A novel therapeutic approach encapsulating brain-derived neurotrophic factor in nanoparticles for treating sensorineural hearing loss.

AUTHOR(S)

Fergal J. Glynn

CITATION

Glynn, Fergal J. (2010): A novel therapeutic approach encapsulating brain-derived neurotrophic factor in nanoparticles for treating sensorineural hearing loss.. Royal College of Surgeons in Ireland. Thesis. https://doi.org/10.25419/rcsi.10801493.v1

DOI

10.25419/rcsi.10801493.v1

A Novel Therapeutic Approach Encapsulating Brain-Derived Neurotrophic Factor in Nanoparticles for Treating Sensorineural Hearing Loss

Fergal J. Glynn

MB, BCh, BAO; LRCP&SI; MRCSI; FRCSI (Oto)

Thesis submitted for the degree Masters in Surgery (M.Ch) to the Royal College of Surgeons in Ireland, Dublin, 2010

Head of Department of Otolaryngology Head and Neck Surgery and Nominator for Thesis: Professor Michael Walsh

Research conducted at the: [1] Bionic Ear Institute Melbourne, 384-388 Albert St, Melbourne, Victoria 3002, Australia.

[2] Department of Chemical and Biomolecular Engineering, University of Melbourne, Victoria 3010, Australia

Supervisors of Research: Dr Justin Tan, Prfs. Frank Caruso & Robert K Shepherd

INDEX PAGE

Chapter One

Introduction

Chapter Two

Materials and Methods

Page

Chapter Three

Results **Page**

Chapter Four

Discussion

DECLARATION

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree Masters in Surgery (M.Ch.) is my own personal effort. The majority of content of this thesis is the work of Fergal J Glynn. My co-supervisor Dr Justin Tan captured images of the apoptotic cells, performed density counts of these cells and statistically analysed the data. Dr Tan also deafened the animals, performed the transcardial perfusion and sectioned the cochlea for histological analysis. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed _____

Student Number _____

Date _____

THESIS SUMMARY

There are approximately 688,000 adults in the UK with severe to profound sensorineural hearing loss. While many people who suffer from hearing loss benefit from the use of a conventional hearing aid, these devices are not effective in patients with profound sensorineural hearing loss. Spiral ganglion neurons (SGNs) are the target cells of the cochlear implant, a neural prosthesis designed to provide important auditory cues to profoundly deaf patients. The ongoing degeneration of SGNs that occurs following sensorineural hearing loss is therefore considered a limiting factor in cochlear implant efficacy. Exogenous application of neurotrophic factors prevents SGN degeneration and can enhance neurite outgrowth. Both the quantity and the quality of surviving SGNs appear to be important for the success of the cochlear implant. The addition of BDNF to the cochlear fluids can prevent degeneration of SGNs after sensory hair cells are lost in adult rodent cochleae. This neurotrophin has to be continuously delivered to maintain neuronal survival as they are rapidly cleared by the body's physiological mechanism. Current available methods of neurotrophin application are limited to delivery over a period of less than one month, and carry risks of wound infection and viral inoculation. Alternative methods of delivery are needed. We developed a biodegradable and biocompatible polyglutamic acid particle which along with glycosaminglycan heparin, successfully sequestered BDNF. This BDNF was shown to be released in a biologically active form over a period of 70 days. Its biological activity was confirmed using the neuroblastoma cell line SH-SY5Y. These particles were then successfully inserted into a deafened rat cochlea and improved SGN survival. This work has shown that the PGA-heparin particles are potential carriers for BDNF, for clinical application in an effort to improve patient outcome with profound sensorineural hearing loss.

ACKNOWLEDGEMENTS

I am forever grateful to my colleagues and mentors at the Bionic Ear Institute (BEI), Melbourne, Australia. Professor Rob Shepherd for affording me the opportunity to come to the BEI and for his endless advice and support. I was always made feel part of the team and help and advice was always freely available. Justin supervised and directed me expertly in uncharted basic science waters, his immence support, encouragement and patience was truly valued. Rachel, Jacqueline, James, Jin Xu, Elisa and Andrew all deserve mention for their help, advice and friendship during my most amazing year in Melbourne.

I would like also to thank Professor Frank Caruso and his team for his collaboration and guidance during my time at the Department of Biomolecular and Chemical Engineering, at the University of Melbourne. Again I was made feel most welcome and support and advice was always in plenty supply. I am most indebted to Yajun for his patience and support during the particle synthesis and his for help during the thesis writing. Juan Mendez Garcia, Andrew and Luke for their help and friendship during my time at the university. I would also like to express my gratitude to Professor Michael Walsh, Professor of Otolaryngology Head and Neck Surgery, R.C.S.I, for acting as nominator and advisor for the thesis.

This research fellowship was supported by the "Ethicon Overseas Travelling Fellowship", Royal College of Surgeons in Ireland, for which I am grateful. I am also grateful to the Royal Victoria Eye and Ear Hospital, Melbourne for a research grant. I would like to acknowledge the National Institutes of Health contract NIDCD HNS-N-263-2007-00053 which supported funding of the experimental costs.

Finally and most importantly, I am particularly indebted to my wonderful wife Sinead, for her endless support, advice, critiquing and help in writing the thesis. As always her good judgment, patience and tolerance have been invaluable.

"Life is not measured by the number of breaths we take, but by the moments that take our breath away."

Shing Xiong

"Yesterday is history. Tomorrow is a mystery. And Today? Today is a gift. That's why we call it the present".

Author unknown

ABBREVIATIONS

ABR	**auditory brain stem response**
AN	**auditory nerve**
APTS	**aminopropyltriethoxysilane**
BDNF	**brain-derived neurotrophic factor**
BMS	**bimodal mesoporous silica**
CN	**cochlear nucleus**
CSF	**cerebrospinal fluid**
DAB	**diaminobenzidine**
DMEM	**dulbecco's modified eagle serum**
DMSO	**dimethyl sulfoxide**
DTT	**dithiothreitol**
EDC	**ethyldimethylaminopropyl**
ELISA	**enzyme-linked immuno-adsorbant assay**
FBS	**fetal bovine serum**
GSH	**glutathione**
HF	**hydrofluoric acid**
IHC	**inner hair cell**
MS	**mesoporous silica**
MOP	**mini-osmotic pump**
NGF	**nerve growth factor**
NT	**neurotrophin**
OHC	**outer hair cell**
OSL	**osseous spiral lamina**
PBS	**phosphate buffered saline**

PEG	**polyethylene glycol**
PGA	**polyglutamic acid**
PLGA	**polylactic-glycolic acid**
PMA	**polymethacrylic acid**
PVP	**polyvinylpyrrolidone**
RA	**retinoic acid**
RC	**rosenthals canal**
SGN	**spiral ganglion neuron**
SNHL	**sensori-neural hearing loss**
TRK	**tyrosine kinase**

CHAPTER ONE

INTRODUCTION

1.1 Anatomy of the inner ear

The inner ear contains the vestibulocochlear organ, which is the organ responsible for the transduction of sound and maintenance of balance. The organ consists of two major components: the membranous labyrinth and the bony labyrinth. The bony labyrinth is a shell of dense bone that surrounds and protects the more fragile membranous labyrinth (Splepecky, 1996). The cochlear component of the bony labyrinth consists of the **otic capsule,** which is the coiled external shell of the cochlea and the **modiolus**, a cone-shaped tube that forms the central axis of the cochlea and houses the spiral ganglia (fig 1.1).

The human cochlea is a spiral bony chamber that makes two and a half turns around the modiolus. This spiral chamber is divided lengthwise into three chambers: the scala vestibuli which has the oval window at its base, the scala tympani which ends in the round window and the scala media which contains the neuro-epithelial sensory cells – the organ of Corti (fig 1.2). The scala vestibuli and scala tympani are joined at the apex of the cochlea by the helicotrema, and are filled with the same fluid, perilymph. The scala media is filled with a different fluid, endolymph. Whereas perilymph is an isotonic fluid which has an ionic composition comparable to cerebrospinal fluid (CSF), endolymph is a unique extracellular fluid, specialised to house the sensory cells of the cochlea (Salt, 2003). Endolymph is formed by the stria vascularis and has a high concentration of K^+ ions (Torres and Giraldez, 1998).

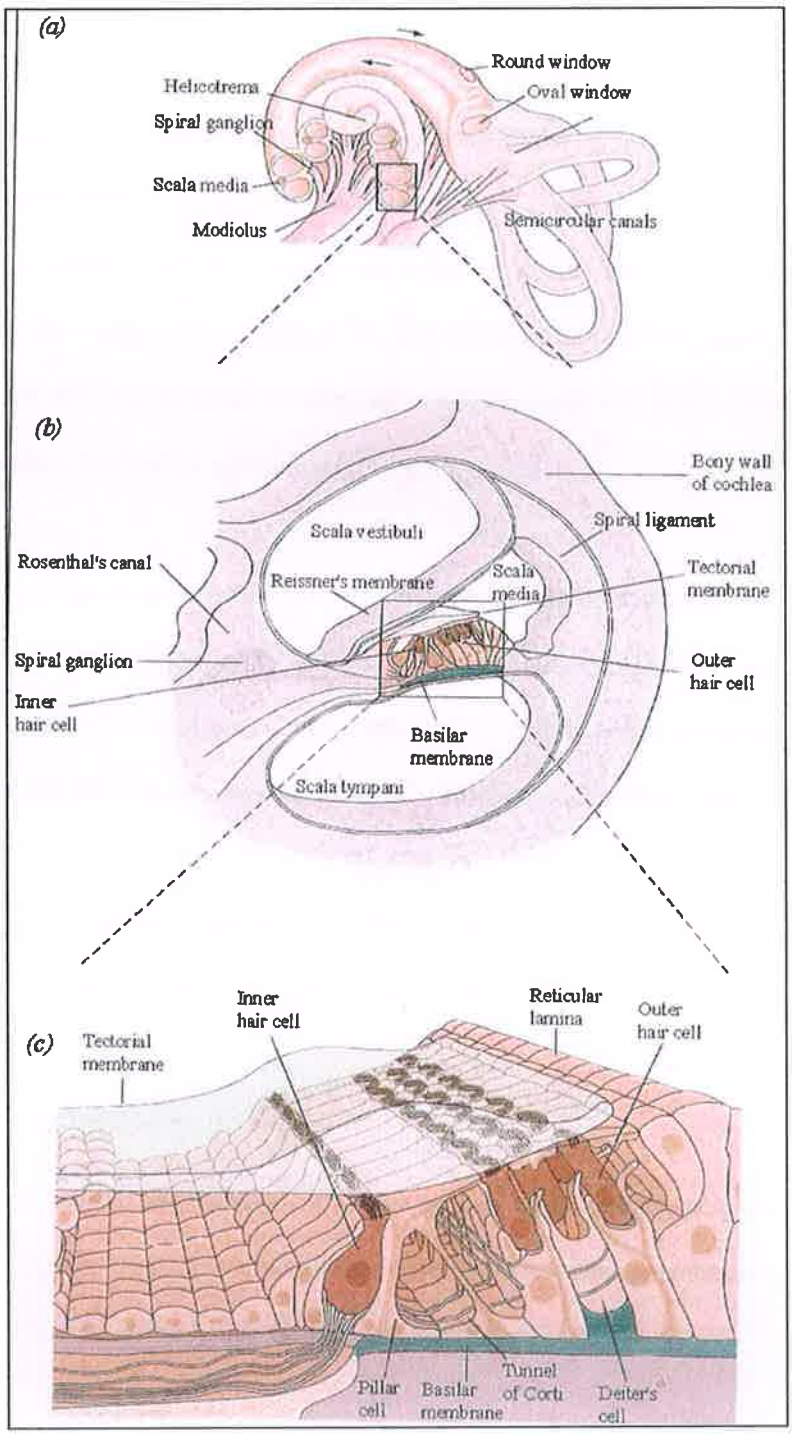


Figure 1.1 Schematic repesentation of the structures of the cochlea illustrating (a) the round window, oval window, modiolus and spiral ganglion, (b) Three scalae, Reissner's membrane, the basilar membrane, the tectorial membrane, the organ of corti and Rosenthal's canal and (c) organ of corti with inner and outer hair cells and supporting cells [Adapted from Hudspeth 2000]

Reissner's membrane separates the scala vestibule from the scala media, whereas the scala media and scala tympani are separated by the basilar membrane (Fig 1.1b). The entire perimeter of the scala media contains tight junctions which prevent any fluid movement from this compartment. In comparison, the medial wall of the scala tympani (the osseous spiral lamina) is reported to contain numerous pores of varying sizes (Schuknecht and Seifi, 1963). These pores are termed canaliculi perforantes and are thought to provide fluid communication routes between the perilymphatic fluid in the scala tympani and compartments within the modiolus housing the spiral ganglion neurons, Rosenthal's canal (RC). (Schuknecht and Seifi 1963; Lim 1970; Glueckert et al 2005; and Rask-Anderson et al. 2006).

At the base of the scala vestibuli is the oval window, to which the stapes is sealed. In comparison, the basal aperture of the scala tympani (the round window) is sealed only by an elastic diaphragm. Adjacent to the round window and connected to the scala tympani is a small canal known as the cochlear aqueduct, which connects the fluid filled inner ear to the CSF of the central nervous system. The precise function of the cochlear aqueduct is unknown however it is reported to assist in the maintenance of fluid and pressure balance between the inner ear and the CSF (Gopen et al., 1997).

1.1.1 Sensory hair cells

The sensory hair cells of the cochlea are located within the organ of Corti, a complex structure attached to the basilar membrane and extending along its entire length. The hair cells of the cochlea are of two types: inner hair cells (IHCs) and outer hair cells (OHCs). The IHCs and OHCs are separated by a tunnel supported by pillar cells known as the tunnel of Corti (fig 1.1c). On the medial side of the pillar cells is a single row of IHCs. IHCs are characteristically goblet-shaped with a centrally placed nucleus. On the lateral side of the pillar cells are three to four rows of OHCs. These cells are characteristically long and cylindrical and have a more basally placed nucleus (Shepherd 2004). Hair cells are so named because of the hair-like stereocilia which protrude from a rigid cuticular plate at their apex. These stereocilia, of which there are 50-150, are arranged in rows based on their height and are mechanically coupled to each other via "tip-links" (Pickles et al., 1984). These tip-links are important for opening and closing ion channels on the sterocilia and for excitation and inhibition of the hair cells. Each human cochlea contains about 15,000 hair cells (Ulehlova et al., 1987), innervated by about 30,000 afferent nerve fibres, extending as dendrites from the primary auditory neurons (Hudspeth, 2000).

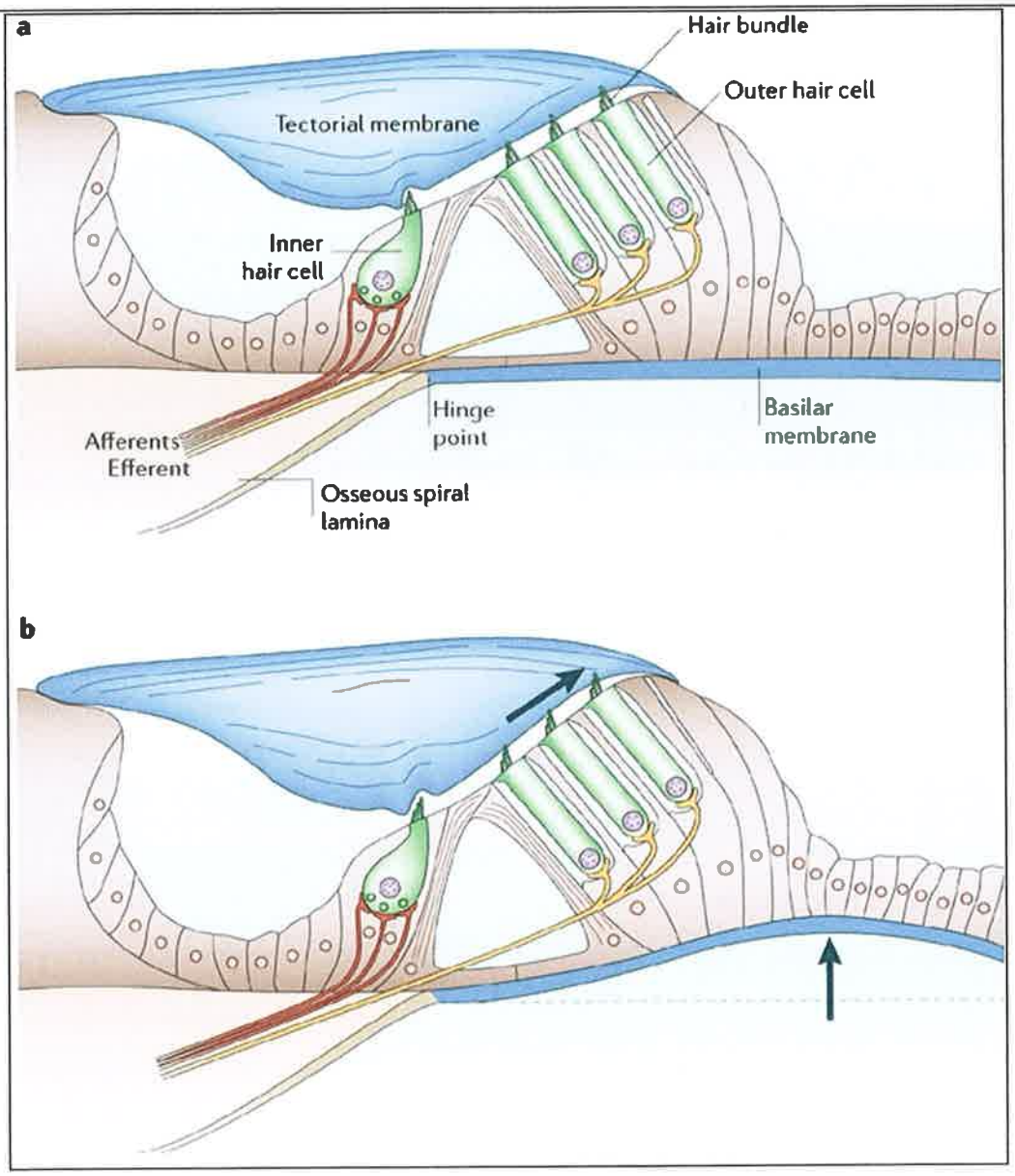


Figure 1.2 Schematic drawing of the organ of corti and tectorial membrane illustrating (a) resting position of the basilar membrane (b) sound wave causing deflection of the basilar membrane, resulting in movement of the sterocillia with respect to the tectorial membrane, leading to depolarisation within the hair cells and innervation of the spiral ganglion neurons (Image adapted from www.naturereviews/neuroscience).

1.1.2 Primary Auditory / Spiral Ganglion Neurons

Auditory information is transmitted from hair cells to the primary auditory neurons also known as spiral ganglion neurons (SGNs), whose cell bodies are located in compartments within the modiolus termed Rosenthal's canal. The SGN cell bodies or somata are separated from the scala tympani and scala vestibuli by a thin layer of bone called the osseous spiral lamina (OSL) (Slepecky, 1996). This layer of bone contains numerous micropores, the canaliculae perforantes. These pores are thought to be the basis of communication between the scala tympani and RC (Shepherd and Colreavy 2004).

SGNs are bipolar neurons with a peripheral dendrite projecting from the cell body to innervate the organ of Corti, and a central axon which projects to the cochlear nucleus (CN) in the brainstem (Shepherd, 2004). SGN's are unequally divided into two types: type I SGNs (90%-95%, numbering approximately 30,000 in normal adults) and type II SGNs (5%-10%) (Spoendlin,1971). Type I SGNs are characterised by their large myelinated cell bodies (diameter of 25-30μm), spherical nuclei and pronounced nucleolus. In contrast, type II SGNs are about half the size of their type I counterparts, are unmyelinated and possess a smaller nucleus and an insignificant nucleolus (Spoendlin and Schrott, 1989).

The peripheral processes of type I SGNs extend toward the organ of Corti, where they pass through tiny openings in the basilar membrane termed habenulae perforate, before terminating on a single inner hair cell. Each inner hair cell is innervated by 20-30 type I SGNs in a convergent manner. In contrast, only a small number of type II SGNs innervates OHCs. This pattern of innervation is divergent, with each type II neuron

synapsing on approximately 10 OHCs (Webster, 1992). In humans, each OHC receives 4-8 afferent endings (Nadol, 1983b).

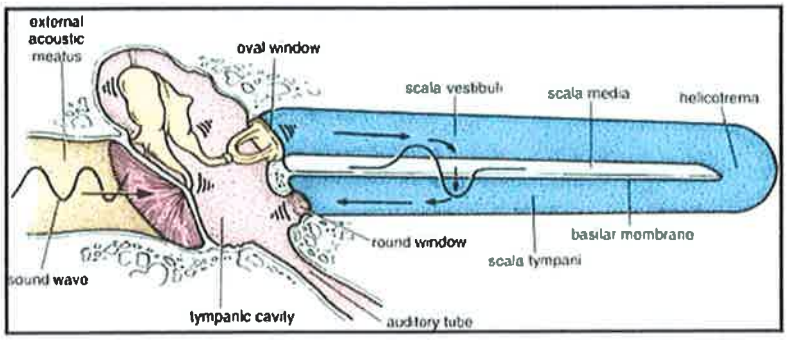
Both type I and type II SGNs possess central axons that project toward the CN in the brainstem. These nerve fibres make up the auditory nerve (AN) and are thus labelled auditory nerve fibres (ANFs). The AN contains roughly 5-10% unmyelinated fibres which arise from type II cell bodies (Ryugo et al., 1991).

A number of studies have used the enzyme horseradish peroxidise to record from and subsequently identify individual primary afferent neurons (Liberman, 1982). These studies have shown that all, or substantially all, of single unit activity recorded from the auditory nerve are from myelinated axons of type I neurons (Robertson, 1984). There has only been one confirmed instance where a recording has been taken from a type II SGN, and in this case the cell was silent (Robertson 1984). Since responses to sound have not yet been recorded from type II fibres, it is not clear what role they play in audition (Young, 1998).

The transfer of sound energy from the middle ear system to the cochlea is via the stapes footplate and oval window. This sound energy sets up a travelling wave in the perilymph of the cochlea and causes displacement of the basilar membrane. The basilar membrane is tonotopically organised in terms of frequency along its length due to its mechanical characteristics, with high frequency sounds causing displacement of the basilar membrane at more basal regions and low frequency sounds at more apical regions (Gelfand 1995). Consequently, the displacement of the basilar membrane causes deflection of the sterocilia of the hair cells at particular frequency locations along the basilar membrane. When the basilar membrane is driven upward, the

stereocilia deflect towards the tallest sterocilia, stretching the tip-links and causing the mechanical ion channels to open. This leads to an influx of potassium via the voltage gradient created by the endocochlear potential, and results in a depolarization of the hair cells and release of the neurotransmitter glutamate into the hair cell/SGN synapse. When the basilar membrane moves downwards the stereocilia deflect towards the smallest stereocilia, compressing the tip-links, closing the ion channels and reducing the probability of potassium ions entering the channel. In this case, the hair cell becomes inhibited and the probability of neurotransmitter release is reduced. The amount of neurotransmitter released affects the activity in the neighbouring SGNs (Pickles 1999, Shepherd 2004).

(A)



(B)

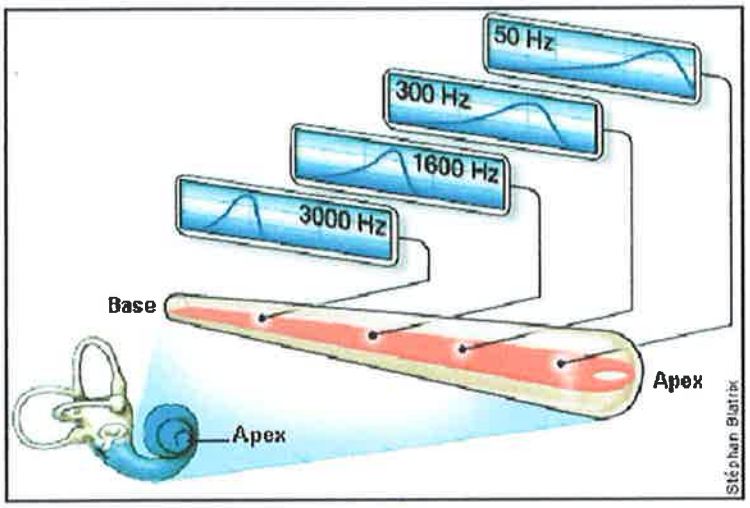


Figure 1.3 (a) shows displacement of the basilar membrane due to the travelling sound wave. (b) Illustrates the tonotopic organisation of the basilar membrane. (Adapted from www.iurc.montp.inserm.fr/cocphys/cocphys.htm)

1.2 Hearing loss and Spiral Ganglion Neuron Degeneration

In mammals, sensorineural hearing loss occurs as a result of permanent damage to sensory hair cells in the cochlea, thereby interrupting the normal pathway for sound signals to be transmitted via the auditory nerve to the brain. Hair cells of the organ of Corti are very sensitive to pathological insult and can be permanently damaged, most commonly as a result of an exposure to loud noise, administration of ototoxic medications, infection and the aging process. In addition to their vulnerability to insult, hair cells are unable to regenerate in mammals once damaged resulting in some degree of hearing loss. Due to the tonotopic organisation of the cochlea, the region(s) along the basilar membrane from which the hair cells are lost or damaged will determine the sound frequency (ies) at which hearing impairment is experienced. A significant loss of or damage to hair cells along the entire length of the basilar membrane can result in a permanent severe to profound hearing loss.

