformulation, PL has been chosen as the polymeric material of interest for the encapsulation of rivastigmine.

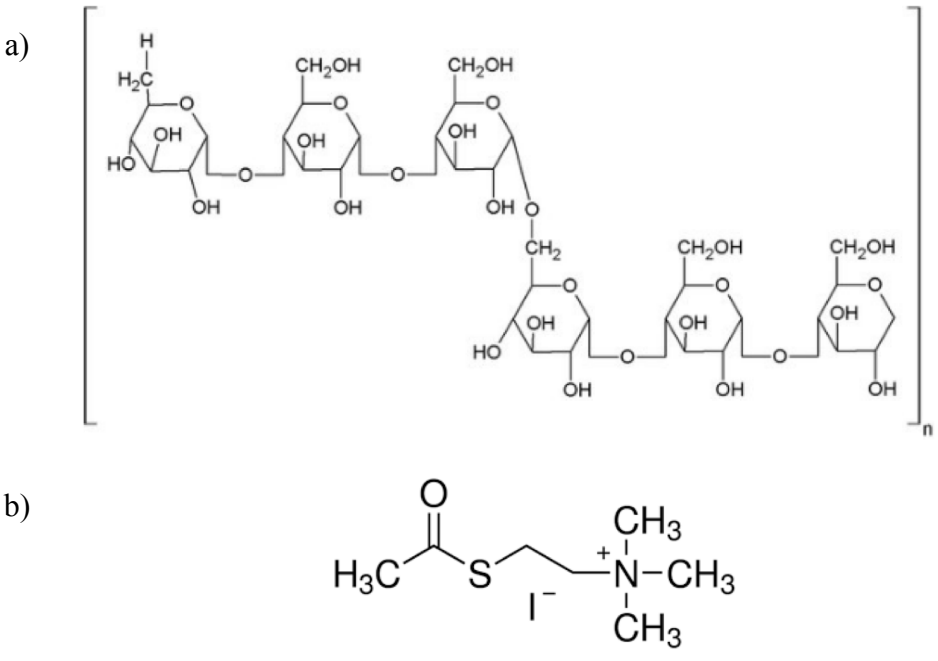


Figure 6.2: Chemical Structure of a) Pullulan Reprinted from (Ferreira et al., 2015) and b) Acetylthiocholine Iodide

In the current study, PL has also been surface modified with an ACh analogue, Acetylthiocholine iodide (ATCh) (Figure 6.2) with the intention of developing a “smart” drug-release model. This way, in the presence of the AChE enzyme, AChE would act on ATCh and break the “cage” to release the encapsulated RT, with the purpose of inhibiting AChE activity and thus ACh degradation. To the best of our knowledge, this will be the first study that surface functionalized PL NP surface with ATCh for the encapsulation of RT.

CHAPTER 7

MATERIALS AND METHODS

7.1 Materials

Pullulan was purchased from J&K Scientific Ltd. Rivastigmine (2 mg/ml) oral solution was purchased from a pharmaceutical warehouse in Izmir/Turkey. Sodium tripolyphosphate pentabasic ($Na_5O_{10}P_3$), ethyl alcohol (CH_3CH_2OH) acetylthiocholine iodide (ATCh) and the reagents for the phosphate buffer, potassium monohydrogen phosphate and potassium dihydrogen phosphate, were purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA. Butyrylcholinesterase from equine serum, chromogenic reagent 5,5′-dithio-2-bis-nitrobenzoate (DTNB), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), were all purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA.

7.2 Methods

7.2.1 Particle synthesis

Prior to NP synthesis, 2% (w/v) PL solution was prepared by the complete dissolution of 2 g PL powder in 100 mL distilled water, and for 0.1 M sodium tripolyphosphate pentabasic (TPP) solution 1.84 g of TPP powder was dissolved in 50 mL of distilled water. PL NPs were obtained upon the dropwise addition of 10 mL 2% (w/v) PL solution to 5 mL 0.1 M TPP solution (distance between the syringe and TPP solution was about 15 cm) stirred at room temperature over magnetic stirring at 100 rpm for 30 minutes, and freeze dried with lyophilisation method. ATCh modified PL NPs were obtained according to the same procedure, with an extra final step following PL addition, which involve the dropwise addition of 625 µL ATCh from a 50 mM stock solution to the formulation. For RT loading, the ratio of PL/TPP remained unchanged, with varying amounts of RT (0.8, 1.0 and 1.2 mL) that were incorporated to PL solution prior to its addition to TPP (Table 7.1).

Table 7.1: Different formulations of Pullulan NPs

Samples	Pullulan (%)	TPP (M)	ATCh added to the medium (µl)	Drug added to the medium (mL)
PL NPs	%2	0.1 M	X	X
RT loaded PL NPs	%2	0.1 M	X	0.8/1.0/1.2
PL/ATCh NPs	%2	0.1 M	625	X
RT loaded PL/ATCh NPs	%2	0.1 M	625	0.8/1.0/1.2

7.3 Particle Characterization

7.3.1 Scanning electron microscopy and energy dispersive x-ray (EDX) composition analyses

Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) composition analyses were carried out to determine the surface morphology of prepared formulations using FEI Quanta 400F (Holland) at METU-BIOMATEN Center of Excellence Ankara Turkey.

7.3.2 Determination of particle size and zeta potential

The particle size, particle size distribution and zeta potential of synthesized NPs were determined by FEI Quanta 400F (Holland) at METU-BIOMATEN Center of Excellence Ankara, Turkey.

7.3.3 Fourier transform infrared (FTIR) analysis

The Fourier transform infrared (FTIR) analysis was carried out for structural analysis to confirm PL-TPP interaction, ATCh modification and RT loading. FTIR spectra were recorded on Perkin Elmer Spectrum-two FTIR Spectrometer in Eastern Mediterranean University. Each sample to be analyzed was used in powder form.

7.4 Determination of the quantity of instant drug release

To measure the release of RT, phosphate buffer was used as a release medium. This have been determined by considering the solubility of drug and the polymeric nano scale system.

Phosphate buffer at pH 7.4 was chosen to mimic the physiological conditions. The masses of samples with varying amounts of RT (1.2, 1.0 and 0.8 mL), were measured using analytical balance. Each sample with a mass of 100 mg was completely dissolved in 10 mL of release medium. UV-Vis spectrum for each sample was measured in the range of 240 to 300 nm as expected λ max was around 264 nm. Absorbance value at λ max for each sample was determined to calculate the concentration of instant drug release. Different concentrations (5, 7.5, 12.5, 15 & 17.5 ppm) of drug were prepared, where these concentrations were directly obtained by dilution from stock solution (100 ppm) and diluted to 2 mL. Different concentrations of RT were analyzed by UV-Vis spectrophotometer to generate the calibration curve shown in Figure 7.1. Calibration curve was later used to determine the amount of RT release from RT loaded NPs.

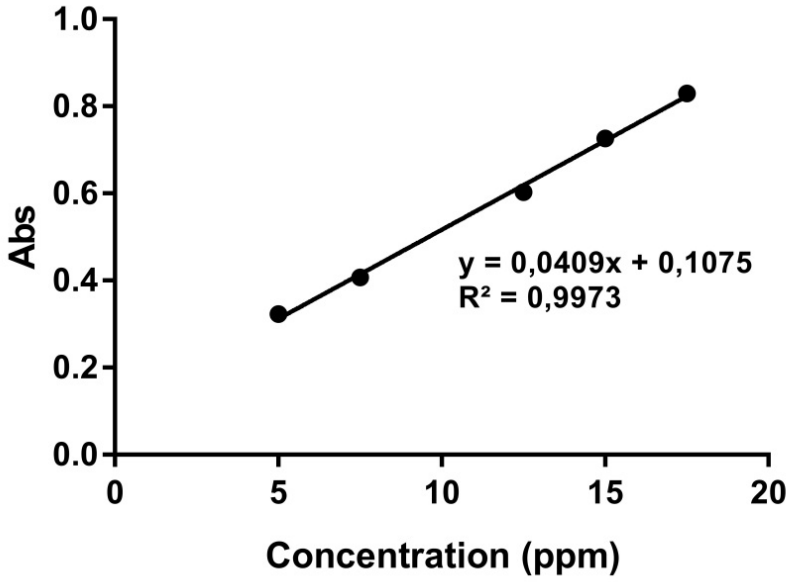


Figure 7.1: Calibration curve for rivastigmine

Determination of percent drug release was also carried out where RT released (%) was calculated as follows;

RT released (%) = $\frac{\text{Released drug (ppm)}}{\text{Loaded drug (ppm)}}$ in 100 mg powdered sample x 100

It also important to note that in order to prove the encapsulation of RT into the pullulan based nano scale system, a similar instant release assay was conducted with slight modifications by using ethanol. Samples with masses of 100 mg were dissolved in 10 mL of ethanol, which is a poor solvent for pullulan, and thus yielding precipitation. Samples were mixed with a glass rod for several minutes to allow the possible drug release. Samples were then centrifuged at 200 rpm for 5 minutes. Supernatant was transferred into clean glass test tube for UV-Vis analysis.

7.5 *In vitro* determination of cholinesterase enzyme activity

Enzymatic hydrolysis of ACh is a crucial step in nerve transport and its impairment is considered as one of the possible underlying mechanisms in AD. Ellman's procedure is a commonly used technique for the determination of ChE activity and thus ACh hydrolysis by AChE and BChE enzymes *in vitro* (Ellman et al., 1961) (as mentioned previously, there are two cholinesterase enzymes; AChE and BChE involved ACh hydrolysis, both of which are inhibited by RT). The original Ellman's procedure is based on the reaction between thiocholine and DTNB (5,5 -dithiobis-2-nitrobenzoic acid), that gives rise to a yellow product ((5-mercapto-2-nitrobenzoic acid and its dissociated forms) that can be monitored spectrophotometrically, where the increase in absorbance is recorded as a measure of ATCh hydrolysis. Therefore, in the current study, the determination of cholinesterase enzyme activity, and thus the inhibitory effect of RT loaded NPs was carried out spectrophotometrically by a slight modification of the Ellman's protocol. Here, the BChE activity in the presence and absence of RT loaded PL/ATCh NPs was investigated.

BChE activity was assayed in a quartz cuvette on a LAMBDA 25 UV/Vis Spectrophotometer. The final reaction volume was 1000 µL and the reaction mixture contained 500 µL 200 mM HEPES buffer, 30 µL distilled water, 100 µL 2.5 mM DTNB, 50 µL BChE enzyme, and 320 µL NP formulation in the presence and absence (control) of RT loaded PL/ATCh NPs (Table 7.2). Components of the reaction mixture (pH 7.5) were prepared as follows; For 2.5 mM DTNB, 9.9 mg DTNB was dissolved in 20 mM MOPS/KOH buffer (pH 7.5), and were brought to a final volume of 10 mL. This produced a final concentration of 2.5 mM DTNB from which 100 µl was picked and added to each

reaction mixture. For BChE enzyme, 1 mg of lyophilized equine BChE enzyme was dissolved in 1 mL of 20 mM buffer, and was diluted 1:100, bringing it to 10 ng/mL, from which 50 µL was added to the reaction mixture. Therefore, the final concentration of BChE in the reaction mixture was 0.5 ng/mL. The lyophilized NPs were prepared to be added to the reaction mixture with distilled water (5 mg/mL). The reaction was initiated by the addition of the NPs. ATCh hydrolysis was monitored at 412 nm for 20 seconds as a result of color development and increase in absorbance at 37 ^{o}C. The cholinesterase activity was calculated according to the equation below.

$$\text{ChE Activity (Unit/L)} = \frac{\Delta Abs412 \text{ X Total Reaction Volume(ul) X } 1000}{13.6 \text{ X Sample Volume (uL)}}$$

ΔAbs_{412}: Absorbance change per minute at 412nm

Total volume: Volume of total activity mixture (1000 µL)

Table 7.2: Test for Enzyme Activity

	CONTROL (µL)	**SAMPLE (µL)**
200 mM HEPES	500	500
Distilled water	30	30
2.5 mM DTNB	100	100
Enzyme	50	50
NP formulation	320	320
Total volume	1000	1000

7.6 *In vitro* coagulation test

As a measure of NP hemocompatibility, parameters of coagulation were assessed *in vitro* by measuring the activated partial thromboplastin time (APTT), prothrombin time (PT) and

international normalized ratio (INR) using Stago-STA Compact machine. Fresh blood, donated by human benefactors provided by Near East University Hospital, Northern Cyprus, was centrifuged using 3.2% (w/v) trisodium citrate in siliconized vacutainer tubes at 1500 rcs for 15 minutes for plasma separation, where 0.025 g of lyophilized samples were submerged into and assessed for coagulation parameters and compared with control/basal values. APTT measures the time it takes for the blood to clot by measuring the integrity of the intrinsic pathway of coagulation cascade while PT measures the integrity of the extrinsic pathway. The INR is also a derivative of PT, and is accepted as the international standard for PT.

