

From DEPARTMENT OF CLINICAL NEUROSCIENCE,  
Karolinska Institutet, Stockholm, Sweden

# ROUND WINDOW MEMBRANE AND DELIVERY OF BIOLOGICALLY ACTIVE AGENTS INTO THE COCHLEA

Amanj K. Saber



**Karolinska  
Institutet**

Stockholm 2010

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ISBN 978-91-7457-067-0

*Dedicated to my family*



## ABSTRACT

Establishing efficient methods for local administration of drugs to the inner ear has great clinical relevance for the management of inner ear disorders. However, the administration route remains a critical issue. The most feasible approach for a non invasive drug delivery to the inner ear is application of medication to the middle ear cavity on the promise that it will diffuse through the thin round window membrane (RWM) separating the inner ear from the middle ear cavity. Gene therapy represents a promising future in otology and offers an exciting therapeutic alternative as it could be used in prevention or management of cochlear disorders. Also for a gene therapy approach, RWM application seems most feasible administration route. Exploring and characterizing the RWM route of administration is thus a fundamentally important area of research for the development of future treatment of inner ear disorders.

The objectives of the thesis were to evaluate the efficacy of two drug and gene delivering vehicles to the inner ear, sodium hyaluronate (HYA) and chitosans, which can be applied to the cochlea. Ultimate aim is to establish an efficient drug delivery system and gene transfection for the inner ear. HYA and chitosans loaded with the ototoxic drug neomycin as tracer for drug release have been instilled into the middle ear of the guinea pigs. Effects on RWM and cochlear hair cells were evaluated after a single instillation of HYA (day 7 and 28), chitosans and saline solution (day 7). The hearing organ was analysed for hair cell loss and the thickness and ultrastructural properties of the RWM were analysed by light and transmission electron microscopy. The *in vitro* transfection efficiency of chitosan was tested by exposing organotypic cultures of the organ of Corti, prepared from postnatal day 2 rats, to chitosan carrying plasmid DNA (pDNA). The *in vivo* transfection efficiency was tested at one day or seven days after infusing chitosan/pDNA polyplexes with the use of osmotic pumps into the cochlea of adult guinea pigs. Tissue analysis was made by immunohistochemistry and RT-PCR.

HYA and chitosans, especially glycosylated derivative, are safe vehicles that can be used for drug transport into the inner ear through the RWM. Both vehicles successfully released the loaded neomycin, which exerted toxic effects on cochlear hair cells in a degree depending on the concentrations used. The vehicles per se had no noxious effect on the cochlear hair cells but they provoked a comparable effect on the thickness and morphology of the RWM. The thickness of the RWM returned to normal 4 weeks after exposure to HYA. Chitosan as a carrier for inner ear transfection, was associated with inconsistent transfection *in vitro* and *in vivo*.

The importance of the RWM as a portal for local therapy of inner ear disorders is highlighted in this thesis by focusing on efficiency and effects of the vehicles, applied to the RWM for delivering biologically active agents into the cochlea.

The difficulties and variability associated with applying substances to the RWM were explored. The results of this thesis add new knowledge concerning mechanisms of passage of biologically active agents through the RWM and may help us to better understand the role of RWM in the local cochlear therapy and problems of local treatment of inner ear diseases.

Key words: Round window membrane, sodium hyaluronate, chitosan, local cochlear drug delivery and gene delivery.

## LIST OF PUBLICATIONS

This thesis is based on following papers. They will be referred to by their Roman numerals (I-IV)

- I. **Saber A**, Laurell G, Bramer T, Edsman K, Engmér C, Ulfendahl M. (2009) Middle ear application of a sodium hyaluronate gel loaded with neomycin in a guinea pig model. *Ear and Hearing*; 30, 81-89.
- II. **Saber A**, Strand P S, Ulfendahl M. Use of the biodegradable polymer chitosan as a vehicle for applying drugs to the inner ear (2010). *European Journal of Pharmaceutical Sciences*; 39,110–115
- III. **Saber A**, Gont C, Dash-Wagh S, Kirkegaard M, Strand P S and Ulfendahl M. (2010) Assessment of *in vitro* and *in vivo* transfection efficiency of the biodegradable polymer chitosan in the inner ear. Accepted for publication in *The Journal of International Advanced Otology*.
- IV. **Saber A**, Manström P, Bramer T, Edsman K, Strand P S and Ulfendahl M. Ultrastructural features of the guinea pig round window membrane following round window membrane administration of sodium hyaluronate and chitosan. (*In manuscript*).