There are several degenerative changes that occur in the peripheral and central auditory systems as a result of sensorineural hearing loss. While the majority of studies describing these pathological changes have been performed in animal models, the findings of these studies are generally consistent with those reported from investigations on human tissue.

The loss of hair cells initiates a series of pathological changes within the cochlea and auditory brainstem and these changes increase with the duration of deafness (Hardie & Shepherd 1999). Studies have shown that there is a significant and progressive reduction in SGN density following the onset of deafness (Leake & Hradek 1988;

Shepherd & Hardie 2001; Hardie & Shepherd 1999), which eventually affects more than 90% of the neurons in the cochlear nerve (Spoendlin 1984). The time course of this degeneration of SGNs varies across species, from weeks to months in rats (Bichler et al 1983; McGuinness & Shepherd 2005) and guinea pigs (Webster & Webster 1978; Miller & Altschuler 1995) to years in cats (Leake & Hradek 1988; Shepherd & Hardie 2001) and extends over an even longer period in humans (Otte et al 1978; Nadol et al 1989). Despite the differing time frames among species, the process of SGN degeneration is generally the same and progresses over time.

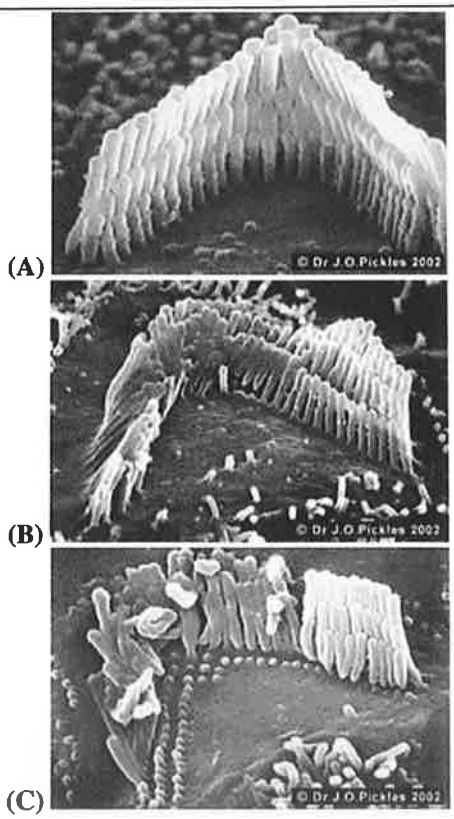


Figure 1.4 (a) illustrates electron microscopy images of normal hair cell sterocillia (b) moderately damaged sterocillia following noise induced hearing loss (c) severely damaged sterocillia following noise induced hearing loss.
(pictures from http://www.musiced.co.uk/features/hearing/index/html)

Degeneration caused by sensorineural hearing loss appears to be due, at least in part, to the removal of neurotrophic support normally received from cells within the organ of Corti (Ylikoski et al 1993; Schecterson and Bothwell, 1994; Ernfors et al 1995. In animal models, this degeneration is characterised by a rapid and widespread loss of the unmyelinated peripheral dendrites within the organ of Corti (Terayama et al 1977). The peripheral dendrites within the habenula become swollen and the adjacent portions of the axons show considerable vacuolisation, indicative of retrograde degeneration (Spoendlin 1984a). This is followed by gradual retraction of these dendrites within the osseous spiral lamina (OSL), with concurrent swelling and degradation of their myelin sheaths. The myelin overlying the ganglion cell soma subsequently breaks down and the cell body begins to shrink prior to complete degeneration (Spoendlin 1984; Leake & Hradek 1988; Nadol et al 1989). The ultimate result is a complete loss of the SGNs and degeneration of the cochlear nerve fibres ascending centrally. Regardless of species, SGN and auditory nerve fibre degeneration is an ongoing process, eventually resulting in very small numbers of surviving SGNs after long periods of deafness (Leake & Hradek 1988; Hardie and Shepherd 1999).

Analogous to animal studies, the degeneration of peripheral processes in human cochleae following sensorineural hearing loss is substantial, with the degree of pathology most pronounced at the base and decreasing apically (Nadol et al 1989; Nadol 1997). Studies of human temporal bones have shown significant variability in SGN numbers following sensorineural hearing loss in adults (Otte et al 1978; Nadol 1989). This variability is reported to be due to several factors including aetiology, extent of pathology and duration of deafness (Nadol 1989). SGN loss is reported to be

more pronounced in older subjects and, supporting observations in animal studies, follows a longer duration of hearing loss (Otte et al 1978; Nadol et al 1989). Clinical studies in children have reported a more even distribution of SGNs throughout the cochlea, and no evidence of ongoing degeneration following deafness in subjects ranging from zero to nine years of age (Miura et al 2002). Nevertheless, the size of the cochlear nerve is significantly reduced in children with sensorineural hearing loss (age one month to 14 years) when compared to normal hearing controls (age two to 16 years).

In addition to the morphological change to the SGNs following hair cell loss, significant physiological changes were also observed. The spontaneous activity of SGNs is significantly reduced following hair cell damage and their response properties to brief electrical stimuli are greatly affected (Shepherd & Hardie 2001). These degenerative alterations in the auditory system can decrease auditory processing via the cochlear implant underpinning the importance of both hair cells and SGNs in determining the clinical performance of cochlear implant patients (Shepherd & Hardie 2001).

Furthermore, the reduction in afferent input to the central auditory system resulting from a severe-profound SNHL can lead to degenerative, atrophic and functional changes in the central auditory system (Hardie 1998).

1.3 The Cochlear Implant

Individuals with a severe to profound sensorineural hearing loss receive minimal if any benefit from conventional digital hearing aids due to their extensive loss of hair cell function. Currently the only clinical treatment for the profound sensorineural hearing loss is a cochlear implant; a neural prosthesis capable of circumventing the damaged hair cells and directly stimulating residual spiral ganglion neurons. The cochlear implant consists of an external microphone and speech processor and an internal implanted receiver-stimulator. The microphone detects sounds and speech from the environment and these signals are then sent to the speech processor, which is responsible for encoding pitch and loudness. The pitch is determined by selecting which electrodes along the array are stimulated and changing the intensity of the stimulation varies the loudness. This code then travels as radio waves via the transmitting coil in the headset across the skin to the internal receiver-stimulator. The internal portion of the implant is surgically inserted into the scala tympani of the cochlea adjacent to the modiolus where the residual SGNs are located. This internal component of the implant consists of an electrode array of 22 platinum bands, which importantly replicate the tonotopic arrangement of the IHCs. The implant package then decodes the signal, and determines how much electrical current needs to be sent to the different electrodes, to directly stimulate the SGNs. This acoustic information is then transferred via the cochlear nerve to the central cochlear nuclei. The absence of implanted batteries and wires through the skin is important for minimising the risk of infection (Clarke 1995).

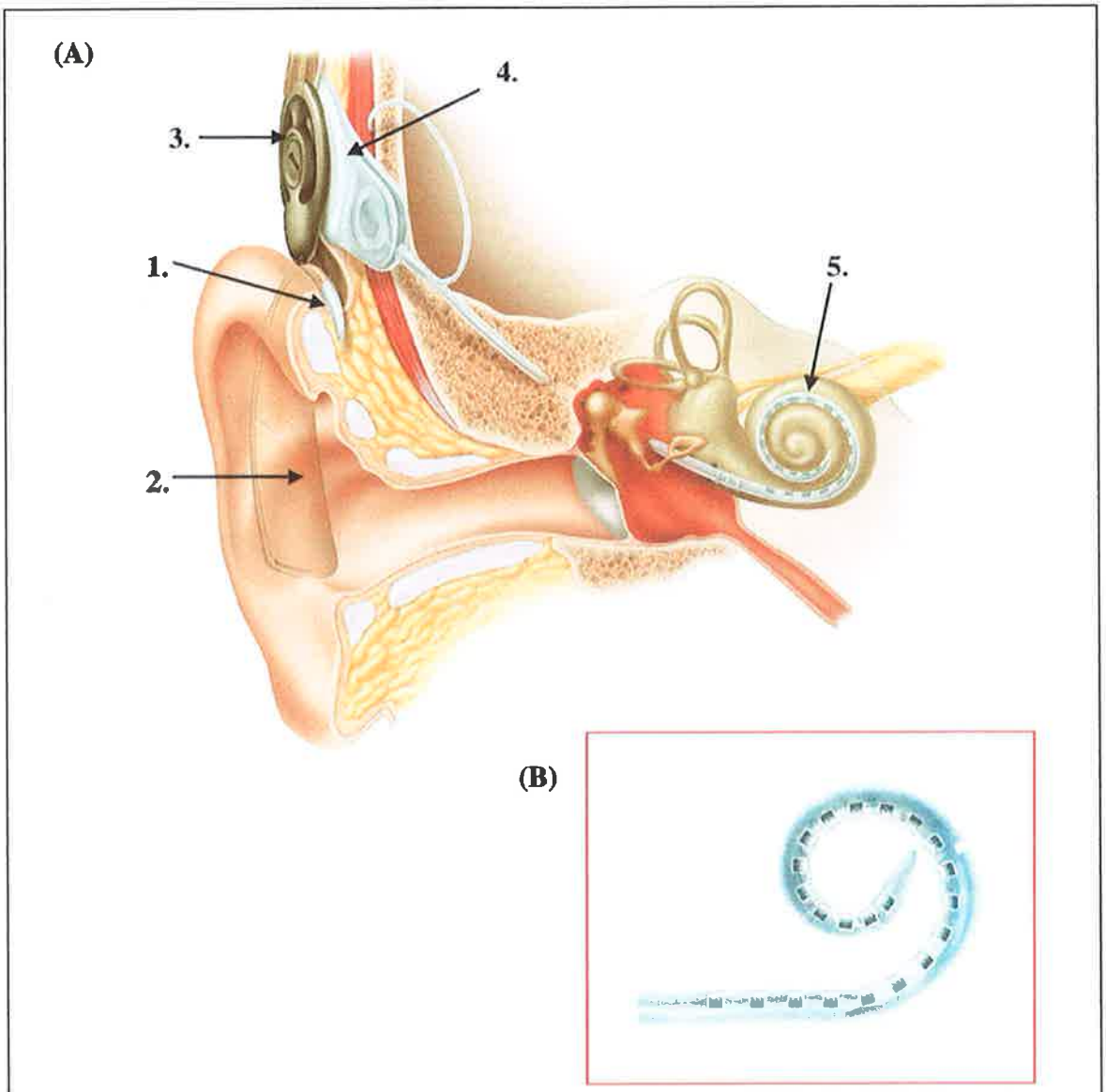


Figure 1.5 (a) shows the components of the cochlear implant 1. The microphone, 2. The speech processor, 3. The transmitter coil, 4. The receiver-stimulator, 5. The electrode array. (b) The electrode array comprising of 22 platinum bands which replicate the tonotopic organisation of the cochlea (images supplied by cochlear Ltd 2010).

As mentioned earlier (section 1.2), the loss of hair cells following deafness causes secondary retrograde degeneration of SGNs. The loss of dendrites, demyelination and shrinkage of excitable neural elements can cause vast changes to the sensitivity, loci and temporal characteristics of neural responses to electrical stimulation (Leake & Hradek 1998). Therefore damage to and loss of auditory nerve fibres may have a detrimental affect on the ability of electrical signals to travel from the cochlear implant to the brain (Otte et al 1978). Indeed, auditory performance of cochlear implant users varies, and may depend on the extent of SGN degeneration in individual patients (Dowell 2002).

Despite the fact that the aetiology of deafness is the best predictor of SGN population density and speech perception outcomes with an implant (Nadol et al 1989), to date there has been no direct relationship found between SGN population and cochlear implant function in humans (Rubinstein & Miller 1999). This is perhaps due to the lack of post-mortem material from cochlear implant patients and the variability intrinsic to measures of histopathology and speech perception assessment. However, animal studies including monkeys (Pfingst et al 1981), rats (Hall 1990) and guinea pigs (Miller et al 1995) have all shown significant correlation between cochlear prosthesis outcomes and the quality and quantity of the surviving SGN population following deafness. One finding in humans, which is consistent with animals, is that the psychophysical dynamic range was found to be significantly correlated with size of the SGN population in a post mortem study of cochlear implant temporal bones (Kawano et al 1998).

1.3.1 Does the survival of spiral ganglion neurons affect patient outcomes with the cochlear implant?

Controversy exists as to whether the number of SGNs influences the clinical performance of cochlear implants. Fayad and colleagues (2006) demonstrated that almost all profoundly deaf patients report benefits after receiving a cochlear implant, however these benefits vary substantially between individuals. Kahn et al. (2005) investigated the perception of single-syllable words using the most recent implant devices, and reported a median patient score of 40%, however individual scores ranged from 0-95%. A significant indicator of cochlear implant performance is reported to be the duration of deafness prior to implantation (Hartshorn et al. 1991; Kileny et al. 1991), although aetiology and experience with the device are also important determinants (Blamey et al. 1996). Presumably the decrease in patient outcomes with an increased duration of deafness is attributed to the ongoing degeneration of neural elements in the cochlea (Hartshorn et al. 1991). While this may seem logical, there are now conflicting reports as to whether this is in fact the case. Whilst animal studies indicate that ongoing degeneration of SGNs may compromise the performance of the cochlear implant (Shepherd & Javel 1997; Hardie & Shepherd 1999) several studies using human temporal bones have reported no such correlation (Blamey 1997) or in fact a negative correlation (Nadol et al 2001; Fayad & Linthicum 2006; Linthicum & Fayad 2009).

When attempting to answer this question it is important to consider several facts. Firstly the clinical findings should be interpreted with caution, given that these studies are unable to provide the necessary controls in the way the animal studies are capable

of illustrating. Moreover these clinical studies do not take into account the performance of any given individual, should they have a greater compliment of SGNs (Rubinstein & Miller 1999). There are significant physiological differences between animals and humans, namely the very slow degeneration of the human auditory nerve in comparison to the rapid degeneration observed in the guinea pig. This may have significant consequences for therapies aiming to improve outcomes for cochlear implant patients.

The varying outcomes with this electrical device suggest that exact performance indicators have yet to be elucidated, and considering that patients with cochlear nerve aplasia do not benefit from electrical stimulation, it is likely that SGN numbers are important (Fayad & Linthicum 2006). It seems reasonable to assume that a good functional outcome using a cochlear implant is dependant on a number of factors, including (but not limited to) the density and integrity of auditory neurons along the length of the modiolus. However this role of the SGNs in the performance of the cochlear implant is (as yet) not fully elucidated and the overall outcomes with this device are likely to be influenced by several factors.

It is therefore important to explore methods for preventing the progressive degeneration of SGNs following the loss of hair cells. The identification of neurotrophins as important survival-promoting and protective agents for SGNs may have important implications for the effectiveness of the cochlear implant and in the future, may lead to pharmacological methods of halting or reversing the retrograde degeneration of SGNs of the inner ear.

1.4 Neurotrophins

During development of the nervous system, neural axons grow towards and form synaptic connections with target tissues which release naturally occurring proteins called neurotrophic factors. The word "neurotrophic" is derived from the Greek terms for nerve (neuro) and nourish (troph) (Dan 2003). As the name suggests, neurotrophic factors support the growth, differentiation and survival of neurons in the developing nervous system (Yuen et al 1996). The quantity of neurotrophic factors released by target tissues has important significance during development, as proposed by the Neurotrophic Factor hypothesis.

This hypothesis states that the production and secretion of neurotrophic factors by target tissues is limited, and neurons that fail to obtain a sufficient supply of such factors will undergo the process of naturally occurring cell death or apoptosis (Oppenheim 1989; Bothwell 1995). This process of eliminating neurons during development ensures that target cells are innervated by the appropriate type and number of neurons (Bothwell 1995).

Extrapolation of this theory suggests that destruction of neuronal tissue will cause degeneration of the respective neuronal population due to a lack of neurotrophic support. Accordingly, exogenous application of such neurotrophic factors is expected to prevent the degeneration that would normally occur, and also maintain the survival of these cells and their axonal processes. Indeed neurotrophic factors can influence the synaptic efficacy and plasticity in the mature nervous system (Lessmann et al 2003) and can promote nerve regeneration after injury (Altschuler et al 1999), with

studies showing neuron survival and regeneration of neural processes in both *in vitro* and *in vivo* models.

1.4.1 The neurotrophin family

Neurotrophins are the best characterised family of neurotrophic factors, and comprise nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5 (NT-4/5). Neurotrophins signal via two receptor types, binding with equal affinity to the p75 neurotrophin receptor and with higher affinity to a specific member of the tyrosine receptor kinase (trk) family. NGF preferentially binds to trkA, both BDNF and NT-4/5 bind to trkB, and NT-3 preferentially binds to trkC (Yuen et al 1996). While trk receptors transmit positive signals such as enhanced survival and growth, p75 transmits both positive and negative signals. Therefore, the two receptors types can either suppress or enhance each other's actions to mediate neurotrophic effects. For example, when activated in the absence of a strong trk signal, p75 induces apoptosis; however when activated in the presence of a strong trk signal, it enhances responses to neurotrophins (Kaplan & Miller 2000).

Neurotrophins in the cochlea, particularly BDNF and NT-3 have many important roles. They are crucial for the development of the auditory system and for the survival and resprouting of mature SGNs in animal models of deafness (Ernfors et al 1996; Staecker et al 1996; Gillespie et al 2003; Miller et al 2003; Wise et al 2005; Rejali et al 2007; Evans et al 2009; Sun & Salvi 2009). Neurotrophic factors produced and secreted by the auditory nerve fibres play a pivotal role in the elongation and guidance of auditory

fibres to their appropriate target within the organ of Corti (Avila et al 1993; Despres & Romand 1994).

BDNF and NT-3 have been demonstrated in many studies to be uniquely important for the normal development and innervation of the inner ear. Studies in rats and mice using in situ hybridisation have revealed higher expression of messenger RNA for both BDNF and NT-3 during the period when SGNs project their processes toward the sensory epithelia of the developing cochlear duct (Pirvola et al 1992; Ylikoski et al 1993; Pirvola et al 1994; Wheeler et al 1994). Simultaneously, the developing SGNs express messenger RNA transcripts for the BDNF and NT-3 receptors, trkB and trkC (Ylikoski et al; Privola et al 1994; Zheng et al 1995).

Further developmental studies using gene knockout mice have also shown that BDNF and NT-3 are important in the developing auditory system. The SGN population of mice lacking the NT-3 gene or the trkC receptor gene was reduced by approximately 85% (Farinas et al 1994; Ernfors et al 1995; Malgrange et al 1998). In addition studies of double mutant mice have reported that the absence of both BDNF and NT-3 or trkB and trkC leads to nearly complete loss of inner ear neurons (Ernfors et al 1995) and a complete loss of all afferent innervation of the ear (Fritzsch et al 1997). Collectively these studies provide strong evidence that the neurotrophins BDNF and NT-3 are produced and secreted by the hair cells and through their high affinity trk receptors, significantly influence the development and survival of SGNs in the inner ear.

1.4.2 The role of neurotrophins following deafness

There is extensive evidence from both in vitro and in vivo experimental animal models of deafness that neurotrophins also play an important role in the survival and maintenance of SGNs. BDNF, NT-3 and NT 4/5 have been reported to promote the survival of early postnatal rat SGNs in culture. BDNF can enhance SGN survival significantly over neurotrophin-free control cultures (Malgrange et al 1996; Marzella & Clarke 1999) and one particular study reported a two fold increase in survival of early post-natal SGN survival in vitro, although the effects were not as potent in comparison to BDNF (Zheng et al 1995; Malgrange et al 1996). The survival effects of BDNF and NT-3 on SGN survival continues in adulthood as shown by in vitro cultures of adult rat SGNs (Lefebvre et al 1994) thus supporting the theory that neurotrophins are required for ongoing trophic support in the auditory system throughout adulthood.

It has also been reported that a combination of neurotrophins can further enhance the survival effects of individual neurotrophins, for example both BDNF and NT-3 can act synergistically to produce more enhanced SGN survival results than either neurotrophin alone (Marzella & Clarke et al 1999).

In vitro studies have also shown that each of these neurotrophins - BDNF, NT-3 and NT-4/5 can protect SGNs from ototoxic effects of aminoglycosides such as gentamicin; therapeutic drugs such as salicylates, and chemotherapeutic agents such as cisplatin (Zheng et al 1995; Zheng & Gao 1996; Duan et al 2002). These findings indicate a role for neurotrophic factors in preventing damaging effects of therapeutic but ototoxic

agents, which are one of the leading causes of damage to the peripheral auditory system leading to hearing loss.

More significantly, in vivo studies have demonstrated that delivery of exogenous neurotrophins to the mammalian inner ear can prevent SGN degeneration that is normally seen following loss of hair cells. BDNF can also provide significant survival effects in vivo preventing SGN degenerating following ototoxic deafness. Staecker et al (1996) reported that intracochlear infusion of BDNF via a mini-osmotic pump over a period of eight weeks yielded a 78% SGN survival rate as compared to only 14-24% survival in the contra-lateral untreated ears. A similar study found that two weeks of treatment with BDNF provided a statistically significant enhancement in SGN survival over untreated ears in a similar deaf guinea pig model (Miller et al 1997). McGuinness & Shepherd (2005) have also shown enhanced survival of SGNs in deafened rats following four weeks of BDNF treatment.

Similar in vivo studies have shown that SGN degeneration following hair cell destruction can be prevented upon exogenous delivery of other neurotrophins to the adult mammalian inner ear. Richardson et al (2005) discovered that a single bolus of NT-3 can provide trophic support to SGNs for up to seven days following deafness in guinea pigs. Studies have shown that an enhanced survival of SGNs in ototoxically deafened ears is also far greater with NT-3 delivery compared to untreated controls (Ernfors et al 1996; Staecker et al 1996).

Although there is significant evidence to show that neurotrophins can prevent SGN degeneration following deafness, one study has revealed that the neurotrophin induced survival effects are not maintained beyond the treatment period. Gillespie et al (2003) have reported that infusion of BDNF into the scala tympani of deafened guinea pigs over a period of four weeks has a survival-promoting effect on SGNs. However they found that cessation of the BDNF treatment led to an accelerated decline in auditory neuron survival, compared to that of the deafened untreated cochlea. This decline was so marked that as early as two weeks after the completion of BDNF treatment, SGN survival rates were not significantly different from untreated specimens. This would suggest that these neurotrophins may need to be delivered over a longer time period in order to be effective.

1.4.3 Potential clinical application of neurotrophins

It is now widely accepted that the withdrawal of neurotrophins that are normally expressed by the auditory hair cells can cause a retrograde degeneration observed in SGNs following hair cell damage (Ylikoski et al 1993; Ernfors et al 1995; Miller et al 2002). Although it has been shown that neurotrophins promote survival of SGNs in mouse, rat and guinea pig models of deafness, it seems likely that they need to be supplied to the cochlea continuously. To date mini-osmotic pumps have been used to administer neurotrophins to the inner ear of experimental animals; however, this method is not clinically viable in humans. For example, the pump and cannula system may provide a direct route for infection into the cochlea. In addition, commercially

available mini-osmotic pumps have a limited life span of a maximum of four weeks, necessitating regular replacement which would further increase the risk of infection to the inner ear. Since the perilymph of the inner ear is continuous with the cerebrospinal fluid of the brain via the cochlear aqueduct, inner ear infection may progress into meningitis in the brain (Wei et al 2006). As such, mini-osmotic pumps are not regarded as a viable option for treating SNHL in humans. It is therefore imperative to now devise alternative ways of administering neurotrophins over longer periods of time using a method that is clinically acceptable.