CHAPTER 8

RESULTS

Synthesized NPs were analyzed on the basis of morphology, elemental composition, particle size, zeta potential, polydispersity index and structural analysis in addition to quantity of drug released.

8.1 Scanning Electron Microscopy (SEM)

Morphological features of synthesized NPs with and without drug and surface functionalization were studied by SEM. The SEM micrographs of synthesized formulations are shown on Figures 8.1-8.4.

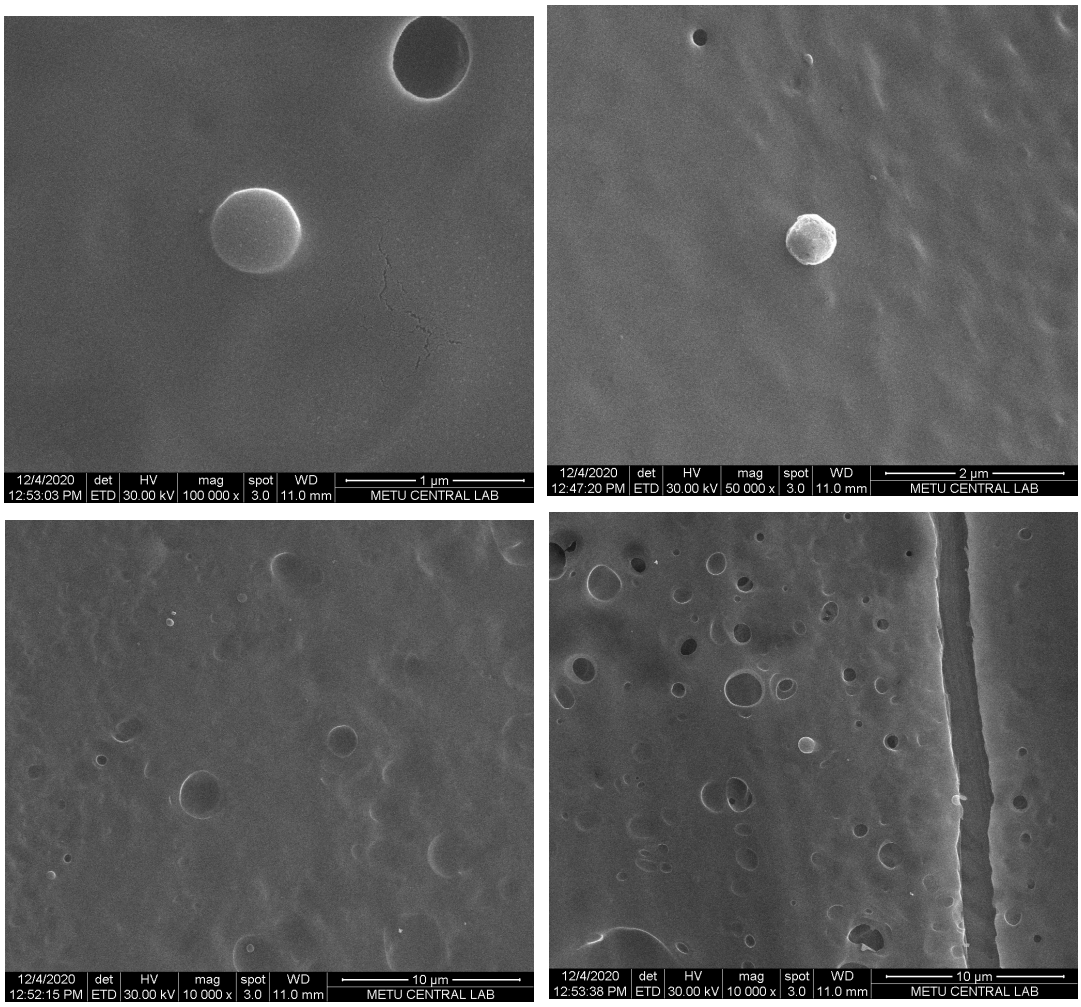


Figure 8.1: SEM micrographs of PL NPs

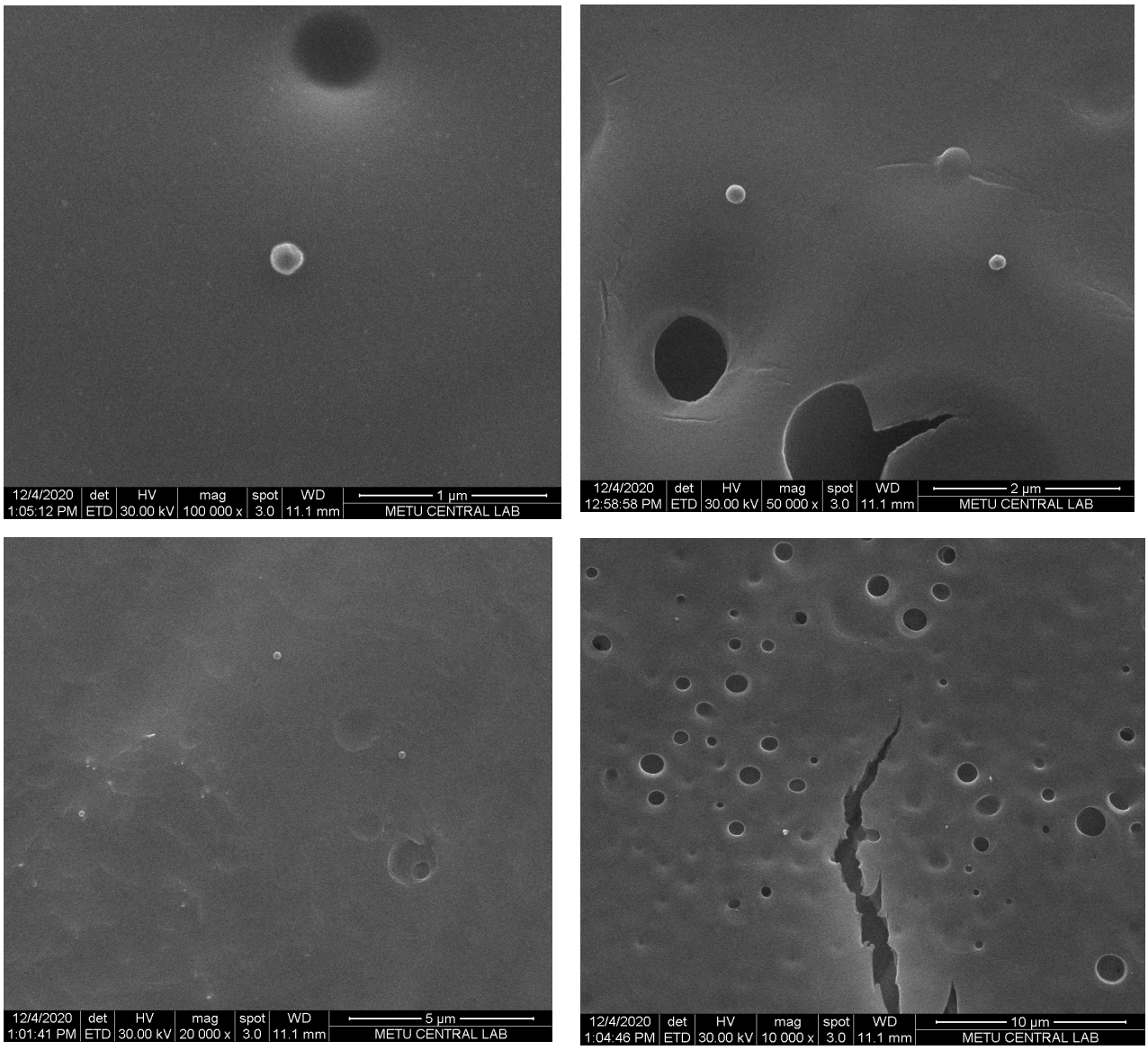


Figure 8.2: SEM micrographs of PL/ATCh NPs

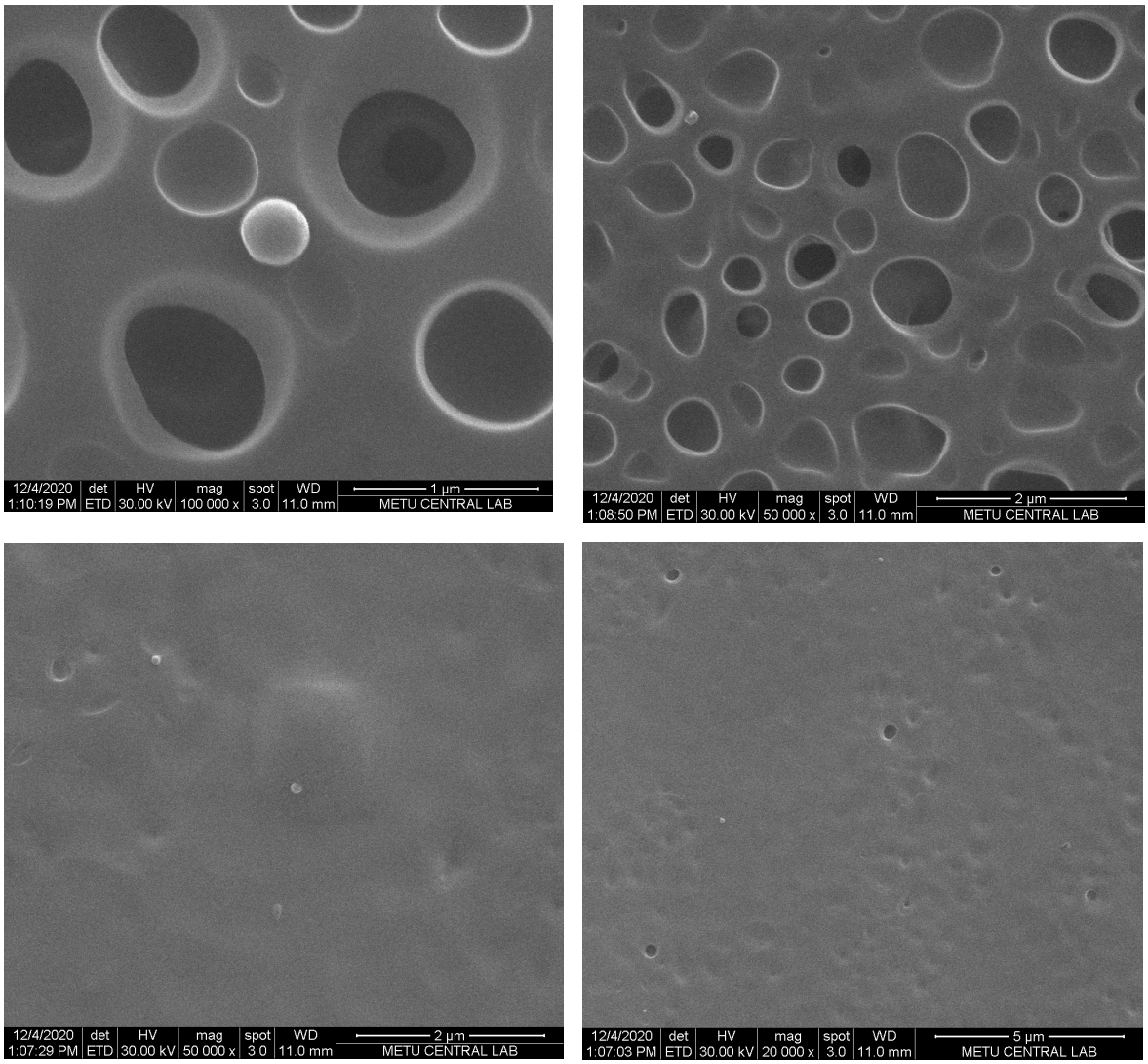


Figure 8.3: SEM micrographs of rivastigmine loaded PL NPs