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## LIST OF ABBREVIATIONS

Ch1	Chitosan 1
Ch2	Chitosan 2
Ch3	Chitosan 3
EDTA	Ethylenediaminetetraacetate
GFP	Green fluorescent protein
HYA	Sodium hyaluronate
IHCs	Inner hair cells
MW	Molecular weight
Neo	neomycin
Neo15%	neomycin 15%
Neo5%	neomycin 5%
Neo2%	neomycin 2%
OHCs	Outer hair cells
PB	Phosphate buffer
PBS	Phosphate-buffered saline
pDNA	Plasmid DNA
RWM	Round window membrane
SB-TCO	Side branch trisaccharide-substituted chitosan oligomers
SGN	Spiral ganglion neuron
SNHL	Sensorineural hearing loss
SM	Scala media
ST	Scala tympani
SSNHL	Sudden sensorineural hearing loss
SV	Scala vestibuli
TEM	Transmission electron microscopy



# 1 INTRODUCTION

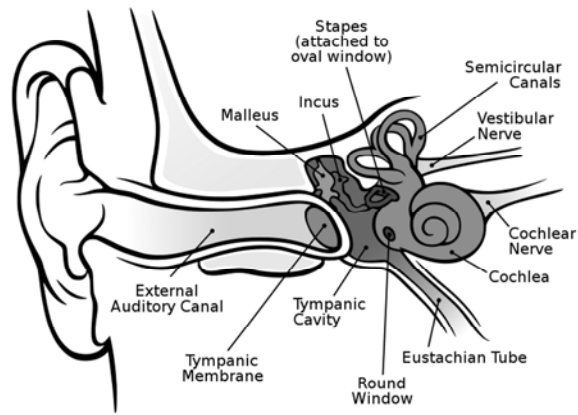
The inner ear, the innermost part of the ear, is embedded deeply in the skull within the temporal bone. This anatomic position protects the inner ear from external injury. However, this isolation has greatly limited the possibility to treat inner ear disorders pharmacologically. Treatment of inner ear disorders through systemic administration is ineffective due to certain physiological and anatomical barriers such as limited cochlear blood supply and the presence of the blood-cochlear barrier. These barriers limit access of the therapeutic agents into the inner ear, compromising the availability of the drugs in adequate concentration for the target cells within the hearing organ, the organ of Corti. In addition, systemic drug administration is associated with increased risk of systemic side effects.

However, the treatment of inner ear disorders represents a promising future in otology. The research into strategies to restore hearing following hair cell death and mechanisms underlying hearing impairment, have provided new alternative biological therapeutic means (local cochlear pharmacological therapy and gene therapy) and led to significant progress in the development of local methods of drug and gene delivery to the inner ear. Accessing the cochlea via the round window membrane (RWM) is probably the most direct and non-invasive approach and has great clinical relevance and potential even in a relatively short perspective. Experimentally, several therapeutic agents have been tested and shown efficacy in prevention of ototoxicity and noise induced sensorineural hearing loss [1-4]. Clinically, there is an increasing interest in local application of drugs e.g. gentamicin and steroids directly to the inner ear across the RWM for the treatment of inner ear disorders such as Ménière's disease, sudden sensorineural hearing loss (SSNHL), and tinnitus [5-12]. For a possible clinical use, RWM administration seems most feasible but we lack an efficient and safe way of administration of drugs into the inner ear. The importance of the RWM for local therapy of inner ear disorders is highlighted in this thesis by focusing on the effects of biocompatible polymers (sodium hyaluronate [HYA] and chitosan) applied to the RWM for delivering biologically active agents into the inner ear.

## 1.1 EAR AND HEARING

The ear is a unique sensory organ that comprises both the hearing organ and the vestibular system, which is responsible for maintaining the body balance. Anatomically, the ear is composed of the external, the middle ear and the inner ear while functionally it is divided into two parts, conduction (external and middle ear) and perception (inner ear) parts (Fig.1). All parts have an essential role in the hearing process.

**Figure 1.** Anatomy of the human ear.  
Adapted from [http://commons.wikimedia.org/wiki/File:Anatomy\\_of\\_the\\_Human\\_Ear.svg](http://commons.wikimedia.org/wiki/File:Anatomy_of_the_Human_Ear.svg)  
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### 1.1.1 The external ear

The external ear composed of the pinna (auricle) and the S-shaped external auditory canal which is about 26 mm long in an adult human.