1.5 Nanoparticles in Medicine: as therapeutic agents in the inner ear

Nanotechnology is the understanding and control of matter generally in the 1-1000 nm dimension range. To give a perspective of size, 1 nm is 1 x 10^{-9} m. The application of nanotechnology to medicine, known as nanomedicine concerns the use of precisely engineered materials at this nano-scale to develop novel therapeutic and diagnostic modalities (Zhang et al 2007; Farokhzad & Langer 2006). As therapeutic delivery systems, nanoparticles allow targeted delivery and controlled release. Many advantages of nanoparticle-based protein/drug delivery have been recognized (Emerich & Thanos 2007); Roy et al 2010). It improves the solubility of poorly water soluble agents, prolongs the half-life of circulating proteins/drugs by reducing immunogenicity, releases drugs/proteins at a sustained rate or in an environmentally responsive manner and thus lowers the frequency of administration, delivers proteins/drugs in a targeted manner to minimize side effects and enables a combination

therapy which generates synergistic effects using two or more agents to suppress body resistance (Zhang et al 2008).

The advancement of inner ear medicine will require the development of a non-traumatic and non-toxic delivery of therapeutic molecules to the cochlea. However, drug delivery to the cochlea presents a number of technical challenges, which have hindered the development of therapeutic strategies for the treatment of sensorineural hearing loss and related ear disorders (Tamura et al 2005). Reasons for this difficulty in drug delivery to the cochlea include limited blood flow to the cochlea and the existence of the blood-labyrinth barrier which limits the transportation of molecules from blood to cochlear tissues (Juhn 2001). The sustained delivery of therapeutic molecules is also critical for the efficient treatment of the cochlea, as bioactive molecules usually require a period of minutes or hours over which to produce their therapeutic actions. Consequently a number of researchers are working to solve these problems by developing methods for local and sustained release of molecules into the cochlea (Lefebvre & Staecker 2002; Ge et al 2007; Scheper et al 2009; Roy et al 2010).

Synthetic biodegradable polymers have become very important as biomaterials for applications in tissue engineering and controlled biomolecular delivery. Among these materials, poly DL-lactic-*co*-glycolic acid (PLGA), polyglutamic acid (PGA), and polyethylene glycol (PEG) co-polymers have been widely utilized either as temporary scaffolds or as carriers for delivery of bioactive molecules (Hornyak 2005; Zhang 2008). They can be easily processed into a desired configuration and their physical, chemical, mechanical, and degradative properties can be engineered to fit a particular need.

Layer-by-layer capsules since their introduction in 1998, have attracted particular interest in the delivery of bioactive molecules. (Donath et al 1998; Caruso & Caruso et al 1998). This is largely because of the ability and ease of these capsules to tailor their properties (e.g. size, composition, porosity, stability and surface functionality). Layer-by-layer capsules can be assembled from a suite of materials including synthetic and naturally occurring polyelectrolytes. They are typically formed by the consecutive deposition of positively and negatively charged polymers where electrostatic forces facilitate layer build up. The colloidal template is then sacrificed thus leaving a hollow polymer capsule.

Templating techniques have also been employed to generate materials with tailored properties. The adaptability of templating permits control over the composition, morphology, and porosity of the structures produced (Caruso et al 2001; Han et al 2000; Ryoo et al 2001). One class of material that has been widely employed in template synthesis is mesoporous silica (MS), largely owing to its large surface area and tunable pore size (typically in the range of 2-50nm), (Kresge et al 1992). It is therefore possible to develop porous structures derived from biomacromolecules and biodegradable polyelectrolytes.

One of the challenges in this thesis was to find a method of encapsulating an acceptable quantity of BDNF into biodegradable biocompatible particles. It was also imperative that the structural integrity of the protein was maintained during the process to allow it be biologically active. The real challenge was to prolong the release of the protein over weeks to exert the maximal effect of the neurotrophin on the SGNs in the cochlea.

RESEARCH OBJECTIVES

HYPOTHESES

1. Brain-derived neurotrophic factor (BDNF) can be incorporated into nanoparticles by chemical synthesis.
2. Under physiological conditions, incorporated BDNF can be released over days and weeks.
3. Released BDNF preserves its chemical integrity.
4. Released BDNF maintains its biological activity.

AIMS

1. To use polymer chemistry to develop biodegradable and biocompatible nanoparticles which incorporate BDNF.
2. To establish physiological conditions that will allow these BDNF-nanoparticles to release its BDNF.
3. To quantify the amount of BDNF released using enzyme-linked immuno-adsorbant assay.
4. To determine its biological activity by testing its ability to differentiate SH-SY5Y neuroblastoma cell lines into neurons.
5. To surgically implant these nanoparticles into the deafened animal rat model and show a difference in SGN survival.

CHAPTER TWO

MATERIALS AND METHODS

Nanoparticle Development

Layer-by-Layer Capsules

2.1.1 Preparation of BDNF loaded-capsules using thiol functionalised polymethacrylic acid (PMA) and polyvinylpyrrolidone (PVP)

PMA-PDA (peroxydisulfuric acid) sample with 12 mol % of thiol groups was synthetised from PMA (15,000 Da) based on a previous report (SF Chong et al 2009). The thiol functionalised PMA (PMA_{SH}) solution is created by: PMA – PDA (Peroxydisulfuric acid) is measured out and dissolved in 0.5M dithiothreitol (DTT) buffer solution (20 mM MOPS pH 8.2) to a final concentration of 100 g/L (see figure 2.1). This solution is incubated at 37 ^{o} C until the solution is clear, (this should take approximately fifteen minutes). The PMA_{SH} solution is diluted to a 2 g/L concentration using 20 mM pH 4 sodium acetate buffer. Polyvinylpyrrolidone (PVP) Mw 360 000 was purchased from Sigma-Aldrich and used as received.

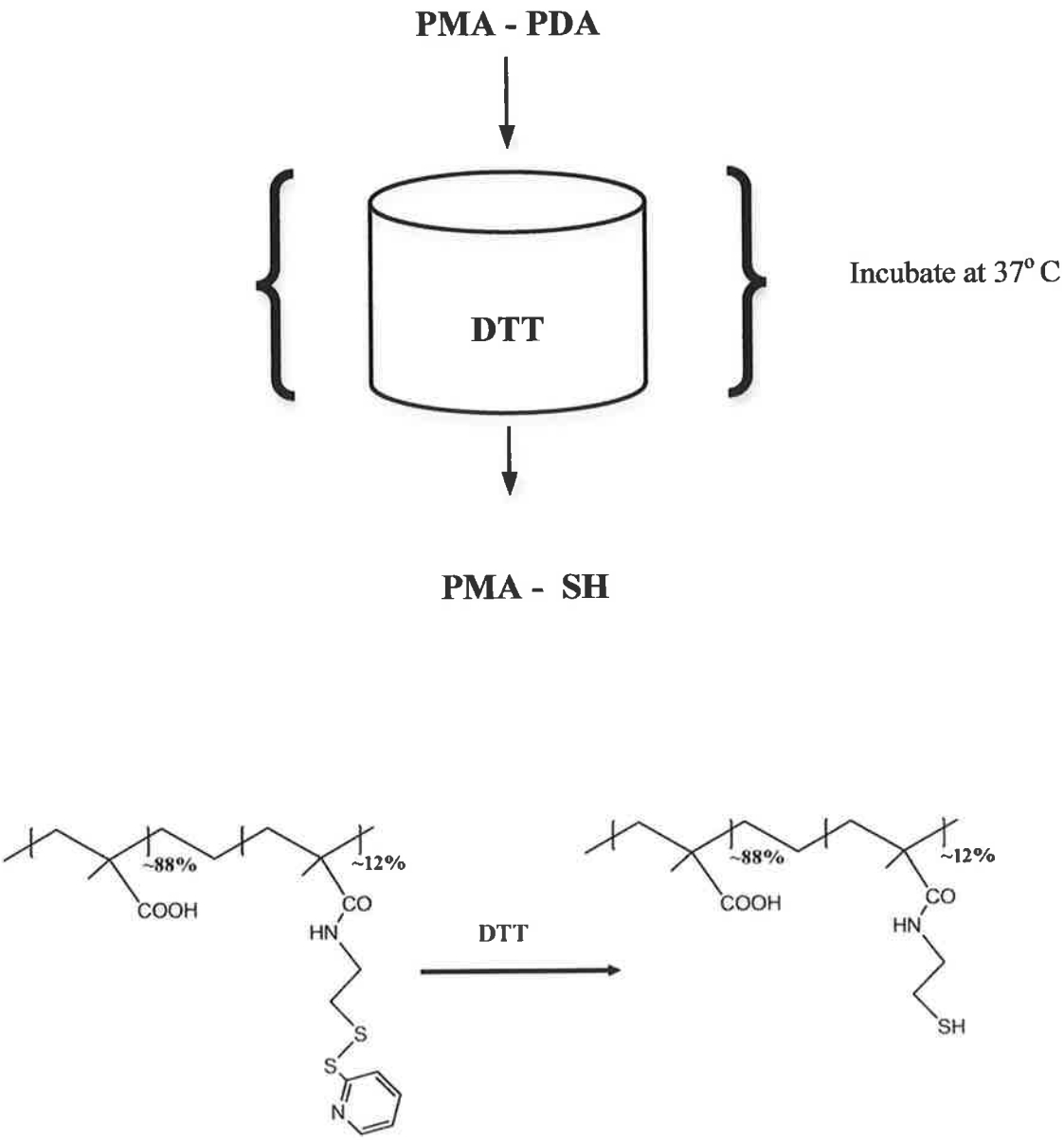


Figure 2.1 Creating thiol-functionalised polymethacrylic acid (PMA_{SH})

Bimodal mesoporous silica (BMS) spheres were prepared (see figure 2.2), briefly, 1.38×10^{-2} mole of CTABr and 2.1×10^{-2} mole of Na_2SiO_3 were dissolved to form a clear solution in 90 mL of Milli-Q water at 30 °C. 6.4 mL of ethyl acetate were then

added. The mixture was stirred for 30 s and allowed to stand at ambient temperature for 5 h. After this period of ageing, the bottle was kept at 90 °C for 48 h in an oil bath. The as-synthesized particles were collected by centrifugation and washed with absolute ethanol three times and then twice with Milli-Q water. After air-drying at room temperature, the particles were heated at 500 °C for 5 h to remove the surfactants. The BMS template was then functionalized with a layer of primary amine groups by 3-aminopropyltriethoxysilane (APTS) grafting, this will change the charge of the particles from negative to positive. In this process, the BMS particles were dispersed in toluene by sonication for 20 min before APTS was added to the suspension. The molar ratio of the BMS particle (calculated as SiO_2)/APTS/toluene was fixed at 5:1:500, and the suspension was refluxed for 24 h. The APTS-grafted BMS particles were separated from the solution by centrifugation, and washed in absolute toluene and methanol twice. Finally, the pellet was dried at 80 °C for 12 h.

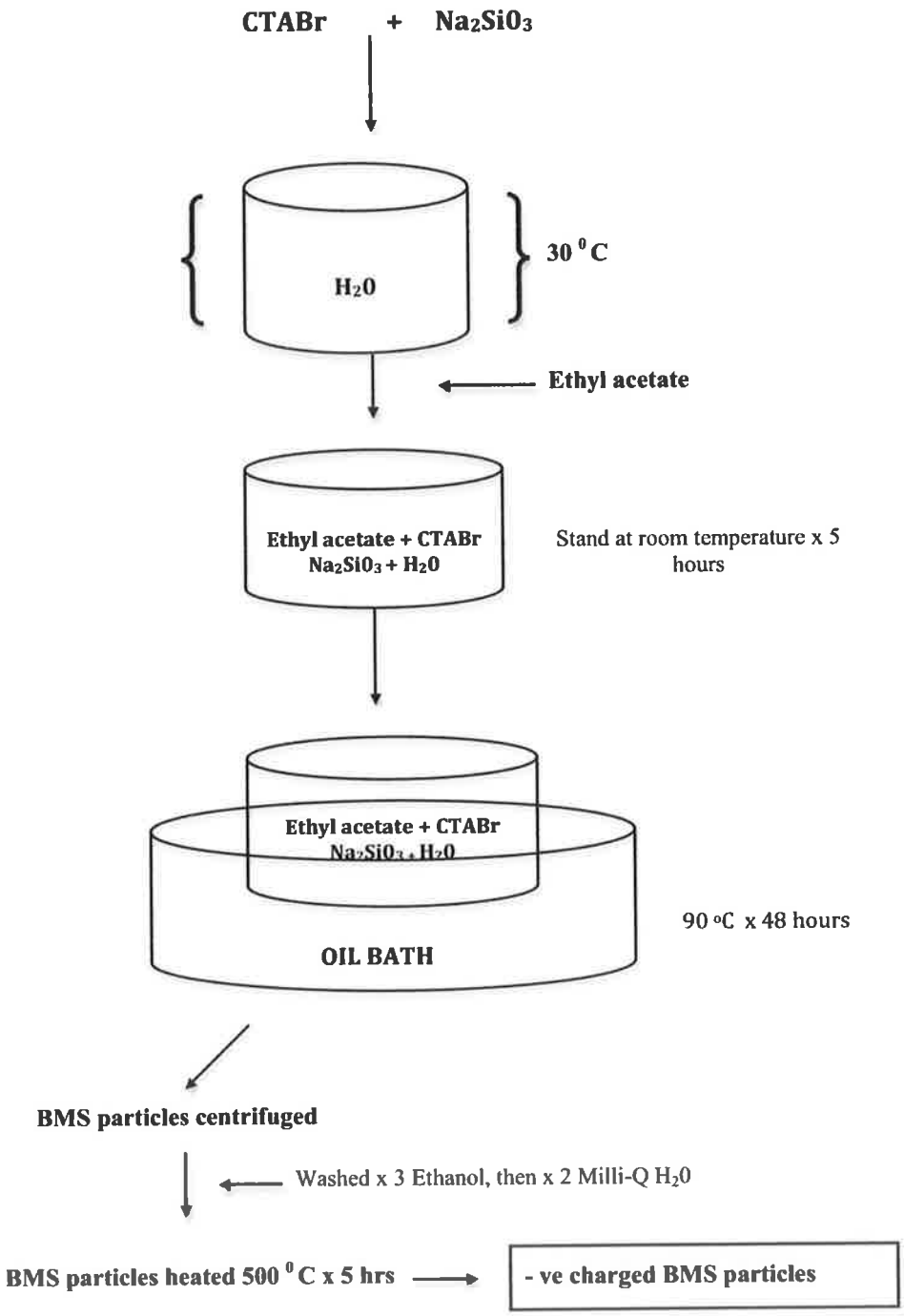


Figure 2.2 Formation of BMS particles

These silica spheres ranged in size from 2μm-3μm and had a bimodal pore structure, that is, smaller pores in the 2-3 nm range and larger pores ranging from 10 – 40 nm with a volume of 1.28 $cm^3 g^{-1}$.

BMS 2.4mg was washed 3 times with milliQ water. These particles were then dispersed in 100μL of the protein Brain Derived Neurotrophic Factor (BDNF) with a concentration of 0.05 mg mL^{-1} in milliQ water at pH 7.9 and allowed to mix at room temperature for 24 h. After protein infiltration the suspension was centrifuged at 1000 *g* and the first supernatant sample was taken and stored for analysis. The solution was then washed 3 times with 20 mM sodium acetate buffer pH 4.0 to remove excess protein.

The protein laden silica particles were then incubated in a 2g L^{-1} of PVP for 15 min. After been washed three times in 20 mM sodium acetate buffer (pH 4.0), the particles were suspended in a 2 g L^{-1} solution of PMA_{SH} for 15 minutes. The particles were again incubated in 2 g L^{-1} solution of PVP for 15 minutes, polymers were sequentially added alternating between PVP and PMA_{SH} until 10 layers had been deposited. In order to provide stability to the capsules at physiological pH the disulphide bridges between PMA_{SH} chains were oxidised. This was achieved by treating the particles with 2 mM chloramine T in 10 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.0) for 1 minute. The particles were washed three times with fresh buffer and dispersed in 50 μL of 20 mM sodium acetate buffer (pH 4.0) to which 200 μL of 2 M HF / 8 M NH_4F (pH 5.0) was added to dissolve the silica core. The resulting capsules were washed by centrifugation (4500 *g* for 5 minutes) and dispersion, which was repeated until the pH of the supernatant was equal to the fresh buffer solution. The capsules containing BDNF were stored in 20 mM sodium acetate buffer solution (see figure 2.3).

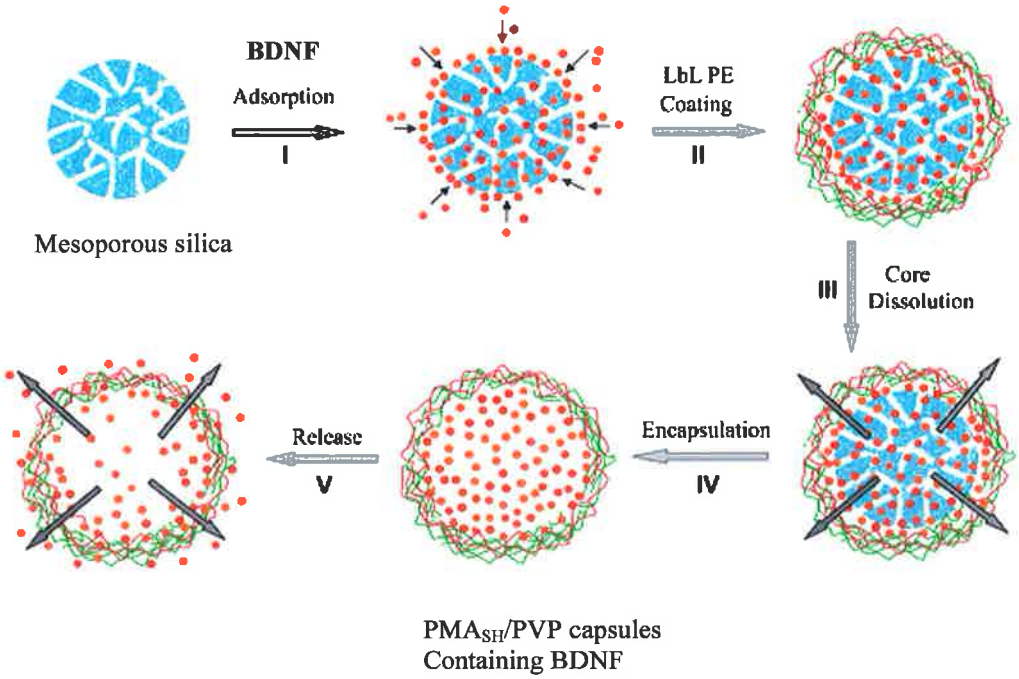


Figure 2.3. Schematic diagram showing the steps in creation of the BDNF loaded PMA$_{SH}$-PVP capsules

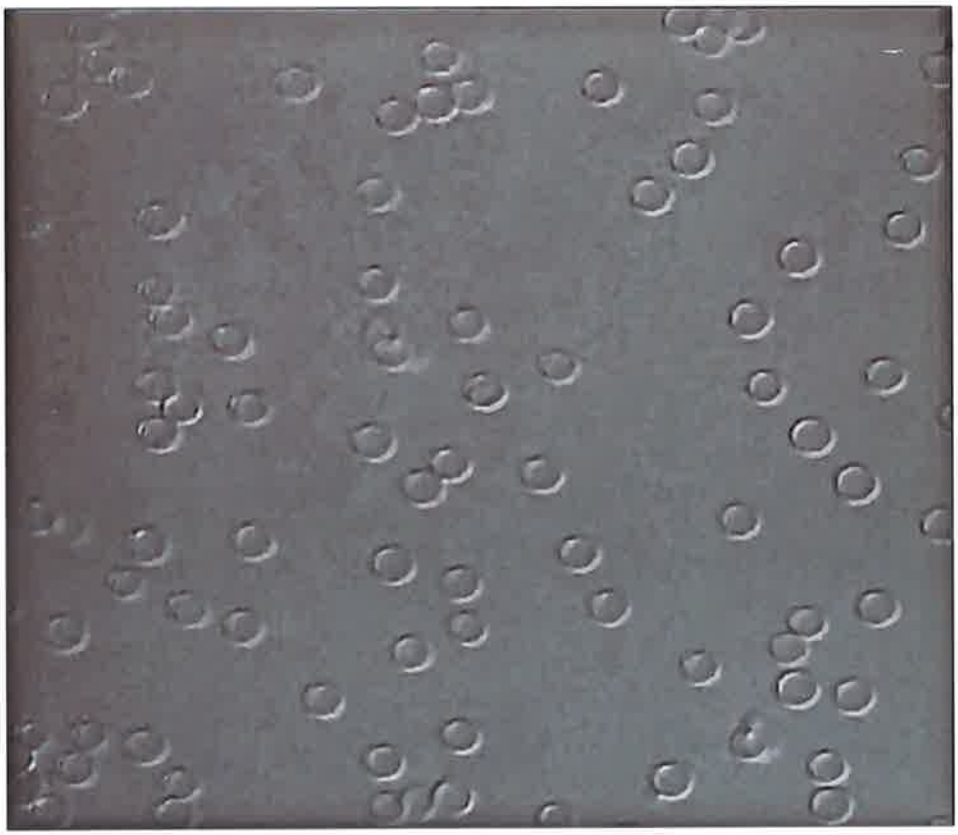


Figure 2.4. PMA$_{SH}$ -PVP capsules with a diameter of 3 μm, x 300 magnification

2.1.2 Preparation of Chitosan / Alginate particles containing BDNF

Mesoporous silica 10mg (SGX 1000 (Phenomenex Ltd, 5 μm in diameter, 100 nm pore size) was washed three times with milliQ water. Chitosan which was 85% deacetylated was purchased from Sigma-Aldrich (catalogue no. 44,886-9) and used as received. 400 μL of chitosan (1 wt%) was added to the silica suspension and allowed to infiltrate for 24 hours. This sample was centrifuged at 1000 *g* and washed three times with pH 4.0 sodium acetate buffer to remove excess polysaccharide. The chitosan was then cross linked with 0.5% glutaraldehyde. 200 μL of the glutaraldehyde solution was added and allowed to react for 24 hours. The suspension was then centrifuged at 1000 *g* and the sample was washed 5 times with sodium acetate buffer to remove all the excess glutaraldehyde solution. The silica core was then dissolved by the addition of 200 μL of 2 M HF / 8 M NH_4F (pH 5.0). The resulting particles were then washed by centrifugation (4500 *g* for 5 minutes) and dispersion, which was repeated until the pH of the supernatant was equal to the fresh buffer solution. The zeta potential of the particles was then measured and was shown to be 18.4 +/- 0.4 mV. In order to facilitate the infiltration of the cationic protein BDNF, the mesoporous chitosan particles were mixed with negatively charged polysaccharide sodium alginate. 250 μL of the medium viscosity sodium alginate purchased from Sigma-Aldrich (derived from *Macrocystis pyrifera* catalogue no. A2033) was added to the chitosan particles. This was allowed to infiltrate for 30 minutes after which the sample was centrifuged at 2500 *g* for one minute. The sample was washed three times with sodium acetate buffer to remove the excess protein. The zeta potential of the chitosan-alginate particles was then measured which revealed an overall negative charge of -39 +/- 5.0 mV.

These particles were then dispersed in 100μL of the BDNF solution with a concentration of 0.05 mg mL^{-1} in milliQ water at pH 7.9 and allowed to mix at room temperature for 24 hours. The sample was centrifuged at 2500 *g* for one minute and washed three times with sodium acetate buffer to remove the excess protein. The particles containing BDNF were stored in 20 mM sodium acetate buffer solution. (see figure 2.6).