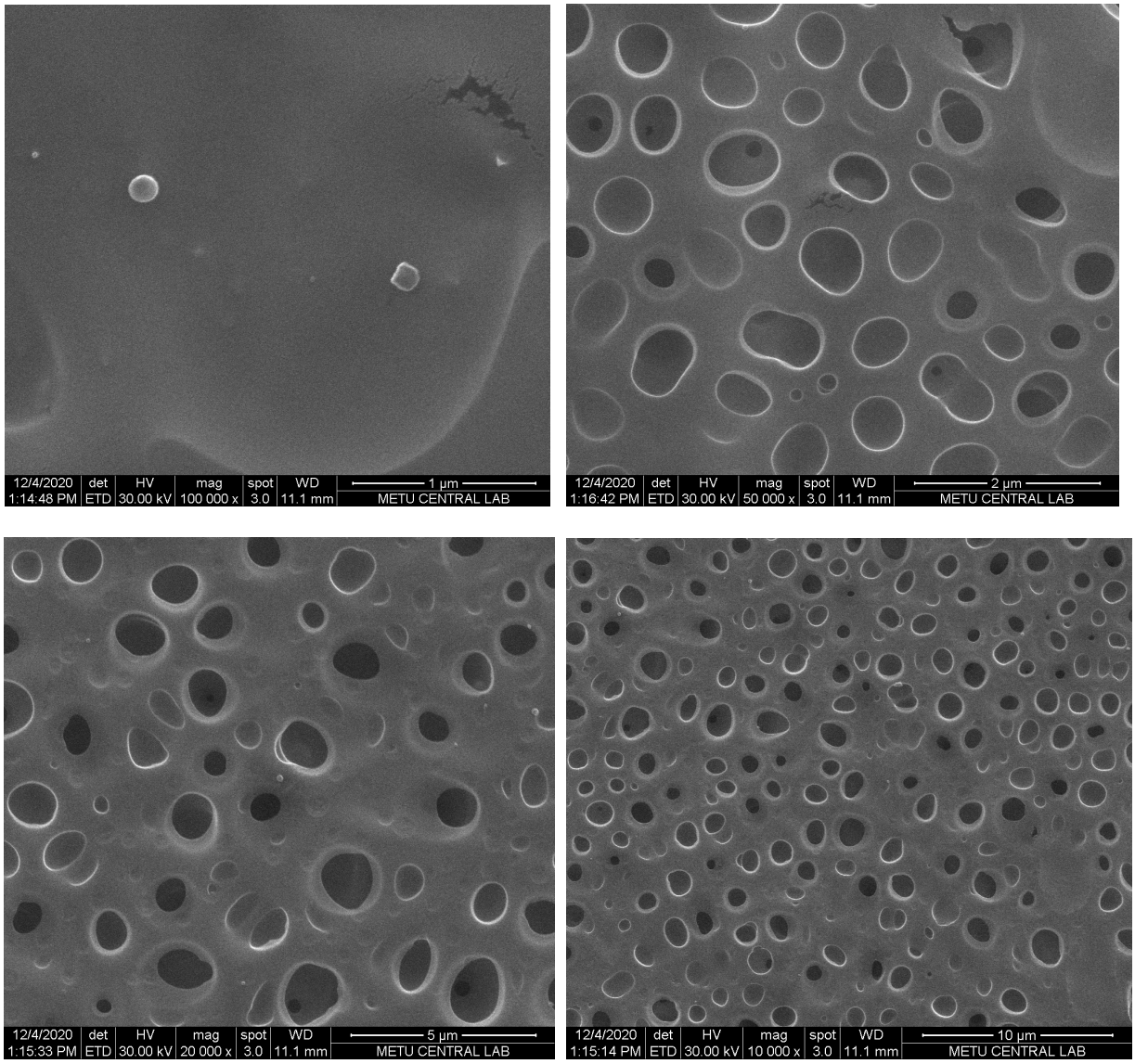
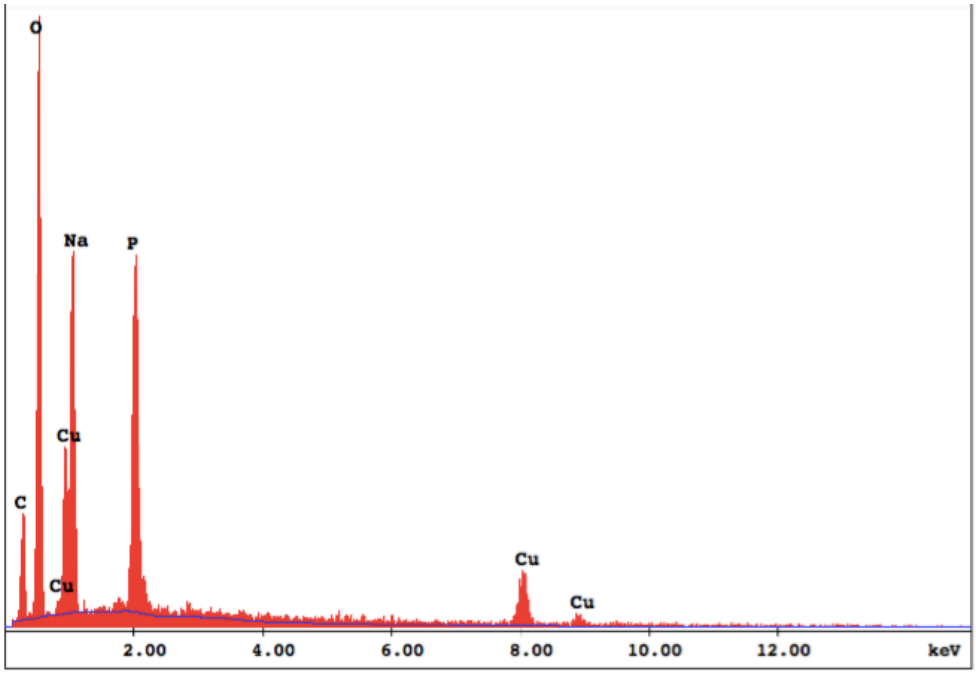


Figure 8.4: SEM micrographs of rivastigmine loaded PL/ATCh NPs

8.2 Energy Dispersive X-Ray (EDX) Composition Analysis

Elemental identification of synthesized NPs was carried out using Energy Dispersive X-Ray (EDX) Composition Analysis. The EDX analysis results are shown on Figures 8.5, 8.6 and Table 8.1.

(a)



(b)

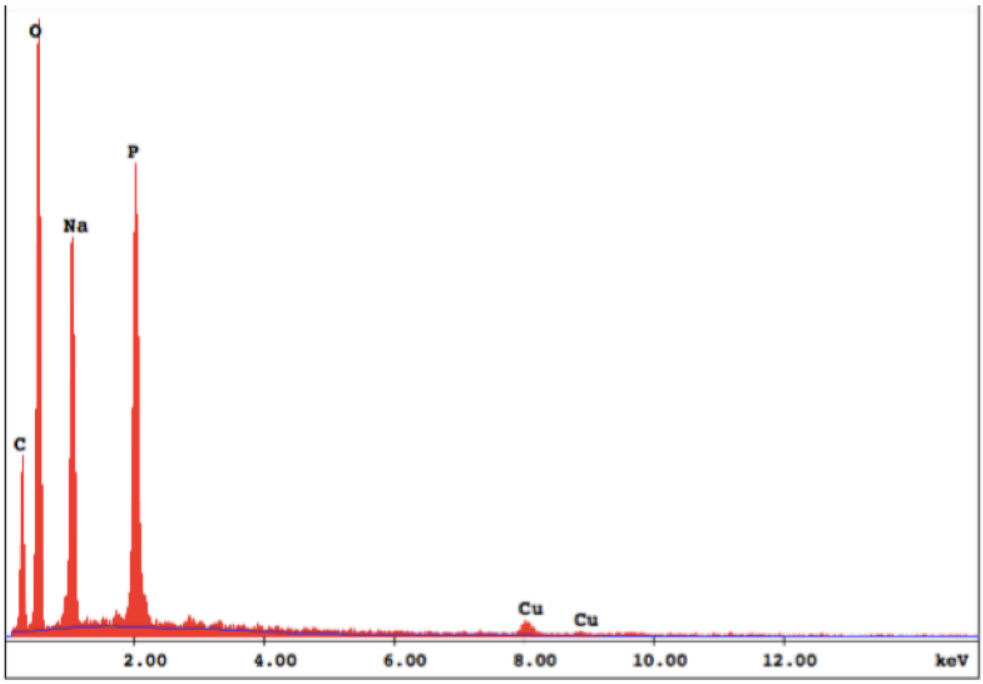


Figure 8.5: EDX elemental analysis results of **(a)** PL NPs **(b)** RT loaded PL NPs

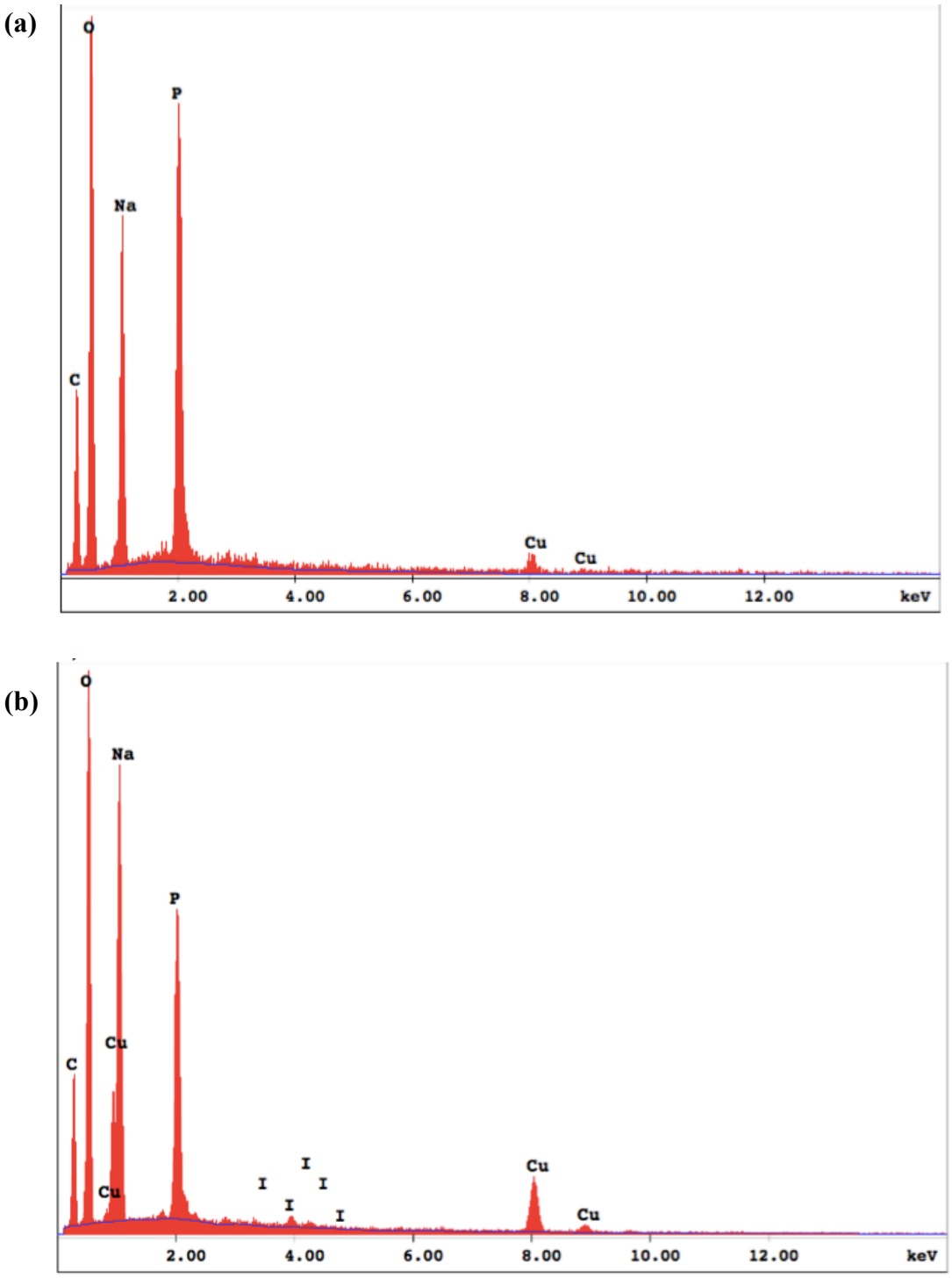


Figure 8.6: EDX elemental analysis results of **(a)** PL/ATCh NPs **(b)** RT loaded PL/ATCh NPs