### 1.1.2 The middle ear

The middle ear or tympanic cavity is an air-filled cavity behind the tympanic membrane. It comprises the tympanic membrane and the three auditory ossicles; the malleus, the incus, and the stapes which transmit acoustic vibrations from tympanic membrane to the inner ear. It is separated by the tympanic membrane from the external ear. Anteriorly it is connected with the nasopharynx through Eustachian tube. The medial wall is formed by the promontory which is the basal turn of the cochlea.

### 1.1.3 The inner ear

The inner ear, a complex structure with delicate anatomy, is embedded and protected deep within the skull in the petrous portion of temporal bone, which is the densest bone in the human body. The inner ear is formed by fluid-filled bony and membranous tubes which are divided into two functionally separate parts, the cochlea (sensory hearing organ) and the equilibrium organs. The bony labyrinth has three sections: the cochlea; the semicircular canals and the vestibule. Within the bony labyrinth there is a second series of delicate membranous tubes, called the membranous labyrinth, having the same parts as the bony labyrinth. The sensory organs contain mechano-receptive cells called hair cells.

#### 1.1.3.1 The cochlea

The mammalian cochlea is snail-shaped organ with a series of fluid-filled compartments and two windows (oval and round). The cochlea transforms mechanical energy into electrical energy by converting fluid movement into neural impulses.

The cochlea is broad at the base and gets narrow towards the apex. In human it is 31-33-mm long with two and a half turns. The length and number of turns differ between species. The interior of the cochlea (Fig.2) is divided by basilar and Reissner's membranes into three fluid-filled compartments; *scala tympani* (ST), *scala vestibuli* (SV) and *scala media* (SM). These compartments wind helically around the central body axis of the cochlea, the modiolus and are filled with two different types of fluids. The SM is filled with endolymph, a filtrate of the perilymph, which has an ionic composition similar to the intracellular fluid with high potassium concentrations and

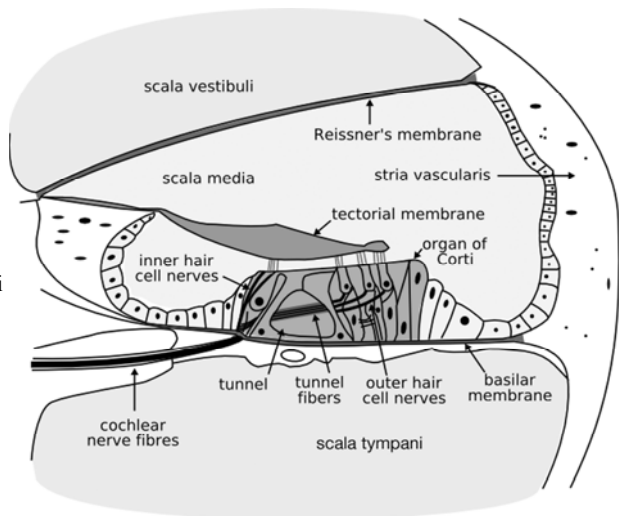
low sodium concentrations. The unique intracellular ionic composition of the endolymph is kept constant by the epithelium of stria vascularis [13-18]. Stria vascularis is responsible for maintaining the high potassium concentration and positive endocochlear potential in SM (80-100 mV). Also the cells lining SM are connected with each other by tight junctions, which help the SM to retain the endocochlear potential [19,20].

ST and SV are filled with perilymph which is similar to cerebrospinal fluid in consistency. The volume of perilymph in the human is about 70  $\mu$ l, with around 40  $\mu$ l in the ST [16]. ST and SV are connected together at the helicotrema, a small opening at the apex of the cochlea.

The basilar membrane stretches between the osseous spiral lamina and lateral cochlear wall. The hearing organ (organ of Corti) is located on it. The basilar membrane is broad, thin and rigid at the base of cochlea while it is narrow, thick and floppy at the apex of the cochlea. Stria vascularis is a highly vascularized and metabolically active tissue forming the lateral wall of SM. The round window is closed by the round window membrane (RWM) which is very compliant, capable of bulging into the middle ear. It separates perilymph in the ST from the middle ear cavity. The oval window closed by the foot-plate of stapes, is located at the beginning of the SV.

**Figure 2.** Schematic illustration of a cross section view through one turn of the cochlea, showing the endolymphatic and perilymphatic spaces, as well as the organ of Corti and its inner and outer hair cells.