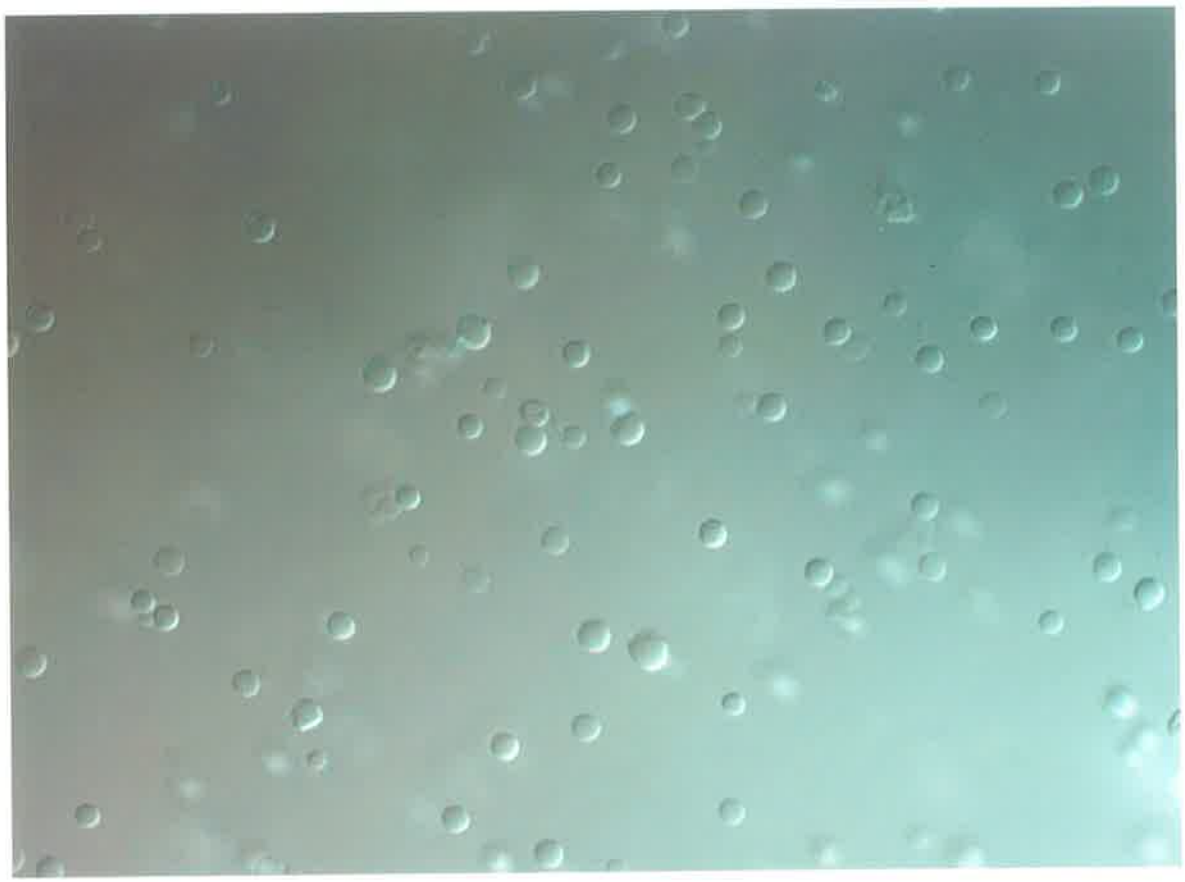


Figure 2.5. Chitosan/Alginate particles 5 μm in diameter x 300 magnification

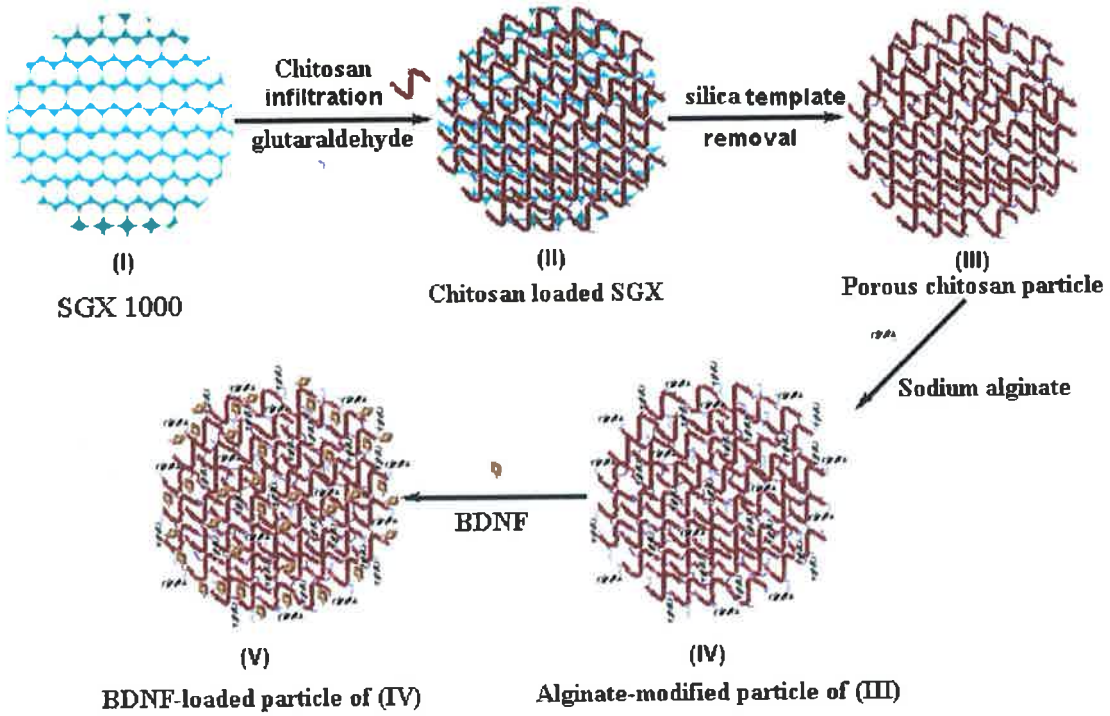


Figure 2.6. Schematic illustration of the synthesis of the Chitosan/Alginate/BDNF particles

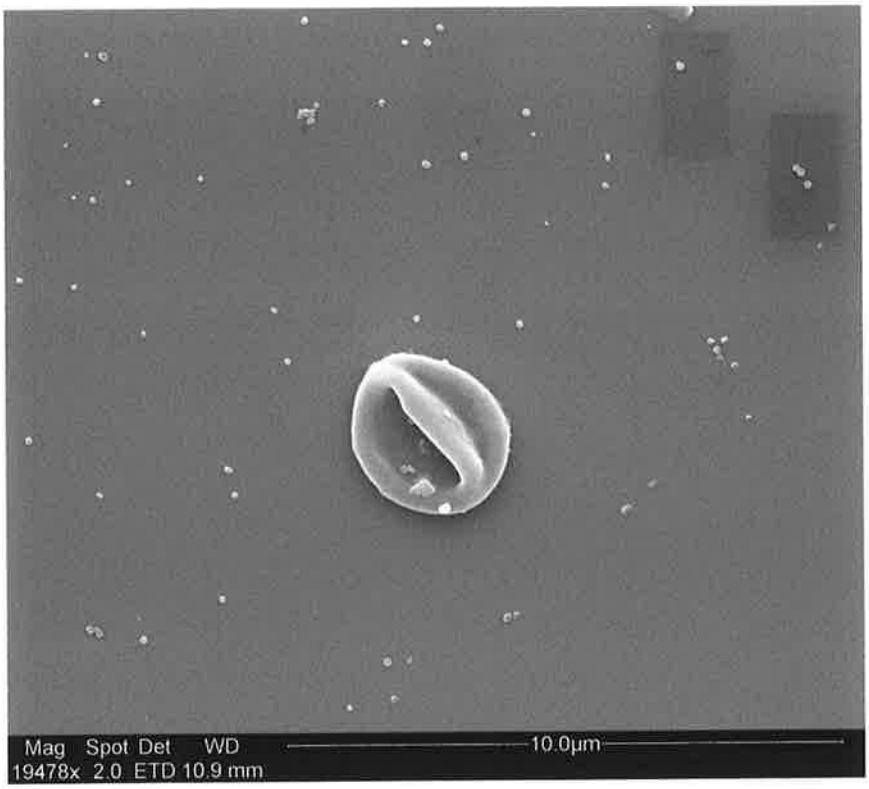
2.1.3 Preparation of Chitosan / Alginate / Thiol-functionalised Polymethacrylic acid (PMASH) / Polyvinylpyrrolidone (PVP) particles containing BDNF

These particles were made in an identical way to the above chitosan-alginate BDNF particles (section 2.1.2). After infiltration of the protein BDNF into the particles they were then incubated in a 2g L^{-1} of PVP for 15 minutes before they were washed three times in 20 mM sodium acetate buffer (pH 4.0), the particles were suspended in a 2 g

L^{-1} solution of PMASH for 15 minutes. Polymers were sequentially added until 6 layers had been deposited.

2.1.4 Preparation of Polyglutamic Acid (PGA) particles containing BDNF

The bimodal mesoporous silica (BMS) spheres were prepared as described in section 2.1.1 paragraph 1. The polypeptide PGA particles were prepared by incubating 2 mL PGA stock solutions (5 mg mL^{-1}) with 10 mg of APTS-modified positively charged BMS particles for 12 h in 20 mM acetate buffer (pH 4.0), followed by removal of excess polypeptide. After covalent cross-linking of the PGA polypeptides by separately adding 0.05 mL of aqueous cystamine (10 mg mL^{-1}) and 0.2 mL of EDC (60 mg mL^{-1}) to the pellet and incubating at room temperature for 6 h. After washing with deionized water, the BMS template particles were removed by exposure to a solution of 2 M hydrofluoric acid / 8 M ammonium fluoride buffer (pH 5) to obtain the replicated PGA particles, which is then dispersed in 1 mL milliQ water. 200 μL of this 10 mg mL^{-1} solution of PGA particles was taken and spun down and the supernatant removed. These particles were then dispersed in 100μL of BDNF with a concentration of 0.05 mg mL^{-1} in milliQ water at pH 7.9 and allowed to mix at room temperature for 24 hours.



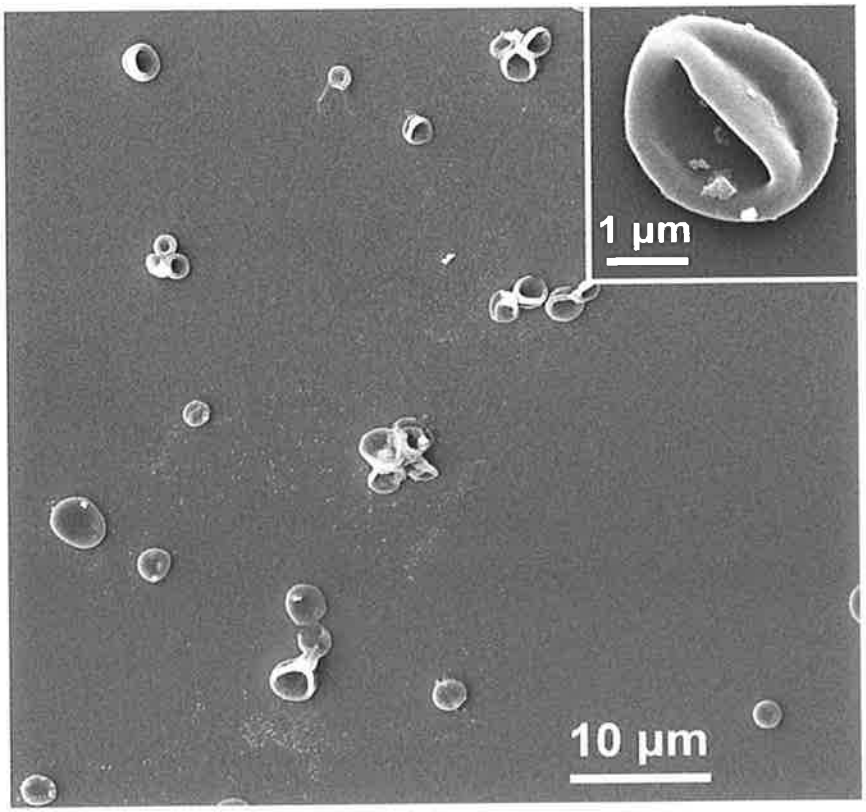


Figure 2.7. Scanning Electron Microscopy images of the polyglutamic acid replica particles

2.1.5 Preparation of Polyglutamic Acid (PGA) / Heparin particles containing BDNF

The PGA particles were prepared as described in section 2.1.4 paragraph 1. 200 μL of 10mg/mL solution of PGA particles were centrifuged and the supernatant removed. Heparin (1000 I.U/ml) was purchased from Mayne Pharmcia Ltd. 35 mL of this heparin solution contains 35,000 iU of heparin. Because 100 iU of heparin is equivalent to 0.2 mg of heparin, we calculated the concentration of heparin in our solution as 2mg mL^{-1}. 1 mL of the heparin solution is added to 2mg of PGA particles. The particles and heparin were mixed gently for 4 hours to infiltrate the heparin. Next they were centrifuged, supernatant removed and washed 3 X with milli-Q water. They were finally stored in 100 μL of milliQ water.

The BDNF was prepared by adding 100 μL of milliQ water at pH 7.9 to the BDNF vial thus creating a 0.1 mg mL^{-1} concentration sample. PGA-Heparin particles were first centrifuged to remove the supernatant. Thereafter 50 μL of BDNF was added to the PGA-Heparin particles and allowed to mix at room temperature overnight. The particles were centrifuged (1000 g x 30 secs) and washed once to remove excess BDNF protein. The BDNF loaded particles were then stored in milli-Q water (see figure 2.8).

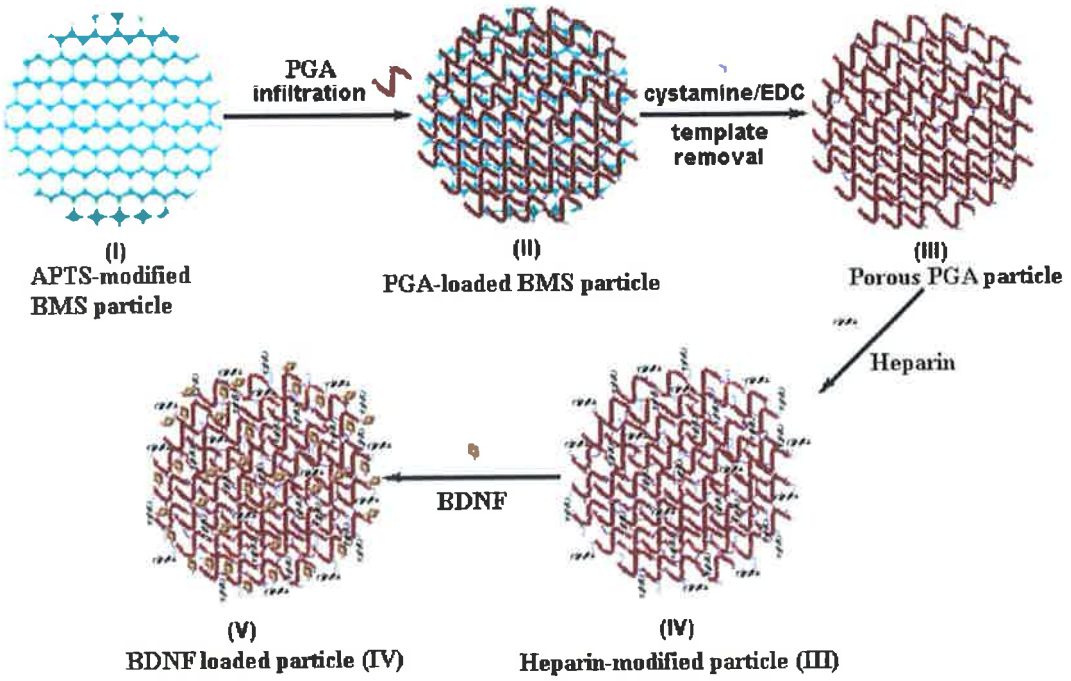


Figure 2.8. Schematic illustration of the synthesis of PGA/Heparin/BDNF particles

2.2 Release Experiments of the Nanoparticles

2.2.1 Release Experiment involving BDNF containing PMA_{SH} / PVP capsules

The entire number of BDNF capsules made from 2.4 mg of mesoporous silica particles were used in the release experiment. The capsules were centrifuged (4500g for 5 minutes) to remove the buffer solution and this was kept for analysis to ascertain if any of the protein had leaked out of the capsules while in storage, this was referred to as time zero. 50 μL of 100 mM phosphate buffered saline (PBS) pH 7.3 at 37 ° C was added to the capsule solution. 50 μL of 20 μM Glutathione (GSH) solution was also added to the capsule solution, creating a final concentration of 10 μM GSH solution. Glutathione is the must abundant non-protein thiol-source in mammalian cells, reaching a concentration of 10 μM in the extracellular space and thus is an important determining factor in controlling cytosolic redox potential[3]. It is believed that reduced GSH is the primary mechanism by which the disulphide bond in the capsules will be cleaved therefore releasing the BDNF protein[4] . The solution containing the capsules, PBS and GSH was placed on an incubator and shaken gently for 3 hours followed by a centrifugation step to remove the supernatant for analysis.
50 μL of the PBS and 50μL of GSH were added to the particle pellet and release of the BDNF was continued for a further 8 and 24 hours. The supernatants harvested at each time point were stored and kept for analysis.

2.2.2 Release Experiment involving Chitosan – Alginate +/- PMASH / PVP containing BDNF particles

The capsules were centrifuged (4500g for 5 minutes) to remove the buffer solution and this was kept for analysis to ascertain if any of the protein had leaked out of the particles during storage, this was referred to as time zero. 100 μL of 100 mM phosphate buffered saline (PBS) pH 7.3 at 37 °C was added to the chitosan-Alginate-BDNF particles. Release of BDNF from these particles using glutathione was described in section 2.2.1. Supernatants containing the released BDNF were harvested at 3, 8, 24, and 48 hours using the same procedure as described in section 2.2.1.

2.2.3 Release Experiment of Polyglutamic Acid (PGA) / Heparin particles containing BDNF

The capsules were centrifuged (4500g for 5 minutes) to remove the buffer solution and this was kept for analysis to ascertain if any of the protein had leaked out of the particles during storage, this was referred to as time zero. 100 μL of 150 mM phosphate buffered saline (PBS) pH 7.3 at 37 ° C was added to the capsule solution. The solution containing the particles and PBS was placed on an incubator and shaken gently. After 3 hours, the particle solution was centrifuged and the supernatant was

removed and kept for analysis. 100 μL of PBS solution was put back into the eppendorf tube with the particles and put back on the incubator. Samples were taken at 8 hours, 24 hours, 48 hours and every day up to 10 days then every second day up to 70 days, each time fresh PBS solution was used.

2.3. Quantifying the Amount of BDNF Released by the Nanoparticles using Enzyme-Linked Immunoabsorbant Assay (ELISA)

BDNF ELISA

Buffers created

Carbonate Buffer

0.025 M sodium bicarbonate with milli Q water, Ph adjusted to pH 9.7

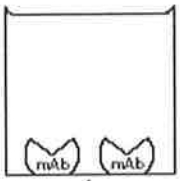
0.025 M sodium carbonate with milli Q water, Ph adjusted to pH 9.7

TBST Wash Buffer

500 ml of a solution containing 20 mM Tris-HCL (pH 7.6), 150 mM NaCl and 0.05% (v/v) Tween 20

3.0.Coating well with anti-BDNF mAb

10 µl Anti-BDNF mAb + 9.99ml carbonate coating buffer (PH 9.7). Add 100 µl per well (96 wells). Cover and place in refrigerator at 4 °C overnight.



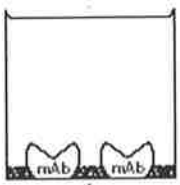
3.1.Block nonspecific binding with Block & Sample 1X buffer

Block and Sample Buffer 1X was prepared by mixing 42.4ml of deionised water with 10.6ml of Block & Sample 5X buffer using aseptic techniques.

The contents of each well was removed by turning the plate upside down and slapping the plate three times on a paper towel to help clear the wells. All wells were vigorously washed with TBST wash buffer.

200µl of this block and sample 1X buffer is added to each well. The wells were incubated **without** shaking for **1** hour at room temperature.

Each well was washed once with the TBST buffer.



3.2.Incubate the immobilized Anti-BDNF mAb with the BDNF standard

A linear BDNF standard curve was generated from 7.8-500pg/ml.

The BDNF standard was supplied at a concentration of 1 µg/ml. This BDNF sample was accurately diluted 1:2,000 in Block & Sample 1X buffer to achieve a concentration of 500pg/ml. This was achieved by transferring 10 µl of undiluted standard into 390 µl of Block & Sample 1X buffer (1:40 dilution) and then

transferring 10 µl of this solution into 490 µl of Block & Sample 1X buffer (1:50 dilution). This produced the final 1:2,000 dilution of the standard.

Following the plate blocking, the contents of the wells were flicked out over a sink and slapped three times over a paper towel to remove the residual liquid. All wells were then washed once with TBST buffer.

Two columns (11 & 12) or 16 wells were designated for the standard curve.

100 µl of Block & Sample 1X buffer was added to wells in rows B through H in the two columns 11 & 12.

200 µl of the diluted BDNF standard (500pg/ml) was added to the first well (row A) in columns 11 & 12 (the columns designated for the standard curve).

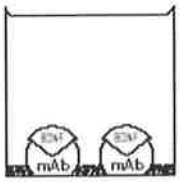
Serial dilutions were immediately performed 1:2 (100 µl/well) down the plate in columns 11 & 12. In the last set of wells (row H) for the standard curve no BDNF was added.

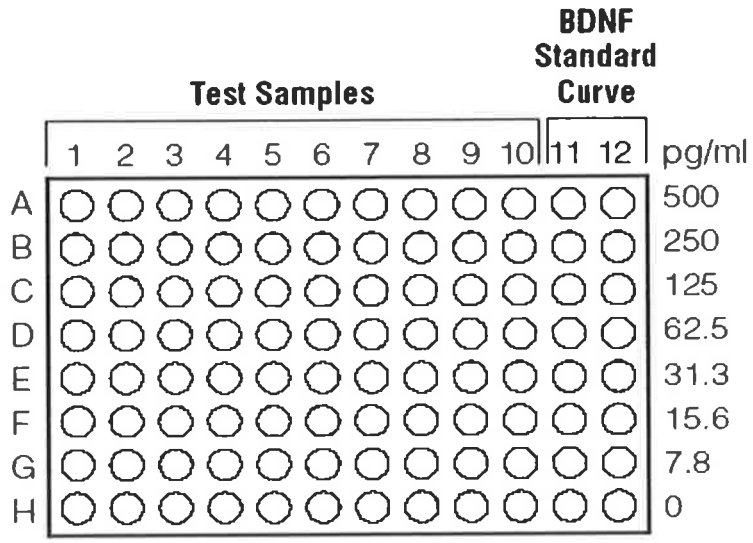
The test samples were then created in each row, 100 µl of sample with serial dilutions until column 10.

Care was taken to add samples quickly to avoid evaporation.

The wells were sealed with a plate sealer and the plate was incubated for **2** hours at room temperature **with** shaking (400 +/- 100 rpm).

After incubation the plate was washed five times with TBST wash buffer.

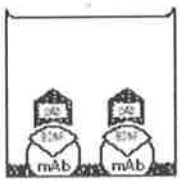




3.3.Incubate with Anti-Human BDNF pAb

In a 15 ml falcon tube, 20 µl of the Anti-Human BDNF pAb was added to 9.98 ml of Block & Sample 1X buffer (1:500 dilution) and was mixed thoroughly. Using a multichannel pipettor 100 µl of the diluted Anti-Human BDNF pAb was added to each well, care was taken not to touch or scratch the surfaces of the wells.

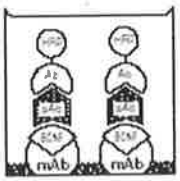
The wells were sealed with a plate sealer and allowed to incubate for **2** hours **with** shaking. After incubation the wells were washed 5 times with TBST wash buffer.



3.4.Incubate with Anti-IgY HRP Conjugate

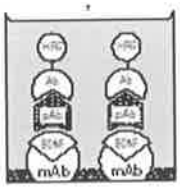
The Anti-IgY HRP conjugate was prepared by accurately adding 50 µl of the conjugate to 9.95ml of Block & Sample 1X buffer (1:200 dilution). Care was taken to mix thoroughly but avoiding excess air bubbles. Using a multichannel pipette, 100 µl of the diluted Anti-IgY HRP conjugate was added to each well. The wells were

sealed with a plate sealer and allowed to incubate for **1** hour at room temperature **with** shaking. After incubation the wells were washed 5 times with TBST wash buffer.



3.5.Colour Development

100 µl of the room temperature TMB One solution was added to each well using a multichannel pipette. This was allowed to incubate at room temperature for **10** minutes. A blue colour formed in the wells. The reaction was then stopped by adding 100 µl of 1 M hydrochloric acid to each well in the same order as the TMB one solution was added. The blue colour changed to a yellow colour upon acidification. The absorbance at 450nm was recorded **within 30** minutes of stopping the reaction using a microplate reader. Care was taken to have the exterior bottom of the plate optically clean and was wiped with 70% ethanol prior to the reading.



Standard Curve

A representative standard curve was then performed in each plate

2.4 SH – SY5Y Neuroblastoma cell lines

2.4.1 Cell Preparation

The SH-SY5Ycells were obtained from the Department of Pathology, University of Melbourne.