Table 8.1: EDX composition analysis of synthesized NPs showing the wt% of individual elements

	Elements (wt%)						
Samples	**C**	**O**	**Na**	**P**	**I**	**Cu**	**Total**
PL NPs	25.09	35.60	16.00	11.14	-	12.17	100
RT loaded PL NPs	31.41	39.32	14.02	12.33	-	2.93	100
PL/ATCh NPs	19.37	28.70	18.75	19.01	-	14.16	100
RT loaded PL/ATCh NPs	28.94	32.55	17.29	8.78	1.28	11.17	100

8.3 Zeta Sizer / Zeta Potential

Particle size, zeta potential and particle size distribution of synthesized NPs are shown in Table 8.2 and Figure 8.7. All of the NPs formed were <50 nm in size. The average size of PL NPs with no ATCh modification and drug loading was found to be 33.34 nm while the PL/ATCh NPs had an average of 34.06 nm. The average size of RT loaded PL NPs and PL/ATCh NPs were 31 nm and 32 nm, respectively. All of the formulations had negative zeta potentials ranging from -0.69 to -9.36 mV.

Table 8.2: Size (nm), zeta potential (mV) and polydispersity index of synthesized NPs

Samples	**Z-Average (nm)**	**Zeta Potential (mV)**	**Polydispersity Index (Pdi)**
PL NPs	33.34	-2.79	0.872
PL/ATCh NPs	34.06	-6.34	0.495
RT loaded PL NPs	31.98	-0.69	-0.43
RT loaded PL/ATCh NPs	32.65	-9.36	0.468

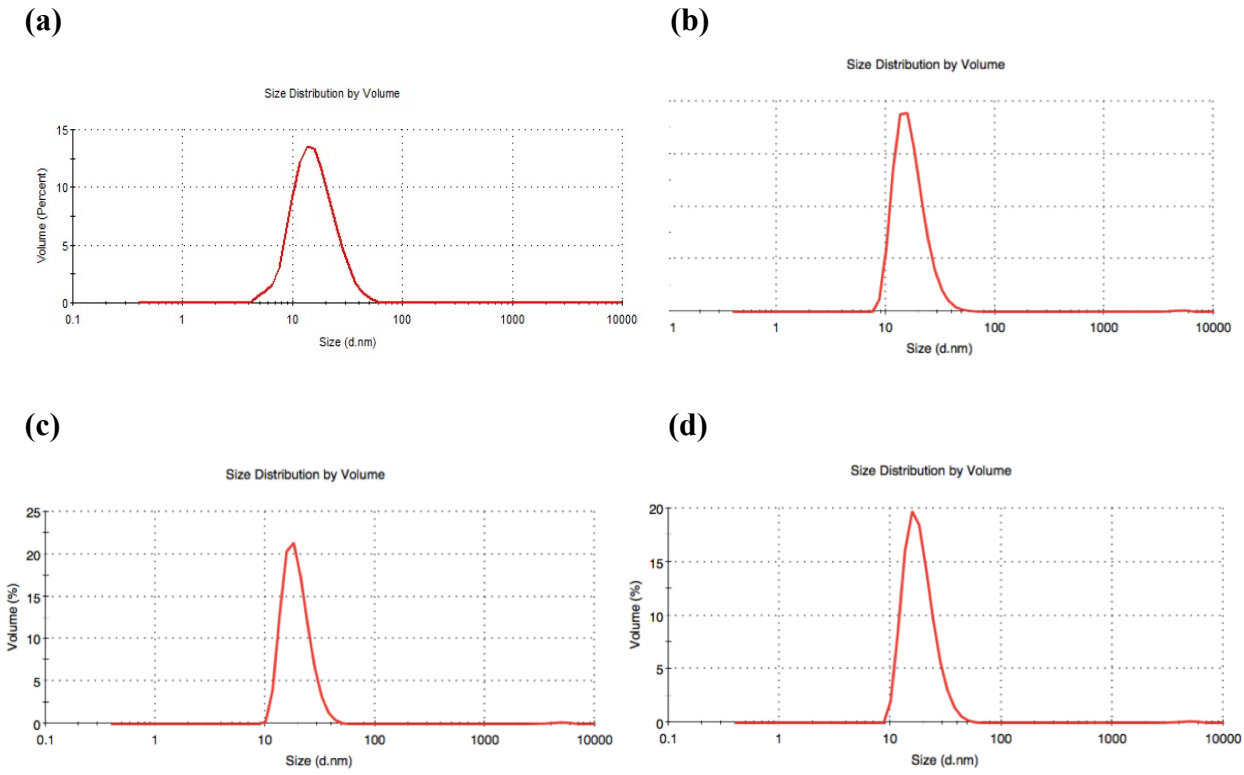


Figure 8.7: Sized distribution by volume for **(a)** PL NPs **(b)** PL/ATCh NPs **(c)** RT loaded PL NPs **(d)** RT loaded PL/ATCh NPs

8.4 Fourier transform infrared (FTIR) analysis

FTIR spectra of synthesized particles are shown in Figures 8.8- 8.11.

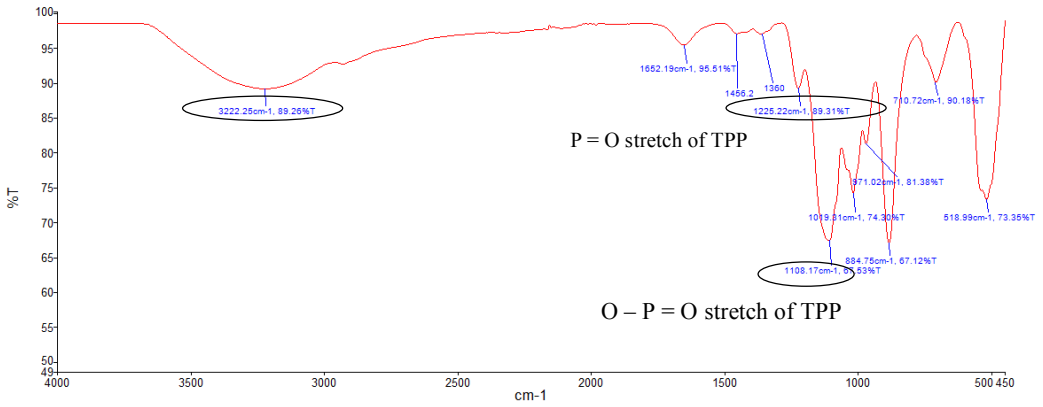


Figure 8.8: FTIR Spectra of PL NPs

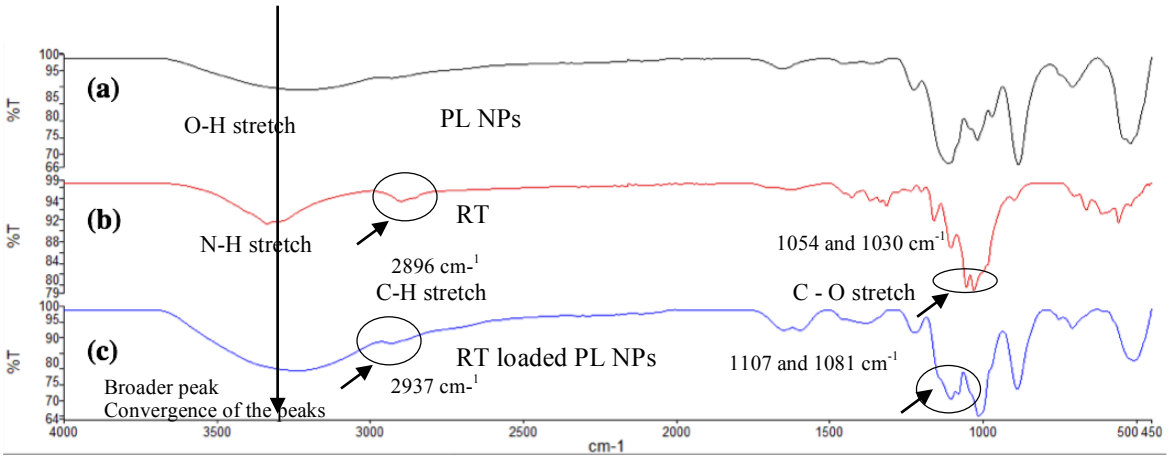


Figure 8.9: FTIR Spectra of **(a)** PL NPs **(b)** RT and **(c)** RT loaded PL NPs

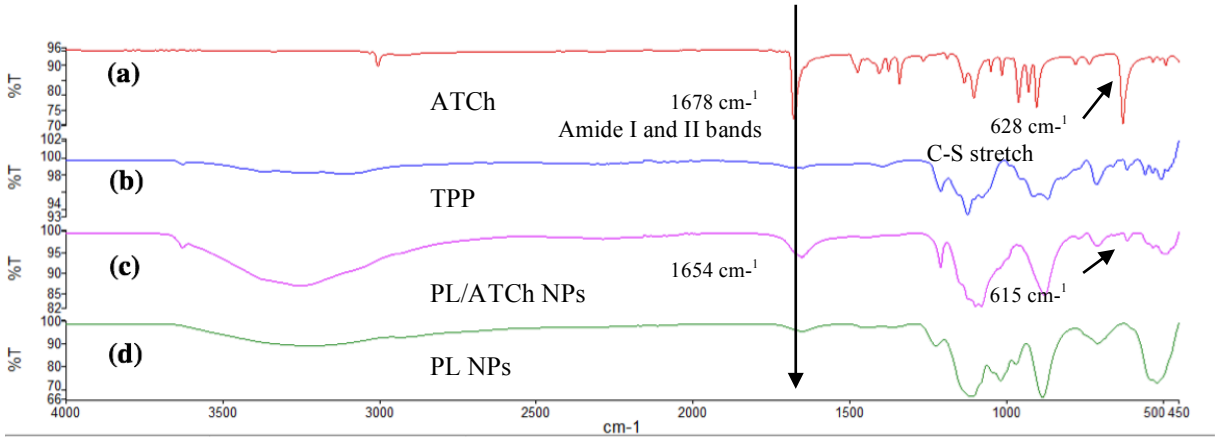


Figure 8.10: FTIR Spectra of **(a)** ATCh **(b)** TPP **(c)** PL/ATCh NPs and **(d)** PL NPs