Adapted from: <http://commons.wikimedia.org/wiki/File:Cochlea-crosssection.png>. Released under the GNU Free Documentation License.



### 1.1.3.2 Organ of Corti

The hearing organ is very delicate and complicated. It rests on the basilar membrane and is covered by a gelatinous like membrane, the tectorial membrane which overlies the organ of Corti (Fig.2). Organ of Corti is formed by few thousands of hair cells and supporting cells. The hair cells lie within a matrix of supporting cells and are separated from each other by the supporting cells. The supporting cells ensure efficient mechanical contact between the hair cells and basilar membrane.

Hair cells, specialized mechanoreceptors, are very precisely organized into one row of the inner hair cells (IHCs) and three rows of outer hair cells (OHCs). In human there are about 15000-16000 hair cells (3500 IHC and 12000 OHC) in each cochlea while the guinea pig cochlea contains 8500-9500 hair cells (1900 IHCs and 6600 OHCs) [21,22]. The cochlear hair cells have fine stereocilia (80 cilia per cell) on their apical surfaces which project towards and partly embedded in the tectorial membrane [23]. The hair cells are frequency-specific and tonotopically organized; those that respond to

high frequency sound are located at the base of the cochlea and those that respond to low frequency sound are at the apex.

The OHCs are tall cylindrical cells with basally located nuclei and peripherally distributed cytoplasmic mitochondria. The IHCs are rounded cells with central nuclei and evenly distributed mitochondria in the cytoplasm.

#### *1.1.3.3 The spiral ganglion neurons (SGN)*

The cell bodies of the auditory neurons, spiral ganglion neurons (SGN), are situated in the Rosenthal's canal which is a spiral canal in the modiolus.

The bipolar SGN send peripheral processes to the hair cells in the cochlea and axons to the second-order neurons, the cochlear nuclei, in the brainstem. The auditory nerve (cochlear nerve) composed of bundles of bipolar auditory neurons, form part of the eighth cranial nerve (CN VIII). The hair cells form synapses with efferent and afferent nerve fibers. IHCs are connected to the afferent nerve fibers and the OHCs to the efferent nerve fibers [24,25]. There are two types of SGNs. Type I (90-95%) myelinated neuron that participate in the afferent innervation of the IHCs and therefore conveying most of the afferent input in the brain stem. Ten to 20 type I neurons converge on each IHC. Type II (5-10%) unmyelinated neuron innervate the OHCs. Each axon from the type II neuron contacts about 30 to 60 OHCs within the same row [22,26].

#### *1.1.3.4 Blood-cochlear barrier*

Most of the cochlea is separated from the systemic circulation by the blood-cochlear barrier which is similar to the blood-brain barrier [27,28]. This barrier consists of the lining endothelial cells of the capillary blood vessels connected with tight junctions thus substances in systemic circulation face physical barriers to gain access to inner ear. The cell lining of the stria vascularis are connected with tight junctions and constitutes part of the blood-cochlear barrier [29-31]. Thus, a pharmacological substance administered to the systemic circulation, must pass through the capillary endothelium before it enters the inner ear. SM is also surrounded by cells connected by tight junctions. Chemicals entering SM from the vasculature are supposed to either enter via the stria vascularis, or the perilymph. Drugs with high protein binding are less likely to gain access to the inner ear compartments. Drugs that are positively charged tend not to enter the SM. Drugs with high lipidsolubility cross more readily.

## **1.2 HEARING**

Hearing, an important special sense in human beings, is essential for normal development and essential to conduct many activities. The process of hearing begins with the capture of the sound by the auricle that works as a funnel, collecting the sound waves that reach the outer ear as air conducted pressure alterations. Sound passes through the external auditory canal and elicits a vibratory motion at the tympanic membrane. This motion is transmitted through the ossicular chain to the footplate of the stapes. The stapes footplate serves as a piston that pushes and pulls upon the fluid in the inner ear causing a cyclic increase and decrease in the pressure of the scala vestibuli, eliciting a traveling wave along the basilar membrane, from the base to the apex. The movements of the basilar membrane create a shear force relative to the parallel sheet of

the tectorial membrane resulting in movement and deflection of the stereocilia. As the stereocilia move, ion channels open and shut in synchrony with the stereocilia motion. The endocochlear potential and the high potassium concentration of SM provide a unique environment for the hair cells to transform the mechanical motion into electrical potentials. As a result, the hair cells (IHCs) depolarize and release neurotransmitter to excite afferent auditory neurons to convert the mechanical sound energy into electrical impulses that are propagated along the auditory nerve to the brain. The IHCs have central function in hearing and regarded as the primary sensory receptors. The OHCs are responsible for sensitivity and frequency tuning properties of the hearing organ through their active motor function (cell length changes).