These cells were grown using a stock culture medium consisting of: 45 ml Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5 g/l of D-glucose, 2 mM L-glutamate, 110mg/L sodium pyruvate (GIBCO) , 5 ml of fetal bovine serum (FBS; Invitrogen) and 0.5 millilitres of 1% Penicillin/Streptomycin (Invitrogen). Cells were seeded in 50 ml culture flasks in 10 millilitres of culture media.

Cell splitting was performed every 2-3 days using phosphate buffered saline and cells were maintained at 37 $^{\circ}$ C in a saturated humidity atmosphere containing 95% air and 5% CO_2.

2.4.2 Cell Splitting

The culture media is removed from the culture flask.

Phosphate buffered solution (PBS) is prewarmed to 37 ^{0} C. In order to dislodge the confluent cells, ten millilitres of this solution is added to the culture flask and the flask is placed into the incubator at 37 ^{0} C for 5 minutes. The cell suspension is then aspirated up and down to ensure a uniform distribution of the cells in the suspension.

10 ml of the cell suspension were centrifuged for 5 minutes at 1000 rpm. The PBS supernatant was removed and the cell pellet washed with 10 ml of culture media. The cell mixture is then gently aspirated up and down to uniformally distribute the cells, followed by a centrifugation step for 5 minutes at 1000 rpm. Supernatant was removed and 4 ml of fresh culture media was added to the cell suspension before

gentle aspiration to uniformally distribute the cells. 10 ml of culture medium was added to each culture flask together with 1 ml of the cell suspension and mixed gently.

2.4.3 Cell Freezing

The culture media is removed from the culture flask.

Phosphate buffered solution (PBS) is prewarmed to 37 ^{0} C. In order to dislodge the confluent cells, 10 ml of this solution was added to the culture flask and the flask was placed into the incubator at 37°C for 5 minutes. The cell suspension was then aspirated up and down to ensure a uniform distribution of the cells in the suspension.

10 ml of this cell suspension was transferred into a Falcon tube, and centrifuged for 5 minutes at 1000 rpm. The PBS supernatant was removed and the cell pellet washed with 10 ml culture media. The cell mixture was then gently aspirated up and down to uniformly distribute the cells in the suspension.

The suspension was centrifuged again for 5 minutes at 1000 rpm and the supernatant removed. 4 ml of fresh culture media was added to the cell suspension and gently aspirated up and down to ensure uniform distribution of these cells. 1.8 ml of the final cell suspension was mixed together with 200 μL of DMSO, before storage in the freezer at -70 ^{o} C.

2.4.4 Counting cells using the Haemocytometer

The haemocytometer is a device originally designed for the counting of blood cells. It is now used to count other types of cells and microscopic particles. The haemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. The chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bound by the lines is known, and the depth of the chamber is also known. It is therefore possible to count the number of cells or particles in a specific volume of fluid and thereby calculate the concentration of cells in the fluid overall. Each haemocytometer consists of two grids as seen in A below, with 4 primary squares. Each primary square has an area of 1 mm^2 , a depth of 0.1 mm and a volume of 100 nl or 10^{-4} ml. In total there are 8 primary squares. A glass cover slip is placed over the etched slide that will cover all 8 primary squares.

Ten microlitres of cell suspension, which is thoroughly mixed, is added to 10 µl trypan blue solution (O.4%) (Sigma-Aldrich). 10 µl of this mixture is pipetted into each of the two chambers of the haemocytometer. Viable cells are phase bright. Dead cells have their cytosol stained blue. All viable cells are counted in each of the 8 corner primary squares, cells that touch the middle line of the borders are counted.

Cells/ml = (N/8) x 2 x 10^4

N = total number of cells counted from all 8 primary corner squares

8 = averages counts from the 8 squares counted

2 = dilution factor (i.e. 10 µl cell sample + 10 µl trypan blue)

10^4 = volume conversion factor (from cells/0.0001 ml to cells/1ml)

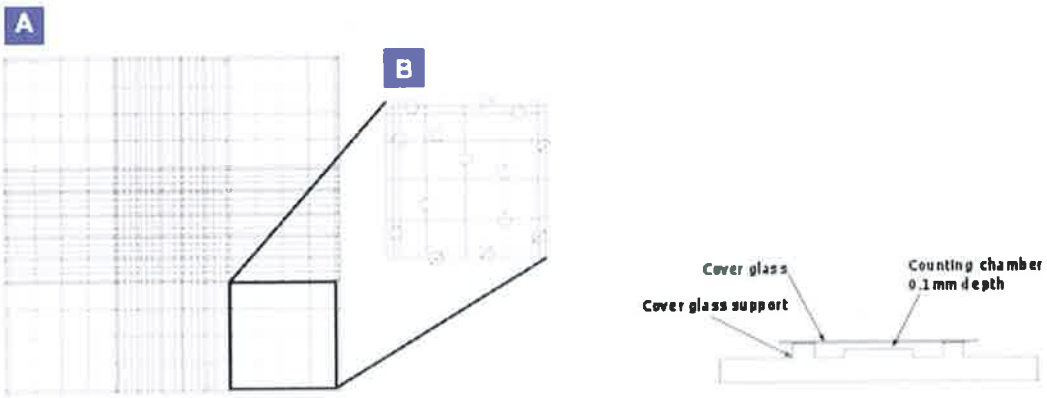


Figure 2.9. Haemocytometer

2.4.5 Differentiation of SH – SY5Y cells into Neurons

Retinoic acid (RA) (mw 300.45) was purchased from Sigma Aldrich. 15mg of RA was added to 1 ml of DMSO creating 50,000 μmol of RA.

The concentration of RA needed was 10 μM. Therefore 10 μL was taken from this stock solution.

Differentiation culture medium containing the retinoic acid was formulated by adding 10 μL of RA into 49.990 ml of DMEM supplemented with 4.5 g/l of D-glucose, 2 mM L-glutamate, 110mg/L sodium pyruvate (GIBCO), 10 % heat inactivated fetal bovine serum (FBS; Invitrogen), 1% Penicillin/Streptomycin (Invitrogen) plus 10 μL of RA.

The SH-SY5Y cells were plated onto an eight well Lab-Tek II chamber slide. The chambers each measure 9mm x 7mm. The haemocytometer was used to count the cells and 800-1600 cells were placed in each well along with 200 μL of culture media with the RA. The cells were then maintained at 37 $^{\circ}$ C in a saturated humidity atmosphere containing 95% air and 5% CO_2. The cells will differentiate into dendrites after one week.

2.4.6 Differentiation of SH-SY5Y cells into neurons using the released BDNF from the nanoparticles

The SH-SY5Y cells were plated onto an eight well Lab-Tek II chamber slide. The chambers each measure 9mm x 7mm. The haemocytometer was used to count the cells and 800-1600 cells were placed in each well along with 200 μL of culture media with the RA. The cells were then maintained at 37 ^{o} C in a saturated humidity atmosphere containing 95% air and 5% CO_{2}.. After one week when the dendrites had appeared in all the cells the culture media was removed from all chambers and replaced with the same culture media except that retinoic acid was omitted. Four chambers had basal media with two different concentrations of standard purchased BDNF. Two chambers had basal media plus the released BDNF from the particles. Two chambers had basal media with no BDNF to act as a negative control. For a positive control we added 2 nM of BDNF into two chambers (Encinas 1999).

From the ELISA data, we determined the concentration of BDNF released by the nanoparticles and added this BDNF to the culture using a final BDNF concentration of 2 nM. The cells were then incubated for a further 1 week changing the media every 3 days.

The cells were fixed after one week and the immunohistochemical marker Apotag® was used to assess apoptosis within the cells.

Basal Control	BDNF 1	BDNF 2	Particle BDNF
Basal Control	BDNF 1	BDNF 2	Particle BDNF

Lab-Tek II chamber slide

2.4.7 Fixing of SH-SY5Y cells

Culture media was removed from all wells in the 8 well Lab-Tek II chamber slide. 100 µL of 4% paraformaldehyde were added to each well and left for 10 minutes. The paraformaldehyde is then removed and 100 µL of phosphate buffered saline (PBS) added to each well, the PBS was left in-situ for 10 minutes and this is repeated 3 X.

The PBS was removed and 100 µl of dilute acidified thionin added to each well and left for 5 mins. The thionin was removed and the wells were washed twice with 100 µl of distilled water. 100 µl of absolute alcohol was added to each well and left for one minute, before it was removed and the dehydration step repeated. 100 µl of Histoclear™ was added and left for one minute, this step was repeated 3 X before mounting the slides in DPX resin.

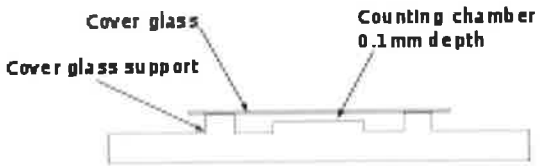
2.4.8 Immuno-histochemical Staining using Apoptotic cell marker Apotag®

ApopTag® Peroxidase In Situ Detection

Cells in each well were fixed for 10 minutes with 100 µl of 1% paraformaldehyde solution, followed by rinsing with PBS 3 X for 5 minutes. They were then post fixed for 5 minutes in an ethanol:acetic acid 2:1 that has been pre-cooled to -20 °C. Each well was then washed 3 X with PBS for 5 minutes. Endogenous peroxidase was quenched with 100 µL of 3% hydrogen peroxide for 10 minutes. Each well was then rinsed 3 X with PBS for 5 minutes. Excess liquid was tapped off gently and the edge of each well blotted to remove the remaining liquid. 20 µL of equilibration buffer was added to each well and allowed to incubate for 1 minute. Working strength TdT enzyme was formulated by adding 70% of reaction buffer to 30% TdT enzyme. 20 µL of this working strength enzyme was added to each well and allowed to incubate in a humidified chamber at 37 °C for 1 hour. 50 µL of stop/wash buffer was then added to each well. Each well was rinsed 3 X with PBS with each rinsing step lasting 1 minute. 20 µL of room temperature anti-digoxigenin peroxidase conjugate was added to the slide and the surface covered. Each well was rinsed 3 X with PBS for 5 minutes. The DAB ($3,3^1$ – Diaminobenzidine) substrate was then formulated as follows: 2 drops of buffer stock solution was added to 5 ml of distilled water and mixed. Next, 4 drops of DAB stock solution was added and mixed followed by addition of 2 drops of nickel solution. 50 µL of this DAB substrate was then added to

each well. The reaction was stopped by placing the slide in a coplin jar filled with distilled water.

The slide was then visualised under the light microscope.



2.5. Animal Rat Model

2.5.1 Deafening Procedure

Adult Wistar rats were used throughout the experiments. The rats were anaesthetized under aseptic technique with a single intraperitoneal injection of 75mg/kg ketamine (Parnell Laboratories NSW -Aus) and 10 mg/kg xylazine (Ilium Veterinary Products NSW Aus). Eye lubricant "lacrilube" (Allergan) was applied to avoid drying out of the cornea. The rats then received subcutaneous injections of loop diuretic frusemide (200 mg/kg body weight) and gentamicin sulphate (420 mg/kg body weight) at different sites.

2.5.2 Surgical Implantation of BDNF Nanoparticles

The surgical implantation of the BDNF nanoparticles was performed 3 weeks after the deafening procedure.

The particles were inserted into cochlea of one ear, thus leaving the other ear as the control. The animals were placed on a heat pad (37 ^{o} C) and were anaesthetised with a single intraperitoneal injection of 75mg/kg ketamine (Parnell Laboratories NSW - Aus) and 10 mg/kg xylazine (Ilium Veterinary Products NSW Aus). This combination produced anaesthesia sufficient to maintain spontaneous respiration and provide effective analgesia. Eye lubricant "lacrilube" (Allergan) was applied to avoid drying of the cornea. Supplemental doses of 0.08 ml of ketamine and xylazine (in a ratio of 2:1) were administered every 20 minutes, or earlier if a pedal or corneal

reflex was detected. A piece of rolled up gauze was placed under the mandible of the animal to gently extend the atlanto-occipital joint. Local anaesthetic 1% lignocaine 0.2 ml was injected subcutaneously in the post auricular area where the incision was to be made. Once anaesthetised the area surrounding one ear was shaved. Under aseptic technique a post auricular incision 2.5cm – 3.0 cm was made and was carried through the subcutaneous and muscular planes (see figure 2.10). A well defined sternocleidomastoid muscle and facial nerve extending anteriorly above the tympanic bulla was easily identified (see figure 2.11). The bony tympanic bulla was exposed (see figure 2.12) and the dorsal region drilled using a high speed 2mm cutting burr, providing a clear view of the stapedial artery with its medial margin lying over the edge of the round window (see figure 2.13). The round window membrane was punctured with a fine needle and perilymph aspirated. A fine 26 gauge canula was used (see figure 2.14), and the tip was introduced through the round window membrane and 50 μL of the BDNF-nanoparticles in PBS was injected into the scala tympani of the cochlea. A small piece of muscle and fascia was used to help seal the hole in the bulla. The subcutaneous tissues and skin were closed using interrupted Vicryl sutures.

Figure 2.10. Adult wistar rat showing slightly extended head position with post-auricular incision

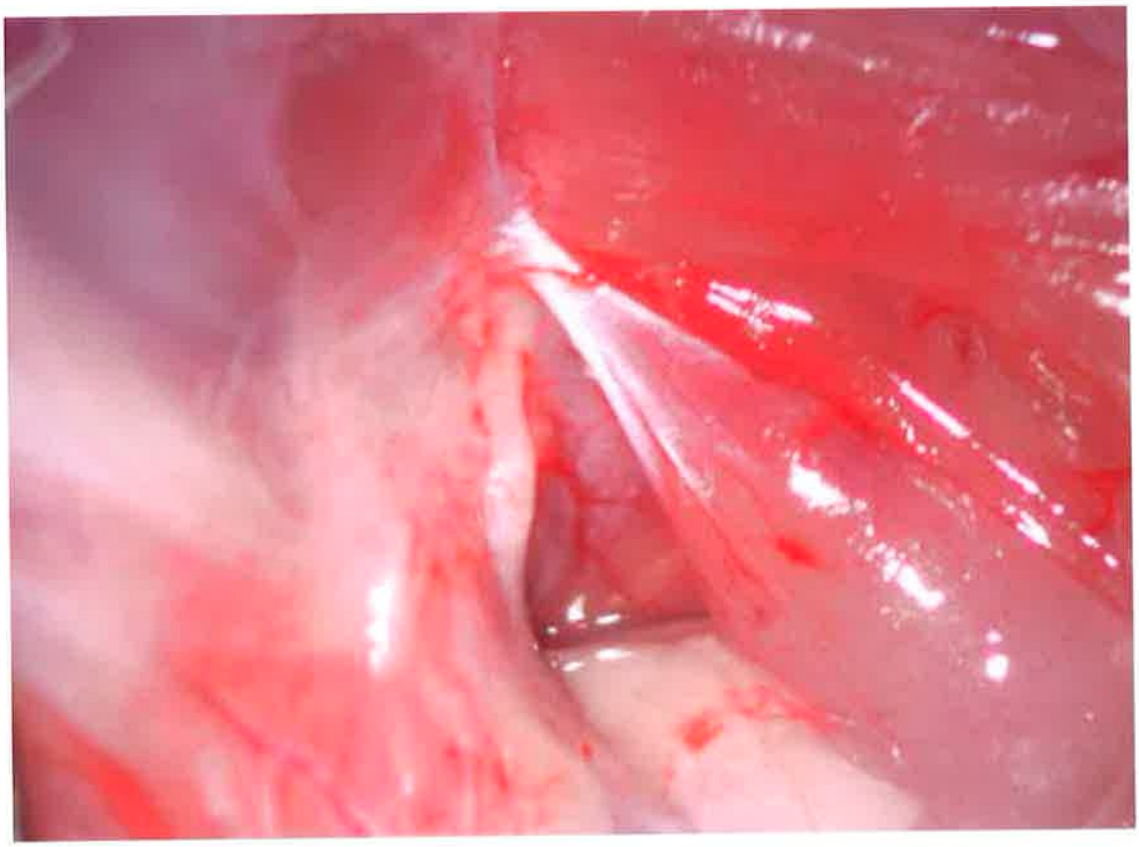


Figure 2.11. Exposed facial nerve and sternocleidomastoid muscle

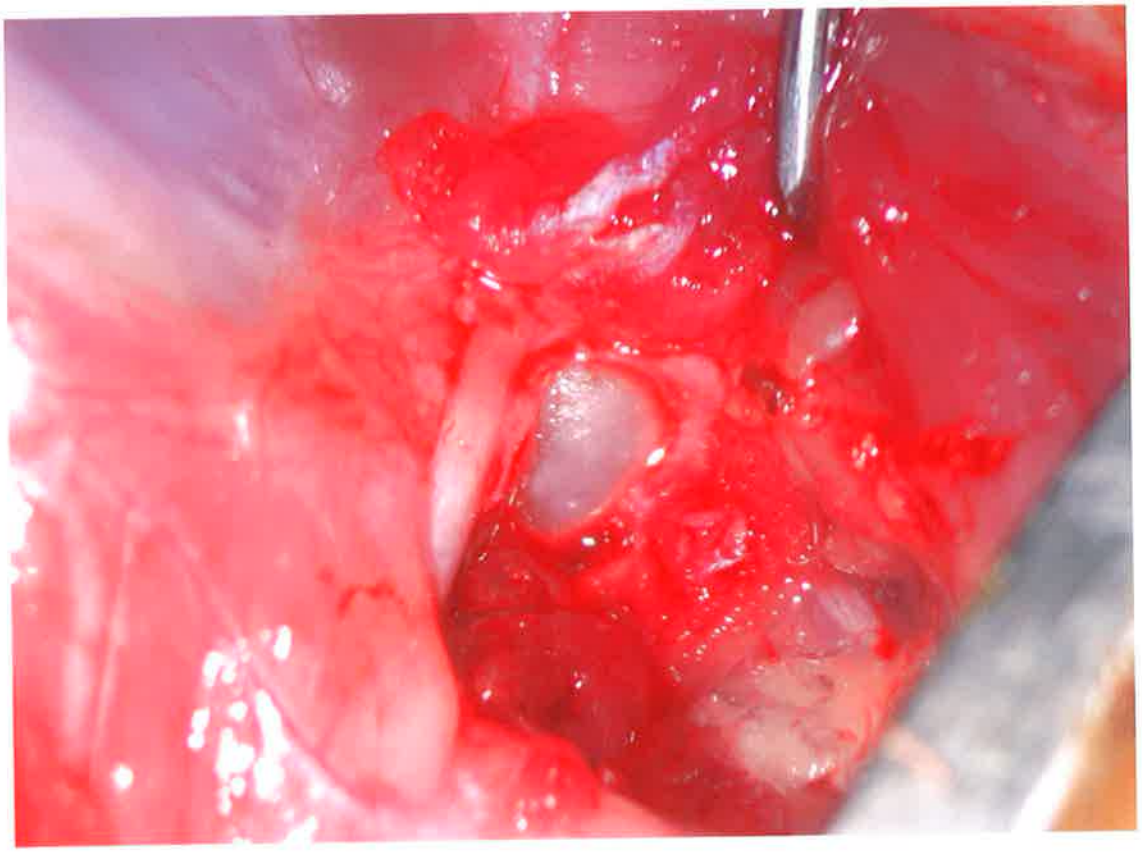


Figure 2.12. Sternocleidomastoid muscle retracted exposing the tympanic bulla adjacent to the facial nerve

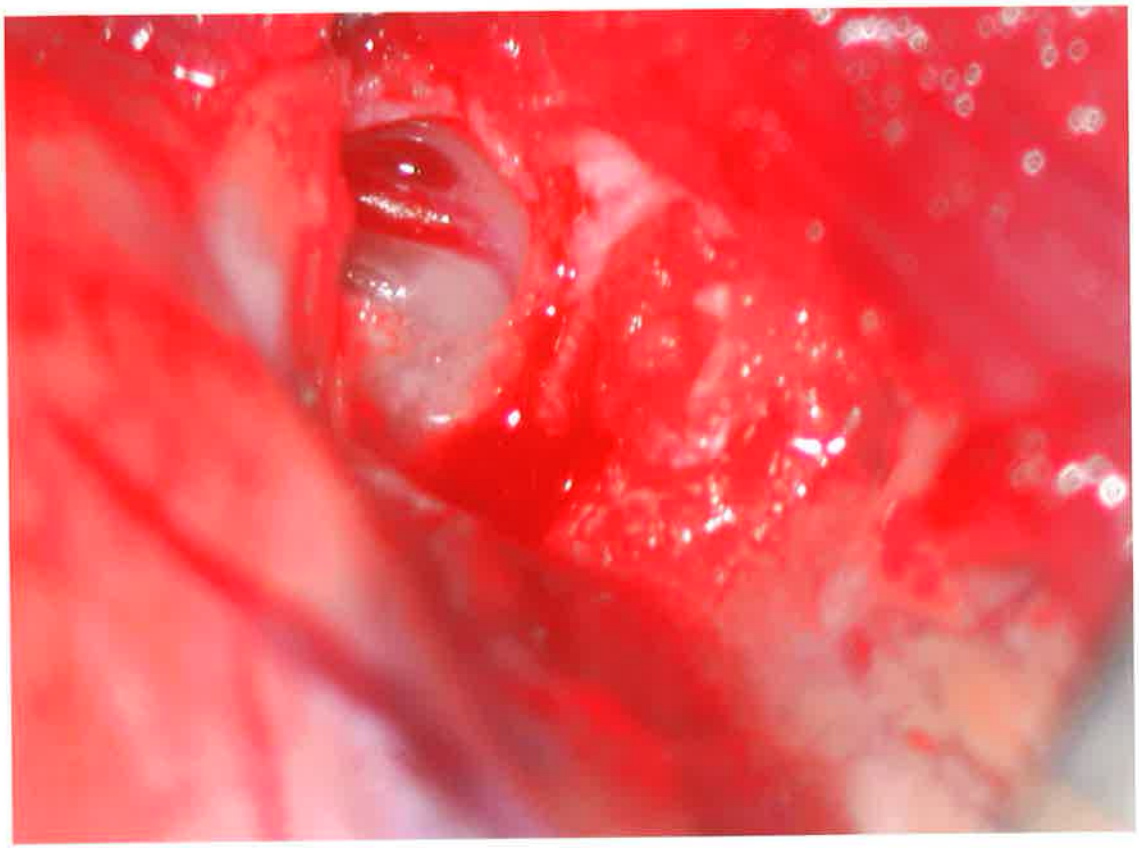


Figure 2.13. Exposed stapedial artery with round window anteriorly

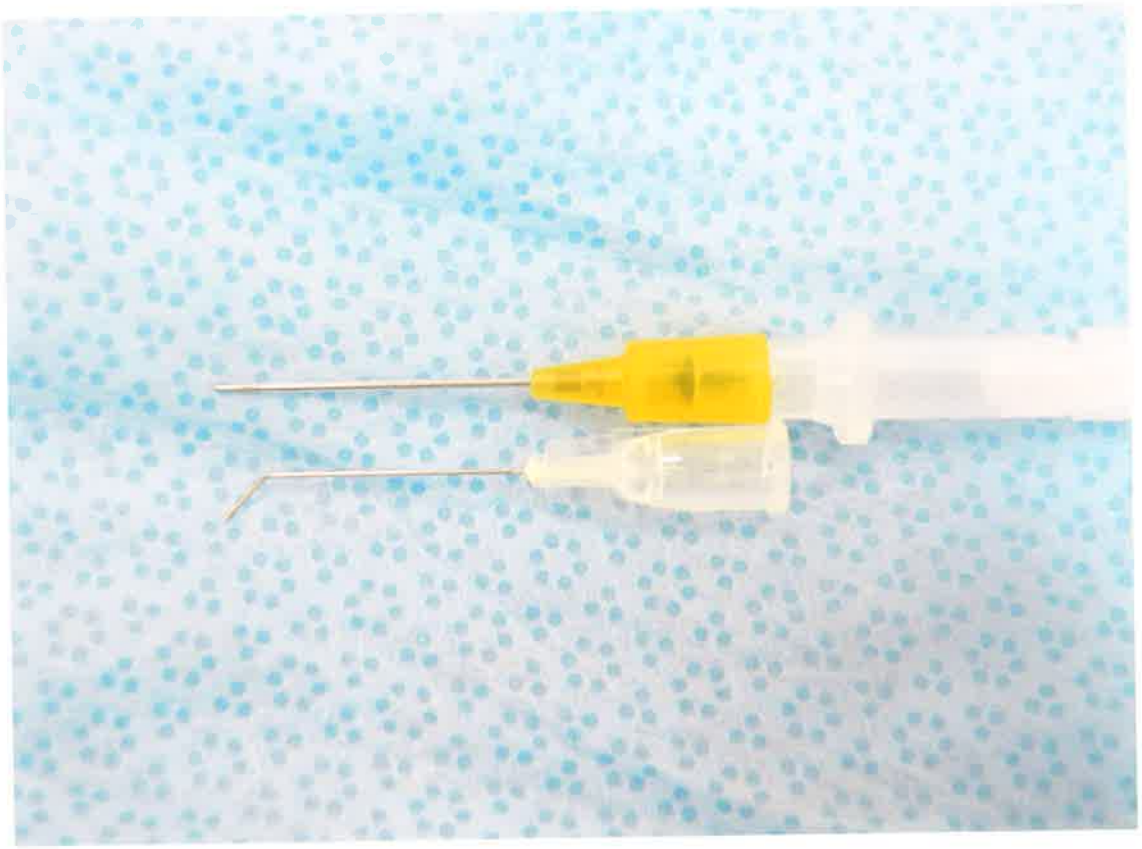


Figure 2.14. Below the 26-gauge cannula used to insert the BDNF-nanoparticles, above the yellow 24-gauge paediatric cannula

2.5.3 Perfusion and Histology

Two months after surgery, the animals were anaesthetised with 75 mg/kg body weight ketamine and 10 mg/kg body weight xylazil. I ml of sodium phenobarbiturate was injected intra-peritoneally. Animals were transfused transcardially with 100 ml of cold 1 X PBS followed by 100 ml of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After perfusion the cochleae were harvested, the oval and round windows opened to allow penetration of the fixative and the cochleae post fixed in the same fixative for 2 hours. They were then decalcified in 4% ethylenediamine tetra-acetic acid (EDTA), embedding in OCT medium for cryosectioning at 10 μm thickness.