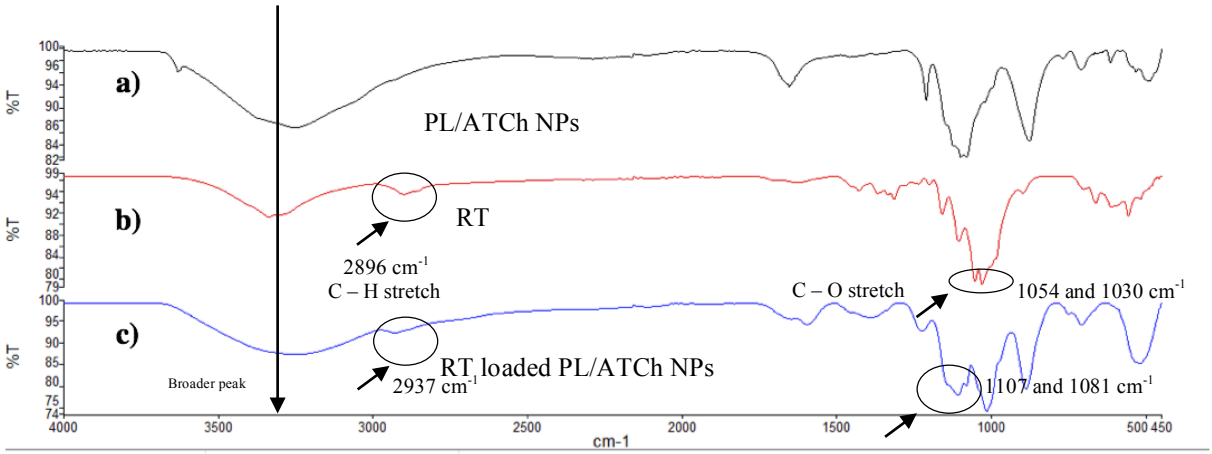
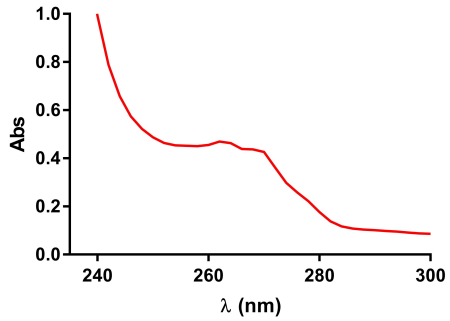


Figure 8.11: FTIR Spectra of **(a)** PL/ATCh NPs **(b)** RT and **(c)** RT loaded PL/ATCh NPs

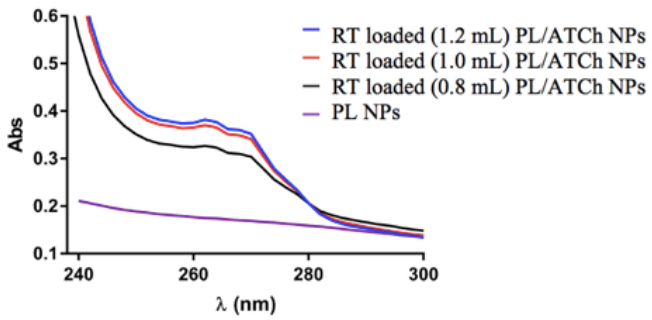
8.5 Quantity of instant drug release

Quantity of instant drug release for all the samples were assayed spectrophotometrically in the range of 240 to 300 nm. The concentration of released RT in the PBS (pH 7.4) was determined by UV spectrophotometer at 264 nm. Figure 8.11 (a-d) shows the drug release of synthesized samples in PBS while Figure 8.11 (e) demonstrates the absence of RT release in ethanol. Absorbance values at 264 nm for 1.2, 1 and 0.8 mL RT containing systems were found as 0.382, 0.370 and 0.327, respectively, where they released the drug into phosphate buffer instantly with a concentration of 6.71, 6.42 and 5.37 ppm. The percent RT released from synthesized formulations were calculated to be 21%, 17% and 14% for 1.2 mL, 1.0 mL and 0.8 mL RT containing systems, respectively. Additionally, no evidence of RT could be found in the supernatant of ethanol solution.

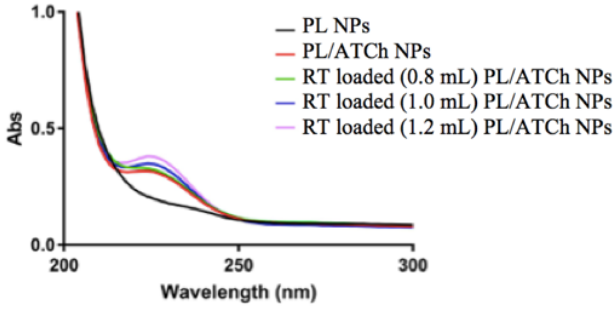
(a)



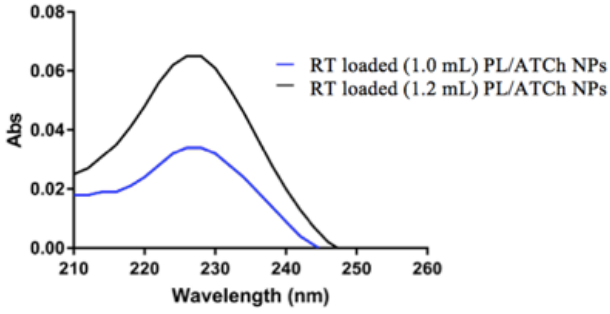
(b)



c)



(d)



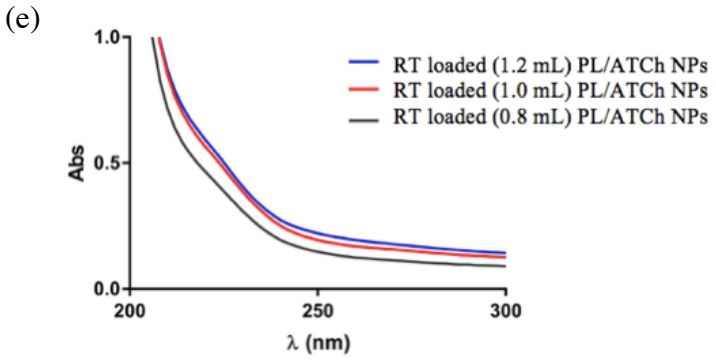


Figure 8.12: Absorbance of **(a)** RT **(b)** PL NPs and RT loaded (0.8, 1.0, 1.2 mL) PL/ATCh NPs **(c)** PL NPs, PL/ATCh NPs, and RT loaded (0.8, 1.0, 1.2 mL) PL/ATCh NPs **(d)** RT loaded (1.0, 1.2 mL) PL NPs; in phosphate buffer and **(e)** Absorbance of RT loaded (0.8, 1.0, 1.2 mL) PL/ATCh NPs; in ethanol

8.6. *In vitro* determination of cholinesterase enzyme activity

The determination of cholinesterase enzyme activity was assessed spectrophotometrically at 412 nm as a measure of enzyme inhibition by AChEI drug containing NPs. PL/ATCh NPs with no drug revealed an absorbance of 0.6837 dA/min, whereas RT loaded PL/ATCh NPs showed an absorbance of 0.4647 dA/min. The ChE activity (Unit/L) for blank PL/ATCh NPs and RT loaded PL/ATCh NPs were found to be 3016 and 2050 U/L, respectively. In other words, it has been demonstrated that RT loaded PL/ATCh NPs decreased ChE enzyme activity by approximately 30%. After monitoring, the color development of both of the samples were photographed as shown in Figure 8.12.

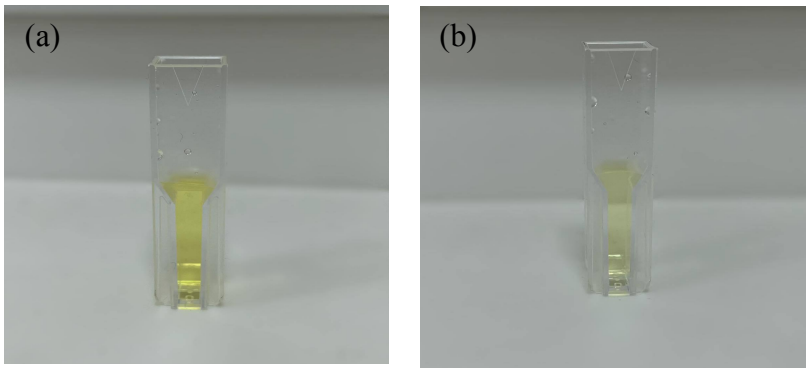


Figure 8.13: Color development of (a) PL/ATCh NPs (b) RT loaded PL/ATCh NPs

8.7 *In vitro* coagulation test

The hemocompatibility of synthesized NPs were assessed by investigating the extent to which they alter the coagulation parameter times i.e. the APTT, PTR and INR. *In vitro* coagulation test results for the control blood and blood with NPs are shown in Table 8.3.

Table 8.3: *In-vitro* coagulation test results

	APTT (sec)	**PT (sec)**	**INR**
Reference values	23.6 – 35.2	11.5 – 15	0.80 – 1.2
Control Blood	32.0	13.3	0.99
PL/NPs	30.3	26.2	2.02
PL/ATCh NPs	47.3	18.1	1.37
RT loaded PL/ATCh NPs	40.3	15.2	1.14

CHAPTER 9
DISCUSSION AND CONCLUSION

In this work, we have aimed to develop a smart nano-mediated drug delivery system that consists of a polysaccharide, PL, and an ACh analogue, ATCh. Biocompatible and biodegradable polymers as encapsulating materials have shown significant potential for the nasal administration of various therapeutics. In addition to its biocompatibility, biodegradability, low immunogenicity and ease of modification, PL was chosen as polymer of interest. The introduction of ATCh as a second component in the formulation was expected to construct a "smart" delivery vehicle, in which the AChEI drug, RT, would be released in time of need i.e. in the presence of the AChE enzyme. To this end, the preparation conditions of this system have been optimized in terms of the volume and concentration of added PL, TPP, and ATCh. Subsequently, RT has been loaded to the formulations to prepare RT-loaded PL NPs. It is important to point out that the NPs were not purified after preparation as all of the added components were biocompatible in nature. This is useful because the direct administration of drug loaded NP dispersions evades the time-consuming purification steps and resulting aggregation problems (Fornaguera et al., 2015).

9.1 Formation of PL and PL/ATCh NPs

The current study demonstrates for the first time the possibility to develop RT-loaded PL NPs. These NPs were prepared by dropwise addition of PL solution and PL+RT solution to TPP solution (pH 8.6), followed by the addition of ATCh to the mixture as means of surface functionalization. It is proposed that PL NPs are formed by a covalent bond formation between the negatively charged lone pairs of oxygen on the hydroxyl group of PL and the partially positive phosphate of TPP. This leads to the formation of phosphorylated PL NPs as the end product (Figure 9.1).

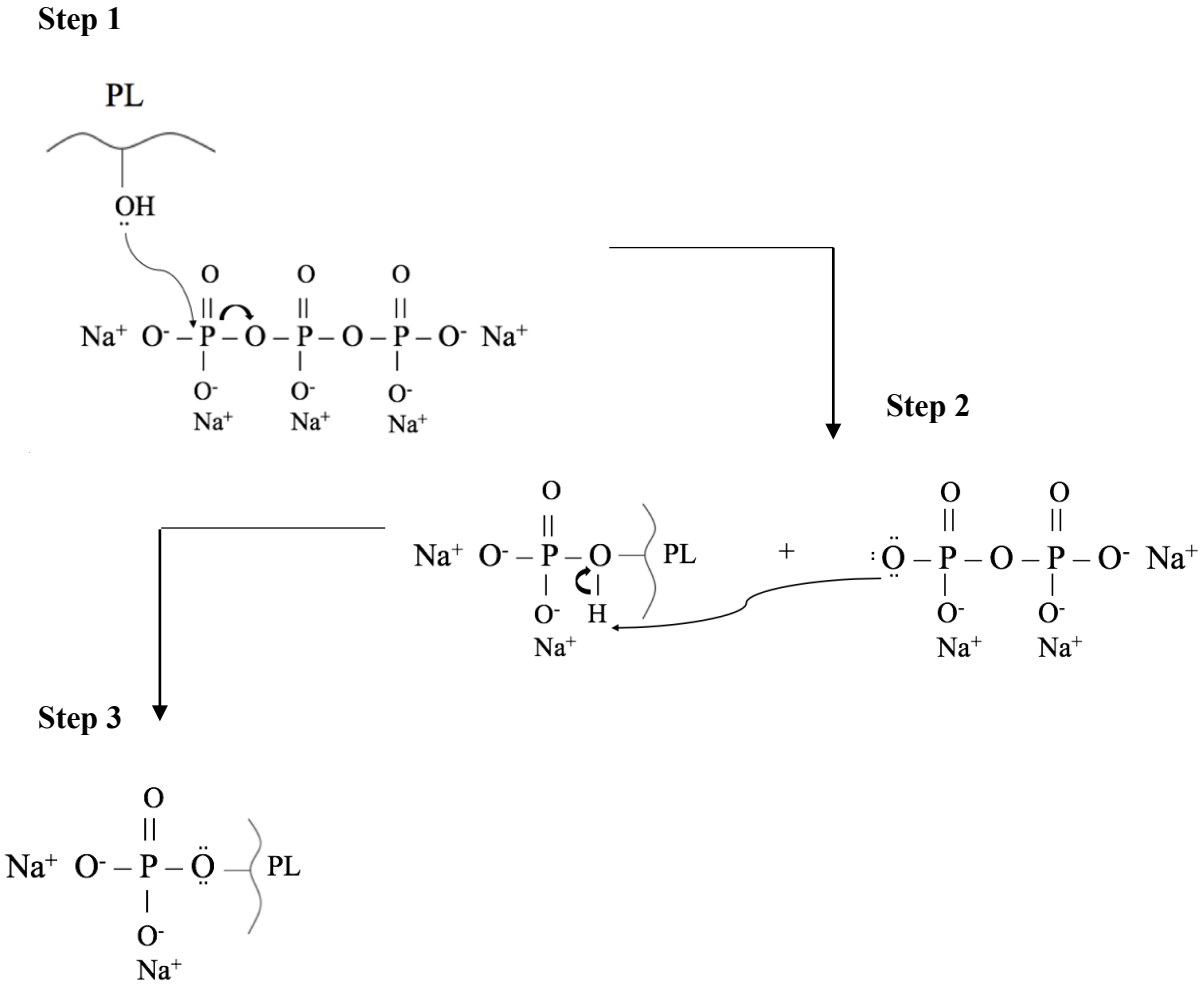


Figure 9.1: Schematic illustration of the proposed chemical reaction mechanism between PL and TPP to form PL NPs