### **1.3 HEARING LOSS**

Hearing loss, the most common sensory impairment in humans, is a major worldwide health problem, affecting people from infancy to old ages. According to the report of World Health Organization in 2006, approximately 280 million people are suffering from hearing impairment [32]. During the pre-lingual period, approximately 1/1000 individuals are affected by severe to profound hearing loss. Pre-lingual hearing loss has a marked effect on speech acquisition, emotional, social and educational development of the children. Later onset of severe hearing impairment seriously compromises the quality of life, as the affected individuals may become increasingly isolated socially. Hearing loss affects one third of adults over the age of 60 and half of the adults above the age of 75 [33,34]. Among the adult population 0.3% manifests a hearing loss greater than 65 dB between the ages of 30-50 and this prevalence is continuously increasing. In developed countries, at least 10% of the population suffers from hearing impairment sufficient to interfere with normal life [35]. Hearing loss is divided into two types, conductive and sensorineural hearing loss (SNHL), depending on where or what part of the auditory system is damaged.

In conductive hearing loss the sound is not conducted efficiently by the external ear and middle ear to the inner ear. This causes a reduced ability to hear faint sounds. Conductive hearing loss often is caused by otitis externa, otitis media, impacted earwax (cerumen), or congenital malformations of the external and middle ear. Conductive hearing loss can usually be corrected either medically or surgically.

SNHL accounts for 90% of all hearing loss cases. This type of hearing loss results from damage at any point between the cochlear hair cells (cochlear) and the auditory cortex in the brain (retrocochlear). The most common affected site is the hair cell. SNHL not only involves a permanently reduced ability to hear faint sounds, but may also involve low speech discrimination. The causes of SNHL include e.g. inflammatory disease, genetic abnormalities, excessive noise, ototoxicity, head injury, infectious diseases, ear infection, tumors and aging (presbycusis). Conductive hearing loss occurs in combination with SNHL, when there are defects in the conductive and the perceptive parts of the ear. The hearing loss is then referred to as mixed hearing loss.

In the human, the hair cells and neurons do not regenerate spontaneously thus restoration of hearing is impossible. Currently, no complete cure exists for SNHL but the treatment have focused on functional improvement such as amplification devices (hearing aids) and devices that stimulate auditory neurons electrically (cochlear implants). These options do not completely restore our ability to hear, but for now, are the best options available.

## **1.4 ROUND WINDOW MEMBRANE (RWM)**

The round window membrane (RWM) closes the round window at the basal end of the cochlea. Despite being called round membrane, it is triangular in shape. RWM is located posteroinferiorly in the medial wall of the middle ear (promontory) within the round window (RW) niche which is created by the promontory bony overhang. In human, the RW niche has a triangular shape with three walls, anterior (1.5 mm), posterior (1.6 mm) and superior (1.3 mm). The average depth of the RW niche is approximately 1.5 mm [36]. To simplify, one can think of the RW niche as a well, with the true RWM being relatively protected from the middle ear at the bottom [37]. In human at the entrance of the RW niche, mucosal folds (false RWM) and fibro-fatty tissues could be present in up to one third of individuals. These could partially or completely cover the RW niche [38]. The false RWM is a three-layers structure but the epithelium on both sides is of the same type [39]. The false RWM and fibro-fatty tissues have not been described in other species. The RW niche in these animals is shallower and almost the entire RWM can be seen [40-42].

### **1.4.1 Electron microscopic features of RWM**

The RWM is thicker at the periphery, thinner towards the central part and has a slight convexity (protrusion) towards the scala tympani [43-45]. The ultrastructure of the RWM consists of three layers: an outer epithelium facing the middle ear, middle layer of connective tissue, and an inner epithelium facing the inner ear [39,43,45-48]. The outer epithelium is of endodermal origin while the middle connective tissue layer and inner epithelium are derived from the mesoderm [41].