2.5.4 Spiral Ganglion Neuron Density

For each animal, every 7^{th} cochlear section was stained with haematoxylin and eosin and examined under light microscopy.

CHAPTER THREE

RESULTS

3.1 Nanoparticle BDNF Release

3.1.1 Thiol functionalised Polymethacrylic acid (PMASH) and Polyvinylpyrrolidone (PVP) Capsules.

BDNF was successfully sequestered into these PMA_{SH} / PVP nanocapsules. The amount of BDNF sequestered however was very low compared to the quantity of BDNF used at the onset. A concentration of 50,000 ng/ml of BDNF was used to infiltrate the mesoporous silica particle prior to the layer by layer development of the nanocapsules. After infiltration of the protein overnight, the concentration of BDNF in the supernatant was 80 ng/ml, showing that there was a high loading of the protein into the mesoporous silica. A pure sample of the reconstituted BDNF (ie 50,000 ng/ml) was also measured using the ELISA kit, which showed a concentration of 49,450 ng/ml, proving that the correct concentration was aceived as per the manufacturing guidelines. Prior to the capsules being used for the release experiments, the supertnatant was taken to measure if any BDNF had leaked out of the capsules during the storage, this was referred to as time zero T_o. Release was measured at 1 hour T_1, 3 hours T_3, 8 hours and 12 hours. A 3 hour release sample of PMA_{SH} capsules with no BDNF was tested as a control and no BDNF was recorded. For each time point two samples were tested to ensure accuracy of results.
Two batches of particles were tested and the results summarized in table 1. As shown in this Table there was some leakage of BDNF out of the capsules during storage. There is also a relative "burst release" of the BDNF, as almost all the sequestered BDNF is released at T_3.

Table 1 BDNF concentrations at various time-points post release for PMASH / PVP / BDNF nanocapsules

PMASH/PVP /BDNF	Batch 1	Batch 2
T_0	2 ng/ml	6 ng/ml
T_1	120 ng/ml	100 ng/ml
T_3	36 ng/ml	20 ng/ml
T_8	6 ng/ml	4 ng/ml
T_{12}	0.5 ng/ml	0.2 ng/ml

3.1.2. Chitosan / Alginate / BDNF Nanoparticles

BDNF was successfully sequestered into these nanoparticles which had a zeta potential of - 39 +/- 5.0 mV. Again 50,000 ng/ml of the protein was used in the infiltration process. After incubation BDNF concentration in the supernatant was again measured and a value of 40 ng/ml BDNF indicated a high protein loading. Samples were again taken at time zero T_0, three hours T_3, eight hours T_8, 24 hours T_{24} and at 30 hours T_{30}. For each time point, two samples were tested to ensure accuracy of results. A 3 hour release sample of Chitosan/Alginate particle with no BDNF was tested as a control, and no BDNF was recorded. Two batches of particles were tested. The results can be seen in table 2.

Table 2 BDNF concentrations at various time-points post release for chitosan/alginate/BDNF nanoparticles

Chiosan/Alginate/BDNF	Batch 1	Batch 2
T_0	214 ng/ml	320 ng/ml
T_3	173 ng/ml	140 ng/ml
T_8	58 ng/ml	48 ng/ml
T_{24}	27 ng/ml	12 ng/ml
T_{30}	22 ng/ml	9 ng/ml

An improvement was seen in the amount of BDNF being released: however a significant amount of BDNF was also found to leak out of the particles during the storage. Although the release is slightly longer than the previous capsules, the majority of the BDNF had been released at 24 hours.

3.1.3. Chitosan / Alginate / BDNF / PMASH / PVP Nanoparticles

Since the majority of the BDNF was released within 24 hours, the method to retard BDNF release was modified. This time Chitosan/Alginate/BDNF particles were coated with, three layers of the polyelectolytes PMA_{SH}/PVP on the outside of the particles in an effort to control the leakage of the BDNF from the nanoparticles. Samples were again taken at time zero T_0, three hours T_3, eight hours T_8, 24 hours T_{24} and at 30 hours T_{30}. For each time point, two samples were tested to ensure

accuracy of results. Two batches of particles were tested. The results can be seen in table 3.

Table 3 BDNF concentrations at various time-points post release for chitosan/alginate/BDNF + PMA_{SH} / PVP nanoparticles

Chitosan/Alginate/BDNF PMASH/PVP	Batch 1	Batch 2
T_0	120 ng/ml	140 ng/ml
T_3	160 ng/ml	130 ng/ml
T_8	65 ng/ml	58 ng/ml
T_{24}	15 ng/ml	11 ng/ml
T_{30}	8 ng/ml	5 ng/ml

3.1.4. Polyglutamic Acid Particles containing BDNF

The BDNF was successfully sequestered into these nanoparticles. Again 50,000 ng/ml of the protein was used in the infiltration process. After incubation, BDNF concentration in the supernatant was again measured and was found to have a concentration of 800 ng/ml BDNF indicating a high protein loading. Samples were again taken at time zero T_0, three hours T_3, eight hours T_8 , 24 hours T_{24} and at 30 hours T_{30}. For each time point, two samples were tested to ensure accuracy of results.

We also tested a 3 hour release sample of PGA nanoparticles with no BDNF in them to act as a negative control and could not detect any BDNF in these samples. Two batches of particles were tested. The results can be seen in table 4.

Table 4 BDNF concentrations at various time-points post release for polyglutamic acid nanoparticles

PGA / BDNF	Batch 1	Batch 2
T_0	45 ng/ml	35 ng/ml
T_3	80 ng/ml	60 mg/ml
T_8	50 ng/ml	42 ng/ml
T_{24}	28 ng/ml	22 ng/ml
T_{30}	14 ng /ml	12 ng/ml

We have an improvement in the relative burst release however the levels of protein released need to be improved.

3.1.5. Polyglutamic Acid / Heparin / BDNF Nanoparticles

BDNF was successfully sequestered into these nanoparticles. Again 50,000 ng/ml of the protein was used in the infiltration process. After incubation the concentration of BDNF in the supernatant was again measured and averaged at 130 ng/ml indicating a high protein loading. Samples were again taken at time zero T_0, three hours T_3, eight hours T_8 , 24 hours T_{24} 48 hours T_{48} and every 24 hours up to 10 days, then every second day up until 70 days . For each time point, two samples were tested to ensure accuracy of results. We also tested a 3 hour release sample of PGA / Heparin

nanoparticles with no BDNF in them to act as a control and could not detect any BDNF in these samples. For the ELISA tests two days samples were mixed together to be measured up to day 15, then 7 days samples were mixed together up to 70 days see Table 5. Three batches of particles were tested. The results can be seen as cumulative amounts of BDNF released in table 5.

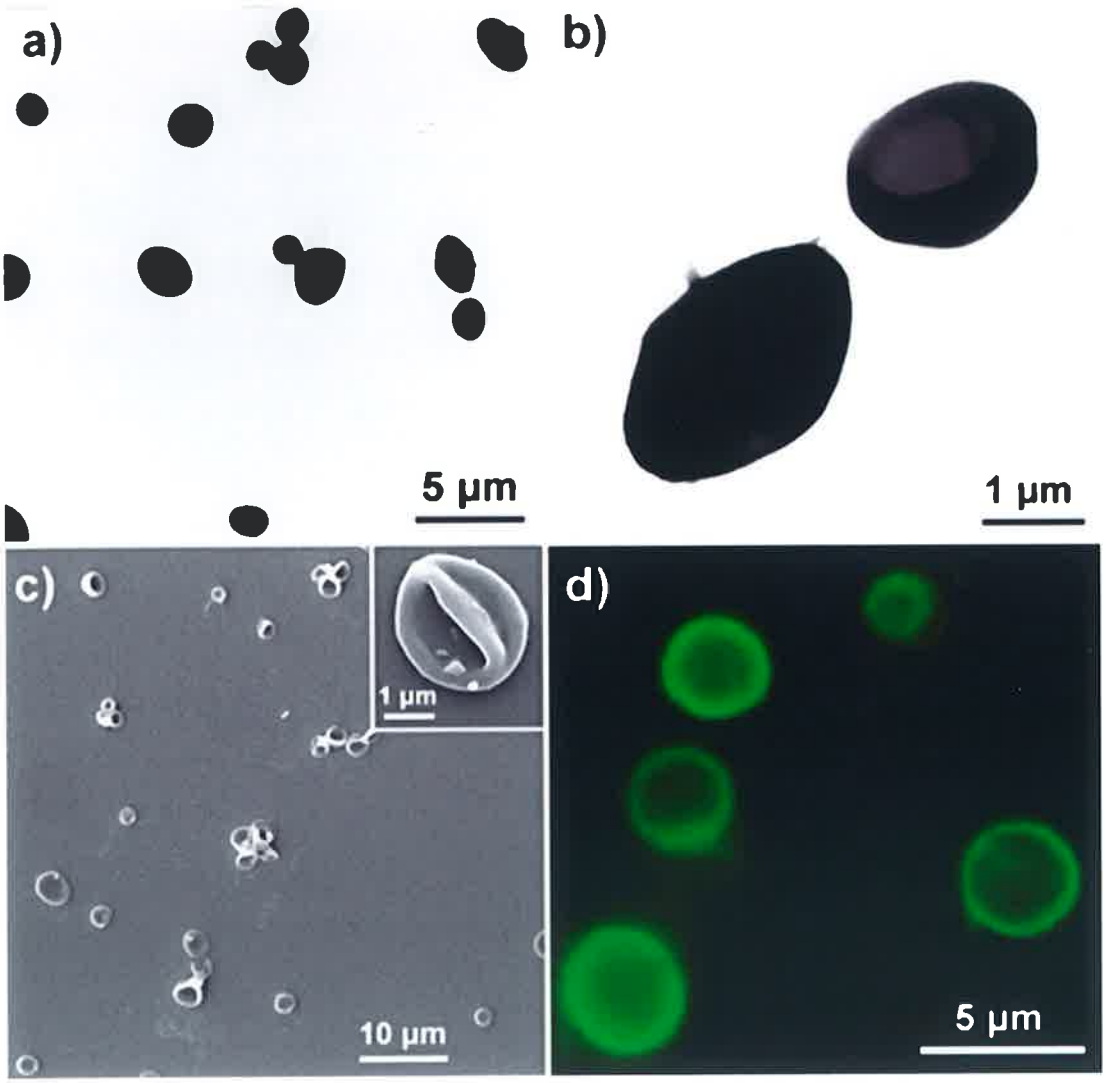


Figure 3.1 TEM images of heparin-modified PGA particles at low (a) and higher (b) magnifications; SEM images of heparin-modified PGA nanoparticles (c); CLSM image of dye-labelled PGA particles (d)

Table 5 Cumulative amount of BDNF released

PGA/Heparin/BDNF	Batch 1	Batch 2	Batch 3
Time zero	25 ng/ml	40 ng/ml	36 ng/ml
3 hours	354 ng/ml	380 ng/ml	440 ng/ml
24 hours	1714 ng/ml	998 ng/ml	982 ng/ml
3 days	2841 ng/ml	1612 ng/ml	1492 ng/ml
6 days	3450 ng/ml	2213 ng/ml	1943 ng/ml
8 days	3932 ng/ml	2750 ng/ml	2463 ng/ml
10 days	4433 ng/ml	3236 ng/ml	2943 ng/ml
13 days	4600 ng/ml	3696 ng/ml	3403 ng/ml
15-20 days	5063 ng/ml	4350 ng/ml	3886 ng/ml
20-27 days	5178 ng/ml	4710 ng/ml	4272 ng/ml
27-34 days	5213 ng/ml	4850 ng/ml	4551 ng/ml
34-41 days	5321 ng/ml	4935 ng/ml	4725 ng/ml
41-48 days	5388 ng/ml	5055 ng/ml	4795 ng/ml
50-55 days	5444 ng/ml	5115 ng/ml	4837 ng/ml
57-62 days	5559 ng/ml	5165 ng/ml	4877 ng/ml
64-69 days	5604 ng/ml	5198 ng/ml	4914 ng/ml

Figure 3.2 Cumulative Release of 3 Batches of PGA/Heparin/BDNF Nanoparticles

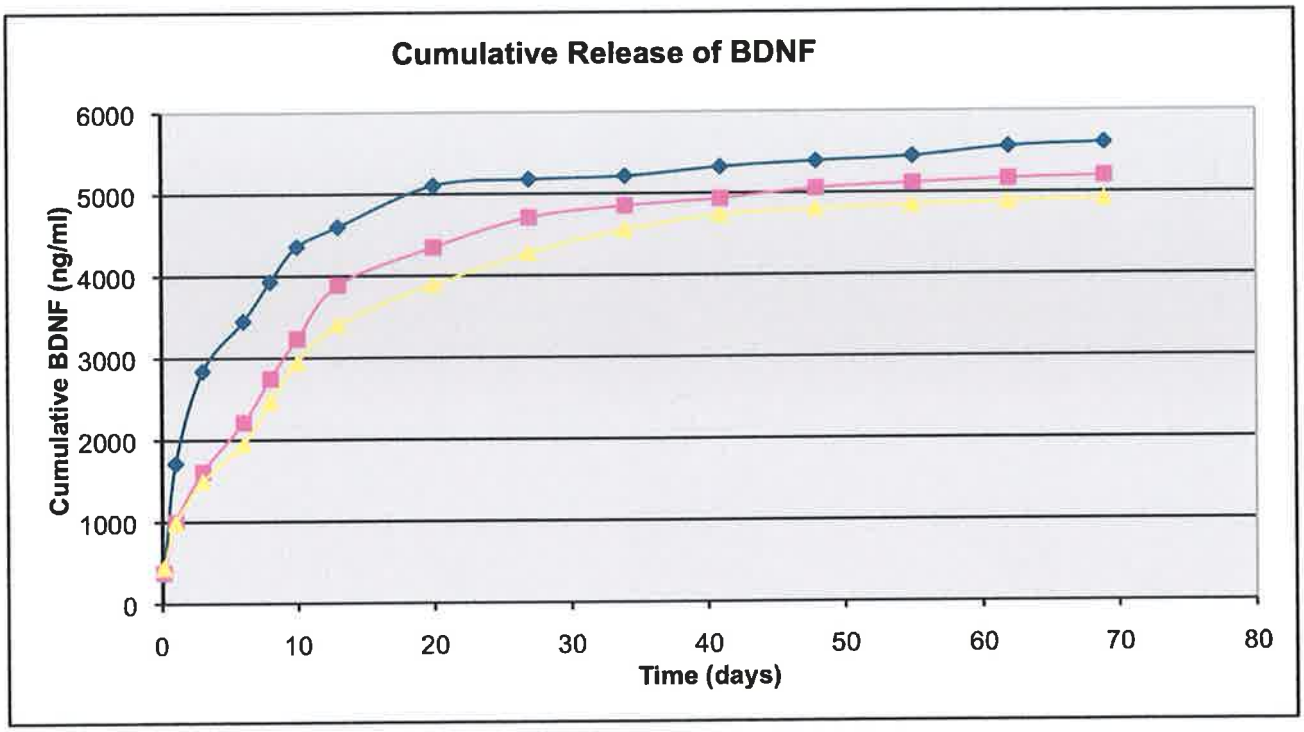
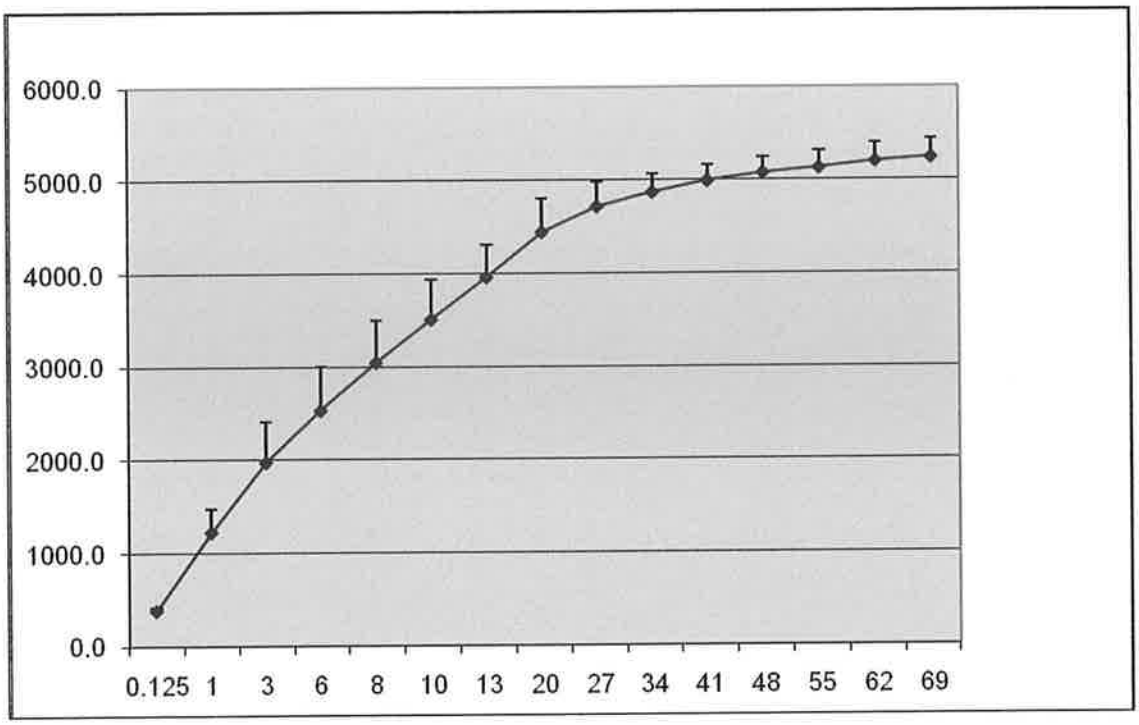


Figure 3.3 Average cumulative values for the 3 batches over time



3.2 SH-SY5Y Neuroblastoma cells

The cells were successfully differentiated using media containing retinoic acid (see figure 3.4).

The media was then changed and three different culture media samples were used to continue growth of these cells; base media with no BDNF (control), media containing commercial BDNF and media containing the BDNF released from the PGA/Heparin/BDNF nanoparticles. We used the apoptotic cell marker Apotag® to test for apoptosis indicating cell death, (See figure 3.5).

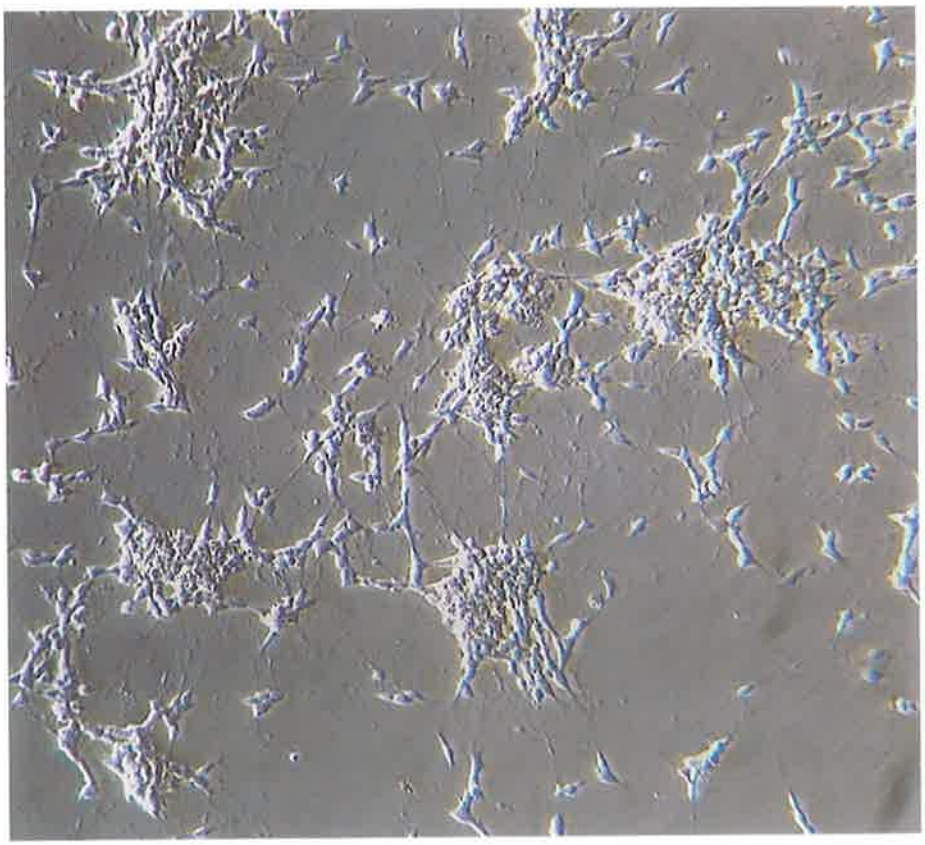
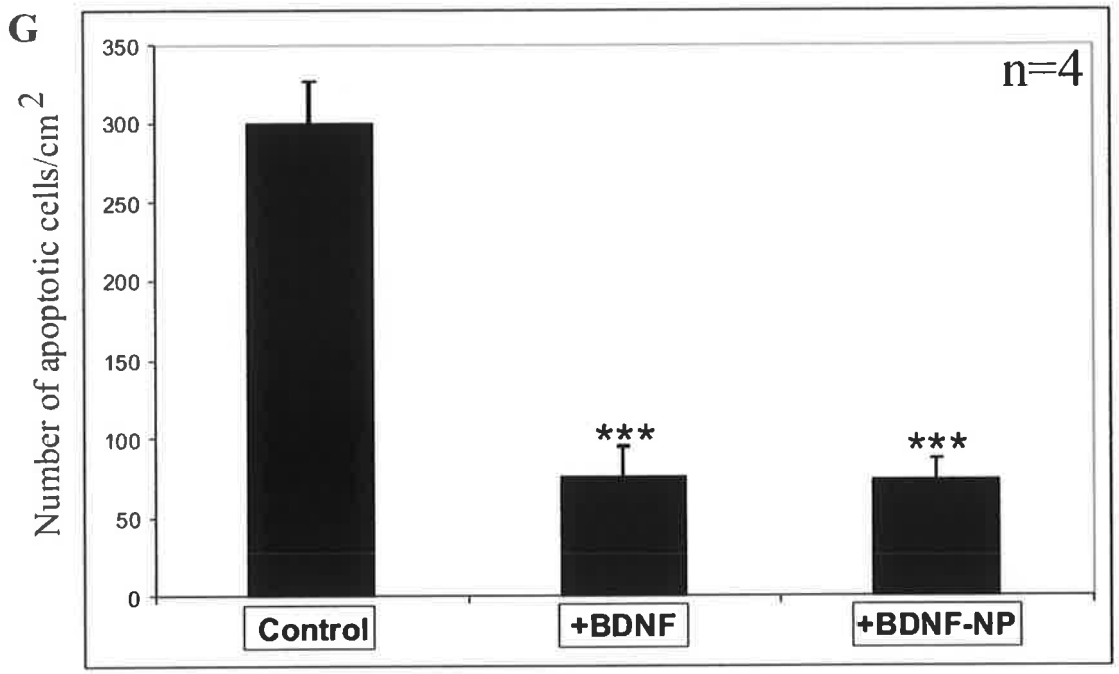


Figure 3.4 SH-SY5Y cells showing neuron outgrowth

Figure 3.6 Number of Apoptotic cells / cm^2



The number of apoptotic cells were counted per centimeter squared under the light microscope. Student's t-test was used and revealed statistically significant difference between apoptotic numbers in the control basal media, compared to the released nanoparticle and commercial BDNF.