9.2 Scanning electron microscopy and energy dispersive x-ray (EDX) composition analyses

The morphology of NPs was observed using SEM. The SEM micrographs of synthesized nanoparticles reveal that the particles were mostly uniform and spherical in shape. They were non-aggregated and separated from each other, indicating a possible stabilization of NPs. Although the spherical shape has been traditionally utilized in the design of NPs in most NP studies due to their ease of preparation, little is known about the influence of particle shape on NP performance, while the effects of particle size and surface chemistry is

widely studied. With advanced nano-synthesis techniques, different shapes and forms of NPs have been developed in recent years. Therefore, NP shape is an important aspect that needs to be investigated for the design of nano-mediated drug carriers. Regarding cell internalization, it is known that NPs having different shapes and sizes display different cell internalization rates as a result of computational modelling and *in vitro* and *in vivo* experiments. The investigation of cellular uptake with varying NP shapes, including nano-spheres, nano-rods and nano-dics, revealed that the shape anisotropy and the initial position of NPs are critical to the interactions between the NP and lipid bilayer that forms the surrounding membrane of cells (Liu et al., 2012). NP uptake is influenced by the contact area between the NP and lipid bilayer, highlighting the significance of NP shape. *In vitro* studies have revealed that unlike the spherical NPs synthesized in the current study, rod shaped NPs possess higher adhesion tendencies compared to spherical NPs. It was also demonstrated that brain targeting of nano-rods yielded a 7-fold increase in brain accumulation that their spherical counterparts (Kolhar et al., 2013). Additionally, in a recent study, the *in vivo* behavior and pharmacokinetic properties of NPs were found to be significantly affected by shape. Rod shaped NPs were found to achieve a longer blood circulation time with increased bioavailability when compared to spherical NPs (Zhao et al, 2017). Therefore, it can be concluded that the employment of rod NPs is more beneficial in the nano-design of drugs that suffer from poor bioavailability.

The energy dispersive x-ray (EDX) composition analysis of synthesized NPs revealed that the elemental composition of PL NPs comprised of carbon, oxygen, sodium and phosphorus elements. As PL is a polymer consisting of only carbon, hydrogen and oxygen, the presence of sodium and especially phosphorus elements provide evidence for the covalent bond formation between PL and TPP. The elemental composition of RT loaded PL NPs were found to be the same with PL NPs. This finding is in accordance with the study design, aimed at encapsulating RT. It suggests the successful encapsulation of RT within the NP core, hence why no evidence of RT i.e. nitrogen was observed in RT loaded PL NPs. It is important to note that the Cu element was generated from the copper grid of SEM sample holder. For RT loaded acetylthiocholine iodide modified PL/ATCh NPs, the results reveal that the elemental composition was the same with the addition of an extra element, iodine, which is considered as proof for ATCh modification on the NP surface. Once again, the lack

of nitrogen suggests the successful encapsulation of RT in RT loaded PL/ATCh NPs. For PL/ATCh NPs with no drug loading, on the other hand, revealed no iodine content, that can be attributed to the random sampling of the NP surface, as the formulation was the same with the drug loaded form with the exclusion of the drug, or can be due to the fact that ATCh might have been encapsulated within the NP core. Additionally, no evidence of Sulphur (S) element was observed in neither of the ATCh containing samples, which strengthens the possibility of ATCh encapsulation along with the drug within the NP core. Nonetheless, the lack of observation of S element could also be masked by Cu that was generated by the copper grid of sample holder. This assumption is based on the fact that although EDX analysis is a very useful method of elemental identification and quantification, it is generally not a particularly sensitive technique with low resolution, making elemental identification difficult (Wolfgong, 2016). Given that an element's concentration in a sample is too low (as in the case for I and S elements), the amount of energy given off by X-rays after hitting the sample becomes inadequate to sufficiently measure its proportion. Furthermore, EDX is unable to detect elements with a low atomic number (hydrogen, lithium and helium) (Wolfgong, 2016) and hence why no hydrogen (H) element could be detected in the synthesized samples. This is due to the fact that hydrogen only has an n=1 shell, which means there are no core electrons to be removed to allow for X-ray emission. Therefore, elemental analysis via EDX results should be treated with caution taking these limitations into consideration.

9.3 Zeta Sizer / Potential

Since the NPs size is a crucial parameter in BBB transport, the aim was synthesizing particles with a mean size of 100-200 nm. As described in the Results section, synthesized NPs were <50 nm in size with a negative surface charge. This is advantageous in terms of the ability to efficiently bypass the BBB when considering *in vivo* applications for the treatment of neurodegenerative disorders. PL NPs had an average size of 33.34 nm while the PL/ATCh NPs had an average of 34.06 nm. The incorporation of RT did not influence the size of PL and PL/ATCh NPs. In fact, it led to a slight reduction in particle size. The average size of RT loaded PL NPs and PL/ATCh NPs were reduced to 31.98 nm and 32.65 nm, respectively. It is known that spherical NPs >200 nm are filtered by the reticuloendothelial system, while particles with sizes <10 nm is rapidly cleared by the kidney. Therefore, it can be concluded

that sizes between 10 nm and 200 nm are the ideal size range in NP design (Liu et al., 2012). Nanometer range particles have easy accessibility in the body by being circulated to different body regions. Extremely small particles i.e. <100 nm particles have been found to have longer blood circulation times (Allemann et al., 1993), with sizes above this value limiting their bio-distribution. Such systems are advantageous in terms of the ability to control the rate of drug administration and duration of action and the targeting of the drug to specific areas (Banerjee et al., 2002). In terms of intranasal means of brain targeting, NPs with sizes < 200 nm have been found to be ideal (Fazil et al., 2012). Furthermore, small sizes also decrease the ability of the innate immune system to detect and eliminate the NPs from systemic circulation. Last but not least, it has been demonstrated that smaller particles have a relatively larger free surface area and thus can provide to a faster release of encapsulated drugs (Gabor et al., 1999).

Zeta potential is another important parameter having an influence over the ability of bypassing the BBB and particle stability. It represents the surface charge of NPs, as such the larger the absolute value of zeta potential, the larger the amount of charge on NP surface. Positively or negatively charged NPs have been found to display more drug release compared to neutral NPs (Tao et al., 2016). Additionally, high negative charges/values of zeta potential indicate that the electrostatic repulsion between particles having the same electrical charge will prevent the aggregation of particles and could stabilize particle suspensions (Feng and Huang, 2001). To this end, most of the studies on brain targeted NP formulations have either moderate (between -1 mV to -15 mV) or high (between -15 mV to -45 mV) zeta potential values (Saraiva et al., 2016) for efficient brain delivery systems. The zeta potentials of synthesized PL NPs were found to be -2.79 mV, where the negativity can be attributed to the lone pair electrons and negatively charged oxygens attached to the phosphate group of TPP, which became -0.69 mV upon RT loading despite its neutral nature. This may be due to the molecular interaction between the polymer and RT, or due to the fact that the electrostatic attraction between the positively and negatively charged groups may be affected by the addition of RT, affecting the zeta potential value. The zeta potential of PL/ATCh NPs have been found to be -6.34 mV upon the introduction of ATCh. The increasing negative surface charge in comparison to PL NPs can be attributed to the electrostatically attached negatively charged iodide ions (I^-) of ATCh. After RT loading, the

surface charge has been found to increase to -9.36 mV, to its maximum value. Thus, RT loaded PL/ATCh NPs possess maximum stability that is attributed to the strong electrostatic attraction of all participant molecules upon the addition of RT. It can be concluded that the values for all of the synthesized NP formulations are highly sufficient to form stable NP suspensions. It is also important to point out that highly charged particles are more likely to be eliminated by phagocytosis, which is the main factor in NP removal. The phagocytosis of neutral NPs, on the other hand, have been found to be significantly reduced *in vivo* (Li and Huang, 2008), which may help with extending the blood circulation time of encapsulated drugs. Therefore, currently, in terms of zeta potential, it is a matter of choice between particle stability and circulation time.

9.4 Fourier transform infrared (FTIR) analysis

The typical absorption bands for the α-configuration of α-D-glucopyranose units in pullulan were observed at 843 cm^{-1}. Absorption at 754 cm^{-1} and 930 cm^{-1} illustrates the two predominant linkages of pullulan, α (1, 4) and α (1, 6))-D-glucosidic bonds. Additionally, infrared spectrum of pullulan showed other features of the polymer at 3308 cm^{-1} (O-H stretch), 2926 cm^{-1} (C-H stretch), 1641 cm^{-1} (O-C-O stretch), 1360 cm^{-1} (C-O-H stretch) and 1147 cm^{-1} (C-O-C) (Appendix I, Figure A1a), as reported earlier (Cheng et al., 2010). For TPP, the spectrum shows the following characteristic bands observed at 1208 cm^{-1} (P=O stretch), 1124 cm^{-1} (symmetric and asymmetric O – P = O stretch), 1077 cm^{-1}(symmetric and asymmetric vibrations in PO_3 group), and 869 cm^{-1} (asymmetric stretching of the P – O – P bridge) (Tomaz et al., 2018) (Appendix I, Figure A1b). The FTIR spectrum of PL NPs exhibit a relatively broader peak at 3222 cm^{-1}, which was linked to the hydroxyl group of PL at 3308 cm^{-1} in the pure PL spectrum, upon interaction with TPP. Additionally, the peaks at 1225 cm^{-1} and 1108 cm^{-1} is attributed to the characteristic P=O stretch and O – P=O stretch of TPP, respectively, providing evidence for the covalent bond formation between PL (hydroxyl group) and TPP (phosphate group) (Figure 8.8). The FTIR spectrum of ATCh reveals a sharp characteristic peak at 1678 cm^{-1} (Appendix I, Figure A1d), attributed to the amide I and II bands associated with the C = O and C – N stretching vibrations, which can also be observed in the FTIR spectrum of PL/ATCh NPs at 1654 cm^{-1} confirming the presence of ATCh in PL/ATCh NPs (Figure 8.10). Furthermore, C – S stretch illustrated at 628 cm^{-1} for pure ATCh is also present at the FTIR spectrum of PL/ATCh NPs at 615 cm^{-1}.

RT spectrum exhibit characteristic peaks at 3337 cm^{-1} (N – H stretch), 2896 cm^{-1} (C – H stretch), 1054 cm^{-1} (C – O stretch) and 660 cm^{-1} (C – H stretch) (Appendix, Figure A1c) (Bhatt et al., 2016). For RT loaded PL NPs, the emergence of the peak at 2937 cm^{-1} (C-H stretch) that was absent in the PL NP and PL/ATCh NP spectrums confirms RT loading in both PL and PL/ATCh NPs (Figure 8.9 and Figure 8.11). The FTIR spectrum of RT loaded particles also reveal a broader peak in the 3500-3000 cm^{-1} range as a result of the convergence of O-H stretch in PL (3308 cm^{-1}) and N-H stretch in RT (at 3337 cm^{-1}). Additionally, the peaks at 1107 and 1081 cm^{-1} represents the C – O stretch of RT confirming RT loading.

9.5 Quantity of instant drug release

Analysis of supernatant of ethanol solution for RT loaded PL/ATCh NPs has been made by UV-Vis spectrophotometer as a way to prove the encapsulation of RT. Ethanol was chosen as the solution of interest due to being a poor solvent for PL and thus yielding precipitation. Consequently, the absence of drug in the supernatant following centrifugation proved the encapsulation of RT within the synthesized NPs. RT was analyzed and two characteristic peaks were observed at 264 nm (λ_{max}) and 270 nm. Therefore, the absorbance value for each sample was determined at 264 nm (λ_{max}) by using the UV-Vis spectrum. Absorbance values at 264 nm for 1.2, 1 and 0.8 mL RT containing systems were found as 0.382, 0.370 and 0.327, respectively. Increase in the amount of encapsulated drug caused an increase in absorbance. These absorbance values were required to calculate the concentration of instant drug release by using the calibration curve. It was found that 1.2, 1.0 and 0.8 mL RT containing NP solutions which correspond to 0.123 mg/ml, 0.125 mg/ml and 0.127 mg/ml of RT released the drug into phosphate buffer instantly with a concentration of 6.71, 6.42 and 5.37 ppm, respectively. Percent drug release were calculated to be 21%, 17% and 14% for 1.2 mL, 1.0 mL and 0.8 mL RT containing formulations. Increasing the amount of drug were found to increase the percent drug release Therefore, the amount of RT in NPs should be increased for further applications. However, it is important to point out that other nano-based studies in literature with better % drug release outcomes have assessed drug release ~ 1/3/6 hours after administration in contrast to the current study, which have examined instant drug release. Consequently, the current 21% drug release represents the instant percentage,

and would presumably produce a better outcome if monitored for longer. Additionally, the fast release rate of NPs can be attributed to small particle size, that is related with smaller diffusion path enabling the easy diffusion of drugs to the NP surface.

9.6 *In vitro* determination of cholinesterase enzyme activity

The cholinesterase activity was assessed for synthesized NPs in the absence and presence of drug incorporation as a measure of assessing the effectiveness of drug loaded NPs in terms of cholinesterase enzyme inhibition. It is important to note that the detection of cholinesterase enzyme activity is based on the following enzyme reactions;

Acetylthiocholine → Thiocholine + Acetate

Thicholine + DTNB → TNB + Mixed Disulfide

This is due to the fact that in the presence of cholinesterase enzyme, ATCh is degraded into thiocholine and acetate. When thiocholine comes in contact with DTNB, it produces TNB and mixed disulfide, where TNB gives off a yellow color. Hence, this color development can be used as a measure of cholinesterase enzyme inhibition to test the inhibitory effect of the drug loaded particles.

RT loaded NPs were expected to produce a significant inhibition of cholinesterase enzyme. As stated in the results section, the absorbance change of PL/ATCh and RT loaded PL/ATCh NPs were 0.6837 dA/min and 0.4647 dA/min, respectively. From these values, the ChE activity for blank PL/ATCh NPs and RT loaded PL/ATCh NPs were calculated to be 3016 and 2050 U/L, confirming the reduced activity of ChE enzyme in the presence of drug loaded NPs. It has been demonstrated that RT loaded PL/ATCh NPs achieved an inhibition of approximately 30% of ChE activity, which is considered as an apparent reduction in ChE activity. This difference in ChE activity can also be observed as a degree of color development in Figure 8.12. The “yellowness” of reaction mixture with RT loaded PL/ATCh NPs was considerably reduced as compared to the enzyme assay with blank PL/ATCh NPs. This means that there was more ATCh available, indicating a degree of cholinesterase enzyme inhibition, suggesting the potential of synthesized NPs in prolonging ACh transmission.

9.7 *In vitro* coagulation test

When a biomaterial comes in contact with blood, there is a rapid adsorption of proteins onto its surface that can elicit adverse reactions through plasma enzyme cascades; one of which is coagulation, also known as clotting, to eliminate the biomaterial. The clotting cascade occurs through 2 distinct pathways; the intrinsic and extrinsic cascade. The extrinsic pathway is activated through an external trauma that triggers blood to escape from the vascular system. The integrity of the extrinsic pathway is measured by PT and INR values. Intrinsic pathway, on the other hand, is activated through an internal trauma of the vascular system *via* platelets, exposed endothelium, collagen and chemicals, assessed by APTT values. The hemocompatibility and biocompatibility of biomaterials is assessed by the extent to which they modify the coagulation parameter times. For a material to be considered compatible, only slight variations in parameter times is accepted. The results reveal that for PL NPs, APPT value was found within the reference values, whereas the PT and INR values were slightly exceeding the reference values. Therefore, it can be suggested that the sample may have interacted with the components of the extrinsic pathway, with no interaction with the intrinsic pathway. For PL/ATCh NPs, all values have been found to slightly exceed the reference values, indicating an interaction with both of the coagulation cascades upon the induction of ATCh. Lastly, RT loaded PL/ATCh NPs reveal that there was only a slight increase in APTT value, while PT and INR values remained within the reference values. Therefore, it can be concluded that despite slight variations in the coagulation parameter times, the results reveal that all NP formulations were promising in terms of biocompatibility with minor effects on the coagulation cascade parameters.

9.8 Limitations of the current study design and nano-mediated drug delivery systems

It is important to point out that the current study poses some limitations. First of all, in theory, even though the synthesized NPs have the capacity to bypass the BBB as a result of having a nano-size and a negative surface charge, BBB passage have not been tested by using *in vitro* BBB models. Therefore, it is essential to test the current study design using *in vitro* BBB models in future studies. Such models are crucial to investigate the transfer of drugs across the BBB. Different models have been developed and employed for a variety of permeation experiments including endothelial cell monolayers, co-culture models, and dynamic models. However, despite aiding to understand the role and dynamics of BBB,

these models suffer from an inability to replicate the real physiological *in vivo* conditions, where no single model can mimic the real word physiological conditions alone (Bagchi et al., 2019).

Secondly, as discussed in section 9.2, the EDX composition analysis carried out for elemental identification is limited in terms of characterizing the chemical composition and thus the chemical structure of samples. The insensitivity of EDX analysis in detecting the S and I elements raises questions on whether ATCh actually takes part in surface modification or whether it is encapsulated within the NPs. However, it is important to point out that the *in vitro* determination of ChE activity depends on ATCh being on the surface of NPs and initiating the chemical reactions of the Ellman's protocol. Therefore, if there were no ATCh on the surface as suspected as a result of the EDX analysis, it wouldn't have been possible to carry out the test. Therefore, it can be concluded that the lack of observation of these elements are credited to the limitation of EDX analysis. Therefore, further characterizations are needed e.g. classical chemical analysis methods, to provide a more convincing data on the chemical composition of synthesized NPs.

Additionally, in the current study design, only instant drug release profiles had been monitored, which may be the underlying reason behind obtaining low percentage drug release. Therefore, to be able to compare the current results with the literature for better interpretation, it is crucial to investigate drug release for longer periods of time e.g. 1/3/6 hours after administration. Moreover, for further drug release experiments, it would be also beneficial to increase the amount of RT incorporated with the intention of obtaining enhanced percentage drug release, as increasing the amount of RT have been found to increase the percentage drug release.

Despite holding a considerable promise, nano-mediated drug delivery systems in general face some complications regarding their use as drug delivery vehicles. It should be noted that *in vitro* and *in vivo* models utilized for nano-technological research are substantial simplifications and generalizations of actual physiological conditions. Such models are therefore restricted in their ability to accurately investigate complex NP interactions of this kind (Landsiedel et al., 2012). To this end, the impact of NPs on disease pathology should

be carefully examined. In addition, efficient brain delivery of NPs loaded with drugs does not mean they are biologically active. (Sahni et al., 2011). As discussed in previous chapters, there are several underlying hypotheses regarding the cause of AD and thus measuring the effect of treatment accurately poses a significant challenge. NPs may also be able to interact and affect the physiology of any given cell in the body. It is therefore essential to establish nanoparticulate systems that releases the drug remotely upon CNS entrance with no complex interactions (Li et al., 2015). In addition, upon systemic administration, NPs interact with bloodstream biomolecules that contribute to their adsorption on NP surface, forming an additional layer referred to as the protein corona. As a result of this layer formation, the surface characteristics and morphology of NPs may be affected, that may have an impact on NP uptake and possible pathological interactions (Pietroiusti et al., 2013). Another problem in need of careful investigation is the possible toxicity of NPs. Current clinical data on NP neurotoxicity are still scarce and mainly based on non-primate animals upon short-term exposure to NPs, with little to no data on the long-term influence of NPs. Therefore, it is difficult to extrapolate the results from *in vitro* models to actual *in vivo* conditions, and hence why this aspect of NPs needs to be carefully evaluated in future research. Moreover, certain NP types cannot be easily eliminated from the body, which may increase the possibility of aggregation and further enhance disease progression (Martin et al., 2013). Taking these in consideration, benefit-to-risk ratio of nano-mediated delivery systems for CNS targeted diseases including AD should be carefully evaluated, addressing the current limitations from a therapeutic point of view.

9.9 Conclusions

The brain is well protected by the surrounding biological barriers, including the BBB, which is responsible for sustaining homeostasis and limiting the entry of potentially toxic molecules, including the ones with good therapeutic value. This in turn affects the brain delivery of drugs with conventional delivery systems. Therefore, failure in developing effective treatment methods for neurological disorders like AD may be attributed due to the inefficiency in drug delivery methods, rather than the therapeutic agents themselves. This has shifted the attention to the development of innovative delivery strategies, one of which is nanoparticle mediated drug delivery.

In this project, a novel nano-mediated delivery system for the anti-AD drug rivastigmine, intended for intranasal administration, improved bioavailability, therapeutic effect and minimized side effects, were developed and characterized. A biocompatible and biodegradable polymer, PL has been successively synthesized and further functionalized with an ACh analogue, ATCh, to develop a smart drug delivery system, and were loaded with RT. RT loaded particles have been shown to be spherical in shape at the desired nano-range ideal for BBB passage, cellular uptake and hence for brain targeted delivery. Synthesized NPs exhibited a negative surface charge, sufficient enough to form stable NPs and inhibit aggregation. Quantity of instant drug release provided evidence for the encapsulation of RT within the NPs, that was instantly released that can be attributed to the small particle size of particles. *In vitro* coagulation tests suggested the NPs to be promising in terms of hemocompatibility, which was expected due to the biocompatibility of the chosen polymer. Last but not least, the *in vitro* determination of cholinesterase enzyme revealed an apparent inhibition of the ChE enzyme in the presence of RT loaded NPs. Therefore, it can be concluded that the synthesized NP formulations are a promising approach for AD treatment, however, further relevant *in vitro* assays would be necessary to a gain a deeper insight of the potential of the RT loaded PL and PL/ATCh NPs as brain targeted drug carriers.

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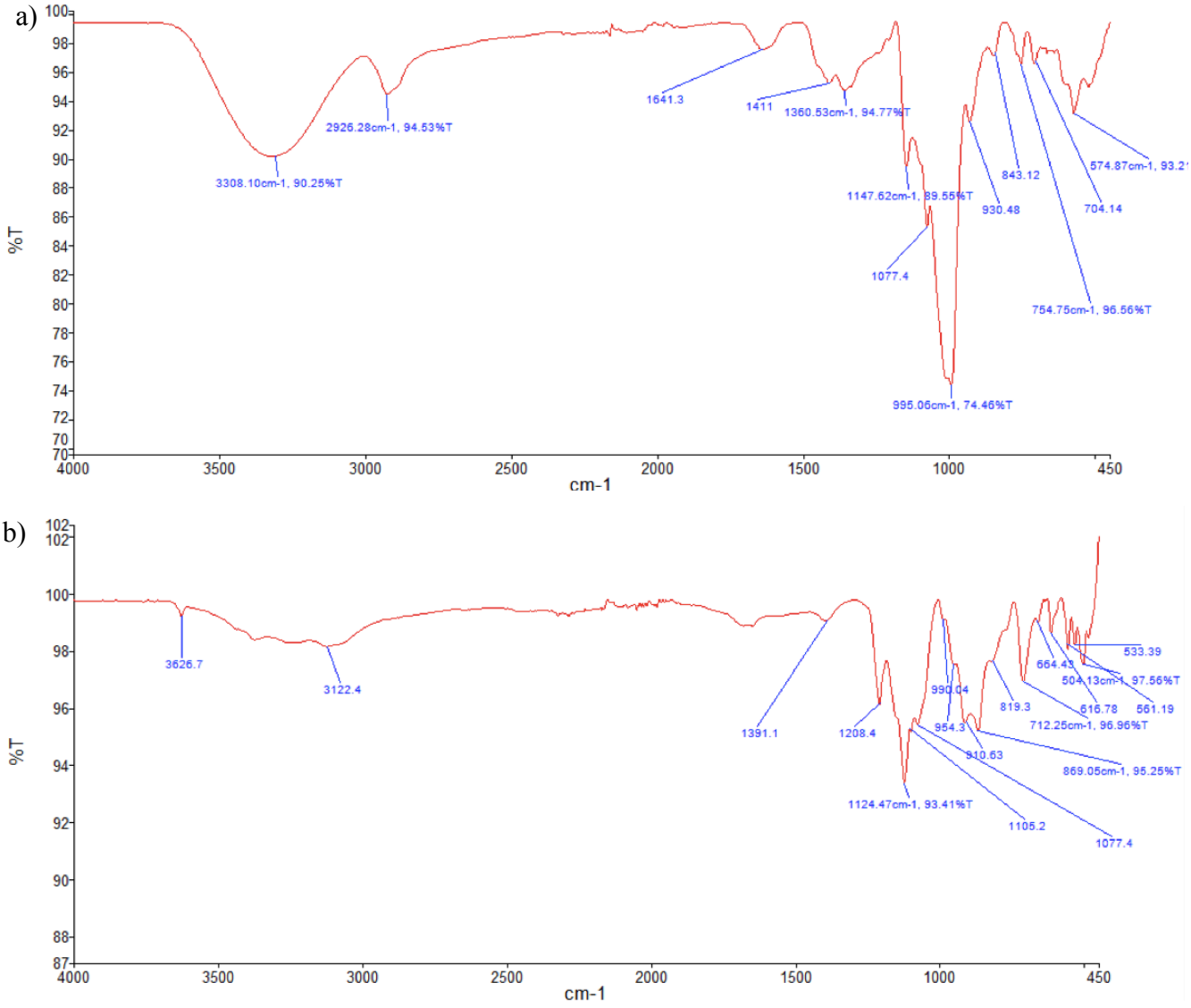
APPENDICES

APPENDIX 1

Raw data for the FTIR spectra of (a) PL (b)TPP (c) RT and (d) ATCh are shown in Figure A1.



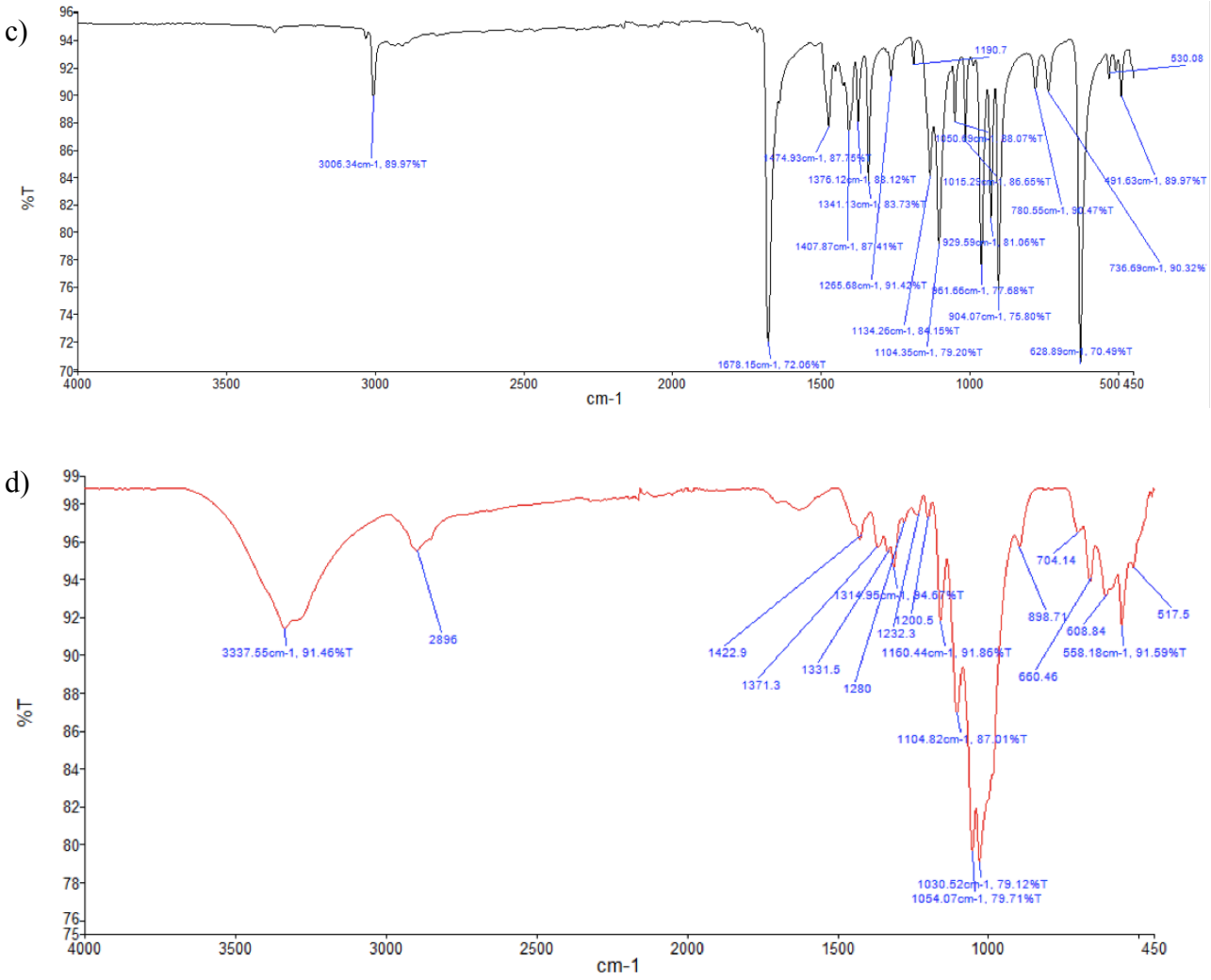


Figure A1: Raw data for the FTIR spectra of (a) PL (b)TPP (c) RT and (d) ATCh

APPENDIX 2

ETHICAL APPROVAL DOCUMENT

NEAR EAST UNIVERSITY

Date: 23/12/2020

To the Graduate School of Applies Sciences,

For the thesis project entitled as "Development of Novel Polymeric Pullulan Nanoparticles for Alzheimer's Disease", the researchers declare that they have an approval from the Near East University, Scientific Research Ethical Board with Decision at 23.01.2020, Meeting No: 2020/76 for project No: YDU/2020/76-955.

Title: Prof. Dr.

Name & Surname: Terin Adalı

Signature: 

Role in the Research Project: Supervisor

Appendix: II: Decision Report for the Research Project YDU/2020/76-955.

EK: 1037-205

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 23.01.2020
Toplantı No : 2020/76
Proje No :955

Yakın Doğu Üniversitesi Mühendislik Fakültesi öğretim üyelerinden Doç. Dr. Terin Adalı'nın sorumlu araştırmacısı olduğu, YDU/2020/76-955 proje numaralı ve **"Hidrojel Ve Polielektrolit Yapılarda Kan Uyumluluğu Çalışmaları"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

1. Prof. Dr. Rüştü Onur (BAŞKAN)
2. Prof. Dr. Nerin Bahçeciler Önder (ÜYE) KATILMADI
3. Prof. Dr. Tamer Yılmaz (ÜYE) KATILMADI
4. Prof. Dr. Şahan Saygı (ÜYE)
5. Prof. Dr. Şanda Çalı (ÜYE)
6. Prof. Dr. Nedim Çakır (ÜYE)
7. Prof. Dr. Nurhan Bayraktar (ÜYE)
8. Doç. Dr. Nilüfer Galip Çelik (ÜYE) KATILMADI
9. Doç. Dr. Emil Mammadov (ÜYE)
10. Doç. Dr. Mehtap Tınazlı (ÜYE) KATILMADI

APPENDIX 3

SIMILARITY REPORT

AUTHOR	TITLE	SIMILARITY
Gülcem Altınoğlu	Conclusion	0%
Gülcem Altınoğlu	Abstract	1%
Gülcem Altınoğlu	Results	9%
Gülcem Altınoğlu	Entire Thesis	13%

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Altinoglu, Gulcem
Nationality: Cyprus
Date and Place of Birth: 26 June 1994, Famagusta
Marital Status: Single

EDUCATION

Degree	Institution	Year of Graduation
M.Sc.	Neuroimaging, Institute of Psychiatry, Psychology & Neuroscience King's College London,	2017
B.Sc.	Medical Neuroscience, School of Life Sciences, University of Sussex	2016

WORK EXPERIENCE

Year	Place	Enrolment
2019-Present	City Island University	Lecturer
2019-2020	Near East University	Lecturer

FOREING LANGUAGES

English Language, fluently spoken and written

International English Language Testing System (IELTS) Overall Band Score of 8.0

PUBLICATIONS

- Altinoglu, G. & Adali, T. (2020). Alzheimer's Disease Targeted Nano-Based Drug Delivery Systems. *Current Drug Targets, 21,* 628-646. DOI:10.2174/1389450120666191118123151

CONFERENCES & CONGRESSES

- Altinoglu, G., Adali, T. & Tulay, P (2019). The anti-cancer activity of Silk fibroin encapsulated Ibuprofen micro-particles. *European Biotechnology Congress 2019*, Valencia, Spain.

- Altinoglu, G., (2019). Neurotechnology for Sleep Disorders. *The 6th International Sleep Medicine and Science Specialist Forum of the International Sleep Science and Technology Association (ISSTA) 2019*, Near East University, Cyprus.

- Altinoglu, G. & Adali, T. (2018). Silk fibroin encapsulated Ibuprofen micro-particles: synthesis and characterization. *2nd International Congress of Biomedical Engineering IBMEC 2018*, Near East University, Cyprus.

- Altinoglu, G. & Adali, T. (2018). Silk fibroin encapsulated Ibuprofen micro-particles: synthesis and characterization. *23rd Biomedical Science and Technology Symposium–BIOMED 2018*, Istanbul Acibadem University, Turkey.

THESES

MS.c
Gulcem, A. (2017). Investigating the Effects of a Neuropeptide (Oxytocin) on Pain Related Processes with Functional Magnetic Resonance Imaging Data, King's College London, Institute of Psychiatry, Psychology & Neuroscience, London, UK.

BS.c
Gulcem, A. (2016). Quantitative Analysis of Mm-AntiNos1 Gene Expression in Adult Mouse Hippocampus, University of Sussex, School of Life Sciences, Brighton, UK.

COURSES GIVEN

Undergraduate:

- Neurophysiology (English and Turkish)
- Neuroendocrinology (English)
- Physiology (English)

COURSES TAKEN

- "Gut-Brain Connection" by Centre of Excellence
- "An Introduction to "Consumer Neuroscience & Neuromarketing" by Copenhagen Business School