The outer epithelium consists of a single layer of epithelial cells, which is continuous with the middle ear mucosa, and basement membrane. The epithelial cells are low cuboidal to flat cells with rounded and centrally located nuclei. The cytoplasm is rich in cellular organelles; mitochondria, Golgi complexes, and rough endoplasmic reticulum [36,44]. Thin and short microvilli are present on the free surface of the epithelial cells, and micropinocytotic vesicles are found both at the apical and the basal sides of the cells. The epithelial cells are connected by interdigitations on their lateral walls and close to the outer cell surface, there are tight junctions but there are no gap junctions between the outer epithelial cells [36,41,43].

The connective tissue layer consists of fibroblasts, collagen fibers, and elastic fibers and it contains blood vessels, lymph vessels and nerve endings [41]. The connective tissue layer is thought to be in conjunction with the mucoperiosteum of otic capsule. There is gradual increase in number of fibroblasts, collagen fibers and elastic fibers towards the inner ear epithelium. Collagen fibers are arranged longitudinally and radially in bundles but there are some fibers irregularly arranged. Blood and lymph vessels traverse from the edges to the center. The blood vessels of the RWM originate from the vascular plexuses of the tympanic mucosa [36,43,45]

The inner epithelial layer is a continuation of the lining of the scala tympani. The epithelial cells are large, flat squamous cells with long lateral extensions which overlaps each other giving the appearance of stratification [45]. The intercellular spaces are wide, and the cell junctions are loose [49]. Sometime the cells appear to be disconnected because of the large spaces. The cells have few organelles and microvilli are absent on the cell surface. The cytoplasm contains small pinocytotic vesicles.



There is no basement membrane between the inner epithelial cells and the connective tissue layer and occasionally pores are seen between inner epithelial cells.

There are no significant differences in thickness of RWM with aging, but there are distinct changes in the ultrastructural characteristics. During infancy, the outer epithelium and the inner epithelium are thick and the connective tissue core is uniformly cellular, with fibroblasts containing large nuclei, irregularly arranged collagen fibers, few elastic fibers; and the ground substance is abundant. With increasing age, the RWM get the described characteristic features. In the elderly, there is an increase in ground substance; elastic fibers become thicker; and thin elastic fibers are absent. [43-45,50].

### **1.4.2 RWM measurements**

In human, the average thickness irrespective of age is about 70  $\mu\text{m}$  ranging from 63-67 $\mu\text{m}$  [45,51] or 40-70  $\mu\text{m}$  [36,43]. The average diameter is  $(2.32 \pm 0.19 \text{ mm})$  with the long diameter being 2.3 mm (1.8-3.2 mm), short diameter 1.9 mm (1.4-2.4 mm). The average surface area has been estimated to be 3.4  $\text{mm}^2$  [36,41].

The RWM of the research animals principally resembles that of the human, but substantial differences in thickness of RWM exist between humans (70  $\mu\text{m}$ ) and other species, being thinnest in rodents. The average thickness of the RWM in small rodents has earlier been reported to be between 10-15  $\mu\text{m}$  in the central part and 70  $\mu\text{m}$  in the periphery [41,51-57], with thickness of 12  $\mu\text{m}$  for rats [58], 10-14  $\mu\text{m}$  for the chinchilla and guinea pigs [54]. In cats, mean thickness of RWM reported to be 20-40  $\mu\text{m}$ ; and in the rhesus monkey, 40-60  $\mu\text{m}$  [44].

### **1.4.3 Function of RWM**

The exact function of the RWM is an area of debate and controversy. It may have several additional functions but its basic function is to act as a release mechanism to permit displacements and fluid pressure changes of the inner ear in conjunction with inward movement of the stapes footplate in the oval window during sound stimulation [44,50].

The RWM is regarded to be the main route for passage of substances from the middle ear cavity into the inner ear, thus it may serve as a barrier to potentially ototoxic substances during infectious processes within the middle ear. [41,44,45,59,60]. It has been also suggested that the RWM act as an alternative way for conducting sound to the cochlea [44,45]. Moreover, animal studies have suggested that the RWM behaves as an active semi-permeable membrane, taking part in secretion into and/or absorption from perilymph [41,46,52]. Experimentally, it is known that the RWM is permeable to different therapeutic agents and biologically active compounds [45,61-63]. Presence of microvilli and organelles in the outer epithelial cells as well as the loose junctions with the long lateral extensions of the inner layer cells suggests that the outer and inner epithelial layers have an absorptive function [45]. Histopathologic changes in the RWM when