3.3 Animal Rat Model

Four Adult Wistar rats were deafened using the protocol outlined in the Materials and Methods section 2.5.1. Three weeks after deafening, the animals were scheduled for surgery. Four animals were operated upon. Two animals succumbed during the general anaesthetic. Two animals had the PGA/Heparin/BDNF nanoparticles inserted through the round window of one cochlea using the untreated cochlea as a control. Two months after the surgery the rats were sacrificed and both cochleae of each animal were examined histologically to assess spiral ganglion density. The side with the BDNF nanoparticles was compared to the control side with no BDNF nanoparticles. In one animal we saw a significant difference in SGN number between the treated cochlea and untreated cochlea (see figure 3.7).

post delivery, both cochleae were harvested and histologically processed. Whereas the fluid-filled scala tympani (St) remained clear in the un-operated cochlea (A), particulate matter could be seen in the BDNF-treated cochlea (B, arrows) and shown in higher magnificationin F (arrow). At low magnification, a higher density of cell bodies within the Rosenthal's canal became apparent in defined locations of the BDNF-treated cochlea (B, boxes D, F, H) when compared to the contralateral, untreated cochlea (A, boxes C,E, G). Pairwise comparison between BDNF-treated and untreated cochleae of the more basal (D vs C), middle (F vs E) and more apical turns (H vs G) showed a higher density of spiral ganglion neurons in the BDNF-treated cochlea (D, arrows). Scale bar (A,B): 200μm. Scale bar (C-H): 20 μm.

CHAPTER FOUR

DISCUSSION

4.1.1 INTRODUCTION

It has been shown that neurotrophins and in particular BDNF can enhance the survival of SGNs in vivo (Malgrange et al., 1996; Marzella & Clarke 1999; Zheng et al., 1995; McGuiness & Shepherd 2005; Song et al., 2009). Due to the significant prospects for clinical application, the strategy for delivering neurotrophins to the inner ear is an issue that needs to be addressed. The current standard delivery technique in animal experimental studies is to cannulate the scala tympani and infuse the neurotrophins via a mini-osmotic pump (MOP) (Prieskorn & Miller 2000). While MOPs are effective in experimental studies, their finite lifespan and the possibility of introducing infection into the inner ear make them unsuitable for clinical use. Such cannulae are niduses for infection which may lead to labyrintitis and potentially meningitis (Wei et al., 2006). In comparison, as a delivery system, the bolus delivery of a therapeutic substance to the cochlea at the time of surgery can be considered reasonably safe, provided the rate of delivery does not cause a mechanical trauma to the cochlea. However there is concern about the longevity of the survival effects on SGNs using a single bolus delivery.

Gillespie et al 2003 have demonstrated that removal of neurotrophin treatment leads to an accelerated decline in SGN survival. This suggests that neurotrophins may need to be delivered for longer periods in order to be effective in the cochlea. At present several alternative methods of neurotrophin delivery are being investigated, including neurotrophin diffusion through the round window (Ernfors et al., 1996; Wise et al., 2005; Gillespie et al., 2004), gene based therapies via viral vectors (Liu et al., 2005; Li Duan et al., 2002; Rejali et al., 2007; Husseman & Raphael 2009) and cell based therapies, in particular the use of Schwann cell transplantation (Pettingill et al., 2008,

Iguchi et al., 2003). These have the potential to provide longer-term delivery of neurotrophins. However it is important to explore other methods of neurotrophin delivery because there are valid concerns on the use of gene and cell based therapies to treat a non-debilitating disease. For example, high virus loading with gene therapy can cause cell toxicity and immune responses (Staecker et al., 2001; Luebke et al., 2001). There is also concern regarding the spread of the viral vector to other sites via the endolymph pathway. Gene-based studies have demonstrated that unilateral viral inoculation of the inner ear can result in gene expression within the contra-lateral cochlea and the CNS (Stover et al., 2000).

In terms of cell transplantation techniques, careful consideration needs to be given to the cell type(s) used, as well as the kind of transplantation. It is important to avoid cells that may have a predisposition to form tumours. Autologous transplantation, where the cells or tissue used for transplantation are taken from the patient's own body, would minimize the immune response and risk of infection. Encapsulation technologies are likely to prove beneficial in preventing migration or dispersal of cells from the transplantation site, as well as immunologically isolating the modified cells from the host and further preventing inflammatory responses.

The focus of this thesis was to create a biodegradable biocompatible particle that would successfully transport the neurotrophin BDNF, and deliver it in a biologically active form over an extended period of time.

In order to achieve this goal it was important to look at the structure of BDNF. The neurotrophin consists of two homodimers of approximately 120 amino acids each,

linked non-covalently by hydrophobic interactions (Fletcher et al., 2006, Rosenthal et al., 1991). The protein is arranged in β-strands, which are held together by four β-hairpin loops. It has been demonstrated that BDNF mediates its biological effect via the hairpin loops. It is a cationic protein meaning it has an overall positive charge (Robinson et al., 1995, O'Leary et al., 2003). In choosing a method of encapsulation of the protein we consider it important to avoid covalently binding the protein to a biocarrier, as this may result in the active moieties of the protein being changed, and thus rendering the protein biologically inactive. (Pumford et al., 1997). All methods used for encapsulation of the protein involved predominantly electrostatic interaction with some hydrogen bonding and hydrophobic interactions.

4.1.2 BDNF ENCAPSULATION TECHNIQUES

The first method of protein encapsulation attempted, was using mesoporous silica (MS) and PMA_{SH} – PVP to create a hollow polyelectrolyte capsule. The reason for using mesoporous silica is its ability to adsorb high quantities of protein owing to its high surface area (up to 1200 $m^2 g^{-1}$) and pore sizes which range from 2-50 nm (Wang & Caruso et al., 2005). Additionally, since the MS carries an overall negative charge, it is reasonable to assume that BDNF will be attracted to the silica core by electrostatic interaction. (Wang et al., 2007). Furthermore, protein degradation is less likely to occur because mechanical forces that may be involved in other methods of encapsulation (e.g. emulsification) have been avoided and no covalent bonding occurs (Zelikin et al., 2006). Coating the MS particles with nanoscale multilayer's of the biocompatible and biodegradable PMA_{SH} – PVP polyelectrolyte's overcomes the problem of protein desorption often encountered with only direct immobilization of

proteins on MS spheres. The multilayer shell also prevents leaching of the protein owing to the relatively weak physical adsorption between the protein and the silicious surface.

PMASH is useful for encapsulation as the disulphide bonds are easily cleaved in the body by glutathione. Glutathione is the must abundant non-protein thiol-source in mammalian cells, reaching a concentration of 10 μM in the extracellular space. It is believed that reduced GSH is the primary mechanism by which the disulphide bond in the capsules will be cleaved, therefore releasing the BDNF protein.

The initial concentration of BDNF used, was 50,000 ng/ml, and after infiltration of the protein overnight, the concentration of BDNF (as measured by the ELISA experiment), in the supernatant was 80 ng/ml, indicating high loading of the protein into the mesoporous silica. Prior to the particles being used for the release experiments, the supernatant after storage was taken and measured to ascertain if any of the BDNF had leaked out of the capsules, this was referred to as time zero. In the two repeated experiments, the results showed 2 ng/ml and 6 ng/ml, indicating some, albeit small leakage of the protein. The amount of BDNF measured at one hour, three hours and eight hours was disappointingly low (see Table 1, 3.1.1 results section). There was a "burst" release of the BDNF, and almost all of the BDNF was released by 12 hours. The values of the BDNF obtained (max 120 ng/ml) were not optimal using this method. Possible explanations for this include; the silica being highly porous and negatively charged had a good affinity with the BDNF, therefore when the silica was dissolved, this high affinity was lost, resulting in BDNF being released

from the capsules, or possibly the multiple washing steps, leading to a loss of the protein.

The second method used to sequester the BDNF, involved the use of the polysaccharides Chitosan and Alginate. Chitosan is made from the N-deacetylated form of chitin, the main constituent in the exoskeletons of crustaceans. Chitosan is a cationic polymer with a highly positive charge density (Anal et al., 2003; Bodmeier et al., 1989). Sodium alginate is an anionic polymer made from the cell wall of brown algae. It forms a viscous gum in combination with water and is of particular use in the food industry (Brownlee et al., 2005). Due to their opposite charge densities, the carboxylate groups on the alginate and the protonated amine groups in the chitosan bonded very well together. The biodegradability and biocompatibility of these substrates, together with their specific interaction with components of the extracellular matrix and growth factors, has led to their ongoing use as bimolecular carriers (Anal et al., 2005; Yuan et al., 2007; Finotelli et al., 2010)

Mesoporous silica was used as a template to infiltrate the chitosan. It was then cross linked to create chitosan strands and the silica core is then dissolved, leaving the porous chitosan particles. As both the protein BDNF and the chitosan are positively charged, the infiltration of the sodium alginate was necessary to increase the carrier's overall negative charge, thereby improving the loading of the protein and thus prolonging BDNF release.

After incubation of the protein, the concentration of the BDNF in the supernatant was 40 ng/ml. This shows very good loading of the protein (initial concentration was

50,000 ng/ml). The time zero samples which assess the leakage of the protein were 214 ng/ml and 320 ng/ml, which illustrates that a significant amount of the protein leaked out of the capsules in storage. Although the 3, 8 and 24-hour values for the released BDNF were higher than the first method, there is still a relative "burst" release of the protein, (see table 2 3.1.2 results section). One possible explanation for the higher values of the released BDNF compared to the PMA_{SH}-PVP capsules, is the fewer washing steps used with the chitosan-alginate particles, resulting in less BDNF being lost. However, the obtained values for the released BDNF are still extremely low. A possible explanation is that the BDNF was reconstituted in milliQ water with a pH of 7.9 and at this pH the particles have a zeta potential of - 39 +/- 5.0 mV, which would explain the excellent loading of the protein. However, once they were stored in acetate buffer with a pH of 4.0, they may acquire a positive charge, which would then repel the protein, resulting in an extensive leakage of the protein.

In an attempt to control the leakage of BDNF from the chitosan-alginate particles, 3 bilayers of the polyelectrolyte's PMA_{SH}-PVP were attached to the outside of the chitosan-alginate particles. Despite this effort, we noticed minimal improvement in the time zero values (120 ng/ml and 140 ng/ml) indicating that at pH 4.0 (conditions needed for layering), there is a probable alteration in charge densities within the particle resulting in a repulsion of BDNF (see Table 3, 3.1.3 Results chapter).

The third method of particle formation involved the use of MS as a template to create nanoporous polymer spheres. The adaptability of the templating permits control over the composition, morphology, and porosity of the structures produced (Ryoo et al., 1999; Wang et al., 2005; Caruso et al., 2001). The process involves sequential solution assembly of the polyelectrolyte polyglutamic acid (PGA) in MS spheres. The assembly involves three main steps. Firstly, the negatively charged polyelectrolyte is immobilized in the MS spheres by solution adsorption. The second step involves the cross-linking of the adsorbed polymer, using a homobifunctional linker cystamine, the cystamine reacts with the substrate carboxyl groups to form an amide linkage which enhances the structural stability of the PGA chains. In the third step the MS template is removed by exposing these particles to a solution of buffered hydrofluoric acid, resulting in the polymer hydrogel replica particles. Polyglutamic acid is a hydrophilic biodegradable, naturally available biopolymer produced by a number of microbial species, most commonly those in the Bacillaceae family (Manocha et al., 2008). Its biological properties such as non-toxicity, biocompatibility and non-immunogenicity qualify it as an important biomaterial in protein delivery applications (Manocha et al., 2008).

The results of this method again showed again good loading of the BDNF because after incubation, the supernatant measured at 800 ng/ml BDNF (initial concentration was 50,000 ng/ml). The time zero samples averaged 40 ng/ml, which is an improvement in the leakage of the protein compared to the method using with chitosan-alginate particles. There was an improvement in the relative burst of the particles (see table 4 3.1.4 Results chapter) however most of the BDNF was still discharged by 30 hours.

The final, and most successful method used, involved the additional use of the glycosaminoglycan heparin. Heparin is a mucopolysaccharide with a molecular weight ranging from 6,000 – 40,000 Da. The average Mw of most commercial heparin preparations is between 12,000-15,000 Da. The polymeric chain is composed of repeating disaccharide units of D-glucosamine and uronic acid linked by interglycoside bonds. Hydroxyl groups on each of the monosaccharide residues may be sulphated giving rise to a polymer that is highly negatively charged. (vcu school of pharmacy www.people.vcu.edu1~urdesai/hep.htm).

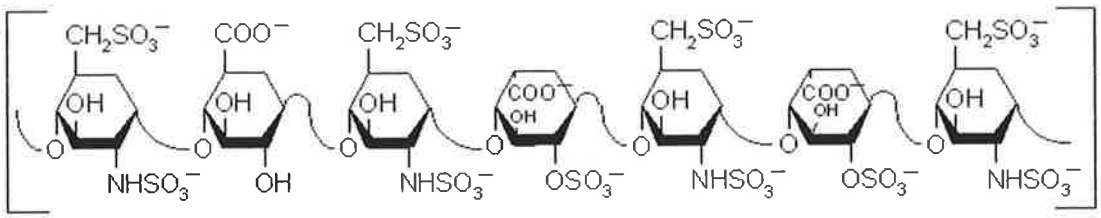


Figure 1 Schematic representation of the heparin molecule

Heparin has been used previously to control the release of heparin-like growth factors (Chung JH et al., 2006) and nerve growth factor (Sakiyama-Elbert SE et al., 2000). However, to the best of our knowledge, it has not previously been shown to prolong the release of non-heparin like growth factors such as BDNF. Following the assembly of the PGA nanoparticles (see section 2.1.4 materials and methods section), we infiltrated the heparin into the particles to give the particles an overall highly negative charge (-40 mV). The positively-charged BDNF was then infiltrated and allowed to react overnight. The supernatant after incubation averaged 130 ng/ml indicating again high protein loading. Time zero values averaged 34 ng/ml,

indicating minimal leakage of the protein out of these particles. The striking difference to the previous methods of encapsulation is the significantly greater amount of BDNF released by the PGA-heparin particles. In the first 24 hours the average amount of BDNF released was 1,231 ng/ml, approximately ten times the amount released previously. The release was also sustained, with an average amount of 3,048 ng/ml released by 8 days, (see graph figure 1 section 3.1.5 Results chapter). Although the majority of the BDNF was released by day 30, there was still a clinically significant level of BDNF released from day 30 to day 70. The prolonged release can be explained by the electrostatic interactions between the positive amine domain in the BDNF, the negative carboxyl groups on the polymer and the highly negative hydroxyl groups on the heparin. The mechanism of release of the BDNF involves hydrolytic disruption of these electrostatic linkages.

The release experiments were conducted with the same number of particles for each experiment (2.4 mg of mesoporous silica crystals used to create each batch). We used phosphate buffered saline (PBS) pH 7.3 at 37°C as the release stimulus because the electrolyte composition closely resembles that of perilymph, the constituent of the scala tympani. PBS also helps maintain a constant pH, osmolality and ion concentration matching those of the human body. It is worth noting that a constant pH and osmolality are important to maintain the nanoparticles, heparin and BDNF in a constant state of ionization. The particles were kept at 37° C for the duration of the release experiment. At each time point the supernatant was removed and stored for analysis, and fresh PBS was added, enabling us to measure the precise amount of BDNF, released at any specific time point.

4.1.3 RELEASED BDNF IS BIOLOGICALLY ACTIVE

To show that the released BDNF was biologically active, we used the third generation neuroblastoma cell line SH-SY5Y. When SH-SY5Y neuroblastoma cell lines are treated with different agents such as retinoic acid or phorbol ester, they differentiate into cells that are biochemically, ultra-structurally and electro physiologically similar to neurons (Kaplan et al., 1993; Pahlman et al., 1995; Abemayor & Sidell 1989). Retinoic acid causes the differentiation of SH-SY5Y cells, and mediates biological responsiveness of the cells to the Trk B ligand BDNF by stimulating the expression of Trk B receptors (Kaplan et al., 1993). The cells were seeded in well chambers filled with a culture media containing 10 μM RA. After one week when the cells had differentiated into neurons, the culture media was removed and the cells were washed 3 times with basal culture media. They were then re-plated and exposed to 3 different culture media. The first was basal media, which acted as a negative control. The second media contained the released BDNF from the nanoparticles, and the third media was the positive control containing an equivalent concentration of commercial BDNF. These cells were allowed to grow for a further 1 week by which time if there was no BDNF in the culture media the cells would undergo programmed cell death (Encinas et al., 1999). The cells were then fixed and stained with the immunohistochemical marker Apotag® to quantify apoptosis within the cells. There was significantly more cells undergoing apoptosis in the basal media (with no BDNF) than in the cells which were exposed to BDNF released from the nanoparticles. This proves that the BDNF released from the nanoparticles was biologically active.

We then proceeded to a pilot study testing the effects of these nanoparticles in animal models. Rats were deafened as described in section 2.5.1 Materials and Methods chapter, and three weeks later, they were given an intracochlear infusion of BDNF-encapsulated nanoparticles in one ear. Two months post-treatment two animals were terminated, their cochleae processed and analysed. The histology of one animal showed a significant difference in spiral ganglion number compared to the untreated control ear. Although there was no quantitative analysis of SGN soma area performed, general observations suggest that SGNs from the BDNF nanoparticle treated cochlea appeared larger than those in the untreated cochlea. The treated ear also showed greater infiltration of leucocyte-like cells in the scala tympani suggesting a host response. In the second animal there was no difference between the SGN number between the treated and control ear. Correspondingly, no leukocyte-like cells were observed in the scala tympani suggesting that nanoparticles were not successfully delivered into the scala tympani possibly due to excessive leakage of perilymph following the injection of the particles at surgery. This gusher effect is likely to flush out the nanoparticles that we delivered. We postulate that the majority of the BDNF-nanoparticles, flowed out with the perilymph, and therefore the trophic effects were lost. It is encouraging that we had a positive result in one animal. It is intended to continue these animal experiments with significantly more animal numbers, in order to verify these results.

We have shown that it is possible to sequester BDNF into a nanoparticle and to have this nanoparticle slowly release the neurotrophin over an extended period of time. We know exogenously-applied neurotrophins are highly effective at protecting SGNs from degeneration, and results from studies combining neurotrophic factor treatment

with chronic depolarization via a cochlear implant are particularly promising (Marzella & Clarke 1999; Song et al., 2009). What is interesting and extremely promising for clinical application is the fact there seems to be a synergistic effect to promote SGN survival when neurotrophins are used in combination with electrical stimulation in the form of a cochlear implant. A study by Kanzaki et al., showed that the combined effects of glial-derived neurotrophic factor and chronic electrical stimulation on guinea pig SGNs in vivo were greater than either treatment alone (Kanzaki et al., 2002).

A similar study by Shepherd et al., on deafened guinea pigs, showed that a combination of electrical stimulation and chronic administration with BDNF (over 28 days), could enhance SGN survival compared to BDNF alone (Shepherd et al., 2005). Interestingly, they found electrical stimulation alone had no effect on SGN survival. This study also showed that auditory brain stem responses (ABR) thresholds were significantly reduced in the BDNF plus electrical stimulation animals compared to the deafened cohorts that did not exhibit SGN rescue. Further analysis showed that the diameter of the neurotrophin treated SGN neurons was increased and that the neurons tended to grow towards the electrode array. This is highly significant as techniques that lead to reductions in threshold at the electrode-neural interface offer significant reductions in power consumption for neural prostheses using transcutaneous radio-frequency links, which are inherently inefficient (Seligman & Shepherd 2004). Longer battery life, smaller components, increased dynamic range and even the potential for smaller more numerous electrodes per cochlear implant array may be realized through such reductions in threshold.

4.1.4 CONCLUSION

In this thesis we have illustrated that BDNF can be sequestered into a nanoparticle. This nanoparticle can then deliver biologically active BDNF over an extended period of time. The use of PGA particles as biocarriers for BDNF provides a very viable method of neurotrophin delivery for possible future clinical applications.

However it remains to be confirmed, if long-term neurotrophin administration will provide ongoing, improved SGN survival. Any side effects relating to prolonged neurotrophin delivery will also need to be ascertained, especially considering that neurotrophin receptors are not specific to SGNs and neurotrophins may therefore elicit effects on other cell types within the cochlea, or potentially throughout the CNS in view of the direct communication between the cochlea and the CNS via the cochlear aqueduct. Alternatively, long term SGN survival may be achieved by combining an initial short term neurotrophin treatment with ongoing electrical stimulation via a cochlear implant. Preclinical trials are required to determine the appropriate time course of neurotrophin treatment and concurrent treatment conditions, such as electrical stimulation from a cochlear implant, as well as optimal dosing regimens to maximize efficacy and minimize toxicity. The application of neurotrophic factors with cochlear implants is an exciting example of a potentially broader application of combining neurobiology and biomedical engineering, in new areas of neural prosthetic research and development.

REFERENCES

Abemayor E, Sidell N (1989) Human neuroblastoma cell lines as models for the in vitro study of neoplastic and neuronal cell differentiation. Environ Health Perspect 80:3-15.

Altschuler RA, Cho Y, Ylikoski J, Pirvola U, Magal E, Miller JM (1999) Rescue and regrowth of sensory nerves following deafferentation by neurotrophic factors. Ann N Y Acad Sci 884:305-11.

Anal AK, Bhopatkar D, Tokura S, Tamura H, Stevens WF (2003) Chitosan-alginate multilayer beads for gastric passage and controlled intestinal release of protein. Drug Dev Ind Pharm. 29(6):713-24.

Anal AK, Stevens WF (2005) Chitosan-alginate multilayer beads for controlled release of ampicillin. Int J Pharm 290(1-2):45-54.

Avila MA, Varela-Nieto I, Romero G, Mato JM, Giraldez F, Van De Water TR, Represa J (1993) Brain-derived neurotrophic factor and neurotrophin-3 support the survival and neuritogenesis response of developing cochleovestibular ganglion neurons. Dev Biol 159(1):266-75.

Bichler E, Spoendlin H, Rauchegger H (1983) Degeneration of cochlear neurons after amikacin intoxication in the rat. Arch Otorhinolaryngol 237(3):201-8.

Blamey P, Arndt P, Bergeron F, Bredberg G, Brimacombe J, Facer G, Larky J, Lindström B, Nedzelski J, Peterson A, Shipp D, Staller S, Whitford L (1996) Factors affecting auditory performance of postlinguistically deaf adults using cochlear implants. Audiol Neurootol 1(5):293-306.

Blamey P (1997) Are spiral ganglion cell numbers important for speech perception with a cochlear implant? Am J Otol 18(6 Suppl):S11-2.

Bodmeier R, Oh KH, Pramar Y (1989) Preparation and Evaluation Of Drug-Containing Chitosan Beads. Drug Dev Ind Pharm 15(9):1475-1494.

Bothwell M (1995) Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev Neurosci 18:223-53.

Brownlee IA, Allen A, Pearson JP, Dettmar PW, Havler ME, Atherton MR, Onsøyen E (2005) Alginate as a source of dietary fiber. Crit Rev Food Sci Nutr 45(6):497-510

Caruso F, Caruso RA, Mohwald H (1998) Nanoengineering of inorganic and hybrid hollow spheres by colloidal templating. Science 282:1111.

Caruso R, Susha A, Caruso F (2001) Multilayered titania, silica and laponite nanoparticle coatings on polystyrene colloidal templates and resulting inorganic hollow spheres. Chem Mater 13;400.

Caruso RA, Antonietti M (2001) Sol–Gel Nanocoating: An Approach to the Preparation of Structured Materials. Chem Mater 13(10): 3272–3282.

Chong SF, Chandrawati R, Stadler B, Park J, Cho J, Wang Y, Jia Z, Bulmus V, Davis TP, Zeliken AN, Caruso F (2009) Stabilization of polymer-hydrogel capsules via thiol-disulphide exchange. Small (22):2601-10

Chung HJ, Kim HK, Yoon JJ, Park TG (2006) Heparin Immobilized Porous PLGA Microspheres for Angiogenic Growth Factor Delivery. Pharmaceutical Research 23(8)1835-1841.

Clarke GP, Sandford M(1995) Interactive computer system for tuning the cochlear 22-channel implant in young children. Ann Otol Rhinol Laryngol Suppl.166:165-6.

Corwin J (1997) Introduction: Inner ear development – initial indications ofs the molecular mechanisms. Semin Cell Dev Biol 8:215-216.

Després G, Romand R (1994) Neurotrophins and the development of cochlear innervation. Life Sci 54(18):1291-7.

Donath E, Sukhorukov GB, Caruso F, Davis SA, Mohwald H (1998) Novel hollow polymer shells by colloid-templated assembly of polyelectrolytes. Angew Chem Int Ed 37:2202.

Dowell RC, Dettman SJ, Blamey PJ, Barker EJ, Clark GM (2002) Speech perception in children using cochlear implants: prediction of long-term outcomes. Cochlear Implants Int 3(1):1-18.

Duan ML, Ulfendahl M, Laurell G, Counter SA, Pyykkö I, Borg E, Rosenhall U (2002) Protection and treatment of sensorineural hearing disorders caused by exogenous factors: experimental findings and potential clinical application. Hear Res 169(1-2):169-78.

Duckert LG, Duvall AJ 3rd (1978) Cochlear communication routes in the guinea pig--spiral ganglia and osseous spiral laminae: an electron microscope study using microsphere tracers. Otolaryngology 86.

Emerich DF, Thanos CG (2007) Targeted nanoparticle-based drug delivery and diagnosis. J Drug Target 15(3):163-83.

Encinas M, Iglesias M, Llecha N, Comella JX (1999) Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. J Neurochem 73(4):1409-21.

Ernfors P, Kucera J, Lee KF, Loring J, Jaenisch R (1995) Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice. Int J Dev Biol 39(5):799-807.

Ernfors P, Duan ML, ElShamy WM, Canlon B (1996) Protection of auditory neurons from aminoglycoside toxicity by neurotrophin-3. Nat Med 2(4):463-7.

Evans AJ, Thompson BC, Wallace GG, Millard R, O'Leary SJ, Clark GM, Shepherd RK, Richardson RT (2009) Promoting neurite outgrowth from spiral ganglion neuron explants using polypyrrole/BDNF-coated electrodes. J Biomed Mater Res A. 91(1):241-50.

Fariñas I, Jones KR, Backus C, Wang XY, Reichardt LF (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature 369(6482):658-61.

Farokhzad OC, Langer R (2006) Nanomedicine: developing smarter therapeutic and diagnostic modalities. Adv Drug Deliv Rev 58(14):1456-9

Fayad JN, Linthicum FH Jr (2006) Multichannel cochlear implants: relation of histopathology to performance. Laryngoscope 116(8):1310-20.

Fekete DM (1999) Development of the vertebrate ear: insights from knockouts and mutants. Trends Neurosci 22:263-269.

Finotelli PV, Da Silva D, Sola-Penna M, Rossi AM, Farina M, Andrade LR, Takeuchi AY, Rocha-Leão MH (2010) Microcapsules of alginate/chitosan containing magnetic nanoparticles for controlled release of insulin. Colloids Surf B Biointerfaces. 81(1):206-11.

Fletcher JM, Hughes RA (2006) Novel monocyclic and bicyclic loop mimetics of brain-derived neurotrophic factor. J Pept Sci 12(8):515-24.

Fritzsch B (1997) The role of neurotrophic factors in regulating the development of the inner ear. Trends Neurosci 20:159-164.

Ge X, Jackson RL, Liu J, Harper EA, Hoffer ME, Wassel RA, Dormer KJ, Kopke RD, Balough BJ (2007) Distribution of PLGA nanoparticles in chinchilla cochleae. Otolaryngol Head Neck Surg 137(4):619-23.

Gelfand SA (1995) Long-term recovery and no recovery from the auditory deprivation effect with binaural amplification: six cases. J Am Acad Audiol 6(2):141-9.

Gillespie LN, Clark GM, Bartlett PF, Marzella PL (2003) BDNF-induced survival of auditory neurons in vivo: Cessation of treatment leads to accelerated loss of survival effects. J Neurosci Res 71:785-790.

Gillespie LN, Clark GM, Marzella PL (2004) Delayed neurotrophin treatment supports auditory neuron survival in deaf guinea pigs. Neuroreport. 2004 May 19;15 (7):1121-5.

Gopen Q, Rosowski JJ, Merchant SN (1997) Anatomy of the normal human cochlear aqueduct with functional implications. Hear Res 107(1-2):9-22.

Glueckert R, Pfaller K, Kinnefors A, Rask-Andersen H, Schrott-Fischer A (2005) Ultrastructure of the normal human organ of corti. new anatomical findings in surgical specimens. Acta Otolaryngol 125(5):534-9.

Hall RD (1990) Estimation of surviving spiral ganglion cells in the deaf rat using the electrically evoked auditory brainstem response. Hear Res 49(1-3):155-68.

Han YJ, Kim JM, Stucky GD (2000) Preparation of noble metal nanowires using hexagonal mesoporous silica SBA-15. Chem Mater 12:2068-9.

Hardie NA, Shepherd RK (1999) Sensorineural hearing loss during development: morphological and physiological response of the cochlea and auditory brainstem. Hear Res 128(1-2):147-65.

Hardie NA (1998) The consequences of deafness and chronic intracochlear electrical stimulation on the central auditory pathways. Clin Exp Pharmacol Physiol 25(5):303-9.

Hartshorn DO, Miller JM, Altschuler RA (1991) Protective effect of electrical stimulation in the deafened guinea pig cochlea. Otolaryngol Head Neck Surg 104(3):311-9.

Hornyak GL (2005) Nanotechnology in otolaryngology.Otolaryngol Clin North Am. 38(2):273-93.

Hudspeth AJ (2000) Hearing. In: Principles of Neuroscience, Fourth Edition (Kandel ER, Schwartz JH, Jessell TM, eds), pp 590-613: McGraw Hill.

Husseman J, Raphael Y (2009) Gene therapy in the inner ear using adenovirus vectors. Adv Otorhinolaryngol. 66:37-51.

Iguchi F, Nakagawa T, Tateya I, Kim TS, Endo T, Taniguchi Z, Naito Y, Ito J (2003) Trophic support of mouse inner ear by neural stem cell transplantation. Neuroreport. 14(1):77-80.

Juhn SK, Hunter BA, Odland RM (2001) Blood-labyrinth barrier and fluid dynamics of the inner ear. Int Tinnitus J 7(2):72-83.

Kanzaki S, Stöver T, Kawamoto K, Prieskorn DM, Altschuler RA, Miller JM, Raphael Y (2002) Glial cell line-derived neurotrophic factor and chronic electrical stimulation prevent VIII cranial nerve degeneration following denervation. J Comp Neurol 454(3):350-60.

Kaplan DR, Matsumoto K, Lucarelli E, Thiele CJ (1993) Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. Eukaryotic Signal Transduction Group. 11(2):321-31.

Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10(3):381-91.

Kawano A, Seldon HL, Clark GM, Ramsden RT, Raine CH (1998) Intracochlear factors contributing to psychophysical percepts following cochlear implantation.Acta Otolaryngol. 118(3):313-26.

Kileny PR, Zimmerman-Phillips S, Kemink JL, Schmaltz SP (1991) Effects of preoperative electrical stimulability and historical factors on performance with multichannel cochlear implant. Ann Otol Rhinol Laryngol 100(7):563-8.

Khan AM, Handzel O, Burgess BJ, Damian D, Eddington DK, Nadol JB Jr (2005) Is word recognition correlated with the number of surviving spiral ganglion cells and electrode insertion depth in human subjects with cochlear implants? Laryngoscope 115(4):672-7.

Kresge CT, Leonowicz ME, Roth WJ, Vartuli JC, Beck JS (1992) Ordered mesoporous molecular sieves synthesised by a liquid-crystal template mechanism. Nature 359:710-711.

Leake PA, Hradek GT (1988) Cochlear pathology of longterm neomycin induced deafness in cats. Hear Res 33,11-33.

Lefebvre PP, Malgrange B, Staecker H, Moghadass M, Van de Water TR, Moonen G (1994) Neurotrophins affect survival and neuritogenesis by adult injured auditory neurons in vitro. Neuroreport 5(8):865-8.

Lefebvre PP, Staecker H (2002) Steroid perfusion of the inner ear for sudden sensorineural hearing loss after failure of conventional therapy: a pilot study. Acta Otolaryngol 122(7):698-702.

Lessmann V, Gottmann K, Malcangio M (2003) Neurotrophin secretion: current facts and future prospects. Prog Neurobiol 69(5):341-74.

Liberman MC (1982) Single-neuron labeling in the cat auditory nerve. Science 216(4551):1239-41.

Li Duan M, Bordet T, Mezzina M, Kahn A, Ulfendahl M (2002) Adenoviral and adeno-associated viral vector mediated gene transfer in the guinea pig cochlea. Neuroreport. 13(10):1295-9.

Linthicum FH Jr, Fayad JN (2009) Spiral ganglion cell loss is unrelated to segmental cochlear sensory system degeneration in humans. Otol Neurotol 30(3):418-422.

Lim DJ (1970) Surface ultrastructure of the cochlear perilymphatic space. J Laryngol Otol. 84(4):413-28.

Liu Y, Okada T, Sheykholeslami K, Shimazaki K, Nomoto T, Muramatsu S, Kanazawa T, Takeuchi K, Ajalli R, Mizukami H, Kume A, Ichimura K, Ozawa K (2005) Specific and efficient transduction of Cochlear inner hair cells with recombinant adeno-associated virus type 3 vector. Mol Ther 12(4):725-33.

Luebke AE, Steiger JD, Hodges BL, Amalfitano A (2001) A modified adenovirus can transfect cochlear hair cells in vivo without compromising cochlear function. Gene Ther 8(10):789-94.

Malgrange B, Lefebvre P, Van de Water TR, Staecker H, Moonen G (1996) Effects of neurotrophins on early auditory neurones in cell culture. Neuroreport 7(4):913-7.

Malgrange B, Rogister B, Lefebvre PP, Mazy-Servais C, Welcher AA, Bonnet C, Hsu RY, Rigo JM, Van De Water TR, Moonen G (1998) Expression of growth factors and their receptors in the postnatal rat cochlea. Neurochem Res 23(8):1133-8.

Manocha B, Margaritis A (2008) Production and characterization of gamma-polyglutamic acid nanoparticles for controlled anticancer drug release. Crit Rev Biotechnol. 28(2):83-99.

Marzella PL, Clark GM (1999) Growth factors, auditory neurones and cochlear implants: a review. Acta Otolaryngol 119(4):407-12.

McGuinness SL, Shepherd RK (2005) Exogenous BDNF rescues rat spiral ganglion neurons in vivo. Otol Neurotol 26(5):1064-72.

Miller JM, Altschuler RA (1995) Effectiveness of different electrical stimulation conditions in preservation of spiral ganglion cells following deafness. Ann Otol Rhinol Laryngol Suppl 166:57-60.

Miller CA Woodruff KE, Pfingst BE (1995) Functional responses from guinea pigs with cochlear implants. I. Electrophysiological and psychophysical measures. Hear Res 92(1-2):85-99.

Miller JM, Chi DH, O'Keeffe LJ, Kruszka P, Raphael Y, Altschuler RA (1997) Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss. Int J Dev Neurosci 15(4-5):631-43.

Miller AL, Prieskorn DM, Altschuler RA, Miller JM (2003) Mechanism of electrical stimulation induced neuroprotection: effects of verapamil on protection of primary auditory efferents. Brain Res 996, 218-230.

Miura M, Sando I, Hirsch BE, Orita Y (2002) Analysis of spiral ganglion cell populations in children with normal and pathological ears. Ann Otol Rhinol Laryngol 111(12 Pt 1):1059-65.

Nadol JB,Jr. (1983) Serial section reconstruction of the neural poles of hair cells in the human organ of Corti.II. outer hair cells. Laryngoscope 93:780-791.

Nadol JB Jr, Young YS, Glynn RJ (1989) Survival of spiral ganglion cells in profound sensorineural hearing loss: implications for cochlear implantation. Ann Otol Rhinol Laryngol 98(6):411-6.

Nadol JB Jr, Shiao JY, Burgess BJ, Ketten DR, Eddington DK, Gantz BJ, Kos I, Montandon P, Coker NJ, Roland JT Jr, Shallop JK (2001) Histopathology of cochlear implants in humans. Ann Otol Rhinol Laryngol 110(9):883-91.

O'Leary PD, Hughes RA (2003) Design of potent peptide mimetics of brain-derived neurotrophic factor. J Biol Chem 278(28):25738-44.

Oppenheim RW (1989) The neurotrophic theory and naturally occurring motoneuron death. Trends Neurosci 12(7):252-5.

Otte J, Schunknecht HF, Kerr AG (1978) Ganglion cell populations in normal and pathological human cochleae. Implications for cochlear implantation. Laryngoscope 88(8 Pt 1):1231-46.

Påhlman S, Hoehner JC, Nånberg E, Hedborg F, Fagerström S, Gestblom C, Johansson I, Larsson U, Lavenius E, Ortoft E, et al (1995) Differentiation and survival influences of growth factors in human neuroblastoma. Eur J Cancer 31A(4):453-8.

Pettingill LN, Minter RL, Shepherd RK (2008) Schwann cells genetically modified to express neurotrophins promote spiral ganglion neuron survival in vitro. Neuroscience.152(3):821-8.

Pfingst BE, Sutton D, Miller JM, Bohne BA (1981) Relation of psychophysical data to histopathology in monkeys with cochlear implants. Acta Otolaryngol 92(1-2):1-13.

Pickles JO, Comis SD, Osborne MP (1984) Cross-links between stereocilia in the guinea pig organ of Corti, and their possible relation to sensory transduction. Hear Res 15(2):103-12.

Pickles JO (1988) An Introduction to the physiology of Hearing, Second Edition: Academic Press.

Pirvola U, Ylikoski J, Palgi J, Lehtonen E, Arumäe U, Saarma M (1992) Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. Proc Natl Acad Sci USA 89(20):9915-9.

Pirvola U, Arumäe U, Moshnyakov M, Palgi J, Saarma M, Ylikoski J (1994) Coordinated expression and function of neurotrophins and their receptors in the rat inner ear during target innervation. Hear Res 75(1-2):131-44.

Prieskorn DM, Miller JM (2000) Technical report: chronic and acute intracochlear infusion in rodents. Hear Res 140(1-2):212-5.

Pumford NR, Halmes NC, Martin BM, Cook RJ, Wagner C, Hinson JA (1997) Covalent binding of acetaminophen to N-10-formyltetrahydrofolate dehydrogenase in mice. J Pharmacol Exp Ther 280(1):501-5.

Rajan R (2000) Centrifugal pathways protect hearing sensitivity at the cochlea in noisy environments that exacerbate the damage induced by loud sound. J Neurosci 20(17):6684-93.

Rask-Andersen H, Schrott-Fischer A, Pfaller K, Glueckert R (2006) Perilymph/modiolar communication routes in the human cochlea. Ear Hear 27(5):457-65.

Rejali D, Lee VA, Abrashkin KA, Humayun N, Swiderski DL, Raphael Y (2007) Cochlear implants and ex vivo BDNF gene therapy protect spiral ganglion neurons. Hear Res 228(1-2):180-7.

Richardson RT, O'Leary S, Wise A, Hardman J, Clark G (2005) A single dose of neurotrophin-3 to the cochlea surrounds spiral ganglion neurons and provides trophic support. Hear Res 204(1-2):37-47.

Rinkwitz S (2001) Development of the vertebrate inner ear. Ann N Y Acad Sci 942:1-14.

Robertson D (1984) Horseradish peroxidase injection of physiologically characterized afferent and efferent neurones in the guinea pig spiral ganglion. Hear Res 15(2):113-21.

Robinson RC, Radziejewski C, Stuart DI, Jones EY (1995) Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer. Biochemistry 34(13):4139-46.

Rosenthal A, Goeddel DV, Nguyen T, Martin E, Burton LE, Shih A, Laramee GR, Wurm F, Mason A, Nikolics K, et al (1991) Primary structure and biological activity of human brain-derived neurotrophic factor. Endocrinology 129(3):1289-94.

Roy S, Johnston AH, Newman TA, Glueckert R, Dudas J, Bitsche M, Corbacella E, Rieger G, Martini A, Schrott-Fischer A (2010) Cell-specific targeting in the mouse inner ear using nanoparticles conjugated with a neurotrophin-derived peptide ligand: potential tool for drug delivery. Int J Pharm 390(2):214-24.

Rubinstein JT, Miller CA (1999) How do cochlear prostheses work? Curr Opin Neurobiol 9(4):399-404.

Ryoo R, Joo SH, Jun S (1999) Synthesis of highly ordered carbon molecular sieves via template-mediated structural transformation. J Phys Chem B 103(37)7743-46.

Ryoo R , Joo SH, Kruk M, Jaroniec M (2001) Ordered mesoporous carbons. Adv Mater 13(9):677-681

Ryugo DK, Dodds LW, Benson TE, Kiang NY (1991) Unmyelinated axons of the auditory nerve in cats. J Comp Neurol 308(2):209-23.

Sakiyama-Elbert SE, Hubbell JA (2000) Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix. J Control Release 3;69(1):149-58.

Scheper V, Wolf M, Scholl M, Kadlecova Z, Perrier T, Klok HA, Saulnier P, Lenarz T, Stöver T (2009) Potential novel drug carriers for inner ear treatment: hyperbranched polylysine and lipid nanocapsules. Nanomedicine (Lond). 4(6):623-35.

Salt AN (2003) Cochlear Fluids Research Laboratory Website, http://oto.wustl.edu/cochlea/. In: Department of Otolaryngology, Washington University, St Louis.

Schecterson LC, Bothwell M (1994) Neurotrophin and neurotrophin receptor mRNA expression in developing inner ear. Hear Res 73(1):92-100.

Schuknecht HF, Seifi AE (1963) Experimental observations on the fluid physiology of the inner ear. Ann Otol Rhinol Laryngol.72:687-712.

Seligman & Shepherd (2004) cochlear implants in: Horch, KW; Dhillon G editors. Neuroprosthetics: Theory and practice World scientific publishing; Singapore 878-904.

Shepherd RK, Javel E (1997) Electrical stimulation of the auditory nerve. I. Correlation of physiological responses with cochlear status. Hear Res. 108(1-2):112-44.

Shepherd RK, Hardie NA (2001) Deafness-induced changes in the auditory pathway: implications for cochlear implants. Audiol Neurootol. 6(6):305-18.

Shepherd RK, Colreavy MP (2004) Surface microstructure of the perilymphatic space: implications for cochlear implants and cell- or drug-based therapies. Arch Otolaryngol Head Neck Surg 130(5):518-23.

Shepherd RK, Roberts LA, Paolini AG (2004) Long-term sensorineural hearing loss induces functional changes in the rat auditory nerve. Eur J Neurosci 20(11):3131-40.

Shepherd RK, Coco A, Epp SB, Crook JM (2005) Chronic depolarization enhances the trophic effects of brain-derived neurotrophic factor in rescuing auditory neurons following a sensorineural hearing loss. J Comp Neurol 486(2):145-58.

Slepecky NB (1996) Structure of the Mammalian Cochlea. In: The Cochlea (Dallos P, Popper AN, Fay RR, eds). New York: Springer-Verlag NY Inc.

Spoendlin H (1971) Degeneration behaviour of the cochlear nerve. Arch Klin Exp Ohren Nasen Kehlkopfheilkd 200(4):275-91.

Spoendlin H (1984) Factors inducing retrograde degeneration of the cochlear nerve. Ann Otol Rhinol Laryngol Suppl 112:76-82.

Spoendlin H, Schrott A (1989) Analysis of the human auditory nerve. Hear Res 43(1):25-38.

Song BN, Li YX, Han DM (2009) Delayed electrical stimulation and BDNF application following induced deafness in rats. Acta Otolaryngol 129(2):142-54.

Staecker H, Kopke R, Malgrange B, Lefebvre P, Van de Water TR (1996) NT-3 and/or BDNF therapy prevents loss of auditory neurons following loss of hair cells. Neuroreport 7(4):889-94.

Staecker H, Li D, O'Malley BW Jr, Van De Water TR (2001) Gene expression in the mammalian cochlea: a study of multiple vector systems. Acta Otolaryngol 121(2):157-63.

Stöver T, Yagi M, Raphael Y (2000) Transduction of the contralateral ear after adenovirus-mediated cochlear gene transfer. Gene Ther. 7(5):377-83.

Sun W, Salvi RJ (2009) Brain derived neurotrophic factor and neurotrophic factor 3 modulate neurotransmitter receptor expressions on developing spiral ganglion neurons. Neuroscience 164(4):1854-66.

Tamura T, Kita T, Nakagawa T, Endo T, Kim TS, Ishihara T, Mizushima Y, Higaki M, Ito J (2005) Drug delivery to the cochlea using PLGA nanoparticles. Laryngoscope 115(11):2000-5.

Terayama Y, Kaneko Y, Kawamoto K, Sakai N (1977) Ultrastructural changes of the nerve elements following disruption of the organ of Corti. I. Nerve elements in the organ of Corti. Acta Otolaryngol 83(3-4):291-302.

Torres M, Giraldez F (1998) The development of the vertebrate inner ear. Mech Dev 71:5-21.

Ulehlová L, Voldrich L, Janisch R (1987) Correlative study of sensory cell density and cochlear length in humans. Hear Res 28(2-3):149-51.

Wang Y, Caruso F (2005) Mesoporous seilica spheres as supports for enzyme immobilisation and encapsulation. Chem Mat 17(5) 953-961.

Wang Y, Yu AM, Caruso F (2005) Nanoporous Polyelectrolyte Spheres Prepared by Sequentially Coating Sacrificial Mesoporous Silica Spheres. Angew. Chem.-Int. Edit 44:2888.

Wang Y, Angelatos AS, Dunstan DE, Caruso F (2007) Infiltration of Macromolecules into Nanoporous Silica Particles. Macromolecules 40:7594-7600

Webster DB, Webster M (1978) Cochlear nerve projections following organ of corti destruction. Otolaryngology. 86(2): ORL 342-53.

Webster DB (1992) An Overview of Mammalian Auditory Pathways with an Emphasis on Humans. In: The Mammalian Auditory Pathway: Neuroanatomy (Webster DB, Fay RR, eds). New York: Springer-Verlag.

Wei BP, Robins-Browne RM, Shepherd RK, Azzopardi K, Clark GM, O'Leary SJ (2006) Protective effects of local administration of ciprofloxacin on the risk of pneumococcal meningitis after cochlear implantation. Laryngoscope 116(12):2138-44.

Wheeler EF, Bothwell M, Schecterson LC, von Bartheld CS (1994) Expression of BDNF and NT-3 mRNA in hair cells of the organ of Corti: quantitative analysis in developing rats. Hear Res. 73(1):46-56.

Wise AK, Richardson R, Hardman J, Clark G, O'leary S (2005) Resprouting and survival of guinea pig cochlear neurons in response to the administration of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3. J Comp Neurol. 487(2):147-65.

Young ED (1998) Cochlear Nucleus. In: The Synaptic Organization of the Brain, Fourth Edition (Shepherd GM, ed), pp 121-157. New York: Oxford University Press.

Ylikoski J, Pirvola U, Moshnyakov M, Palgi J, Arumäe U, Saarma M (1993) Expression patterns of neurotrophin and their receptor mRNAs in the rat inner ear. Hear Res 65(1-2):69-78.

Yuan W, Dong H, Li CM, Cui X, Yu L, Lu Z, Zhou Q (2007) pH-controlled construction of chitosan/alginate multilayer film: characterization and application for antibody immobilization. Langmuir. 23(26):13046-52.

Yuen EC, Howe CL, Li Y, Holtzman DM, Mobley WC (1996) Nerve growth factor and the neurotrophic factor hypothesis. Brain Dev 18(5):362-8.

Zelikin AN, Li Q, Caruso F (2006) Degradable polyelectrolyte capsules filled with oligonucleotide sequences. Angew Chem Int Ed Engl 45(46):7743-5.

Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC (2008) Nanoparticles in medicine: therapeutic applications and developments. Clin Pharmacol Ther 83(5):761-9

Zheng JL, Gao WQ (1996) Differential damage to auditory neurons and hair cells by ototoxins and neuroprotection by specific neurotrophins in rat cochlear organotypic cultures. Eur J Neurosci 8(9):1897-905

Zheng JL, Stewart RR, Gao WQ (1995) Neurotrophin-4/5, brain-derived neurotrophic factor, and neurotrophin-3 promote survival of cultured vestibular ganglion neurons and protect them against neurotoxicity of ototoxins. J Neurobiol 28(3):330-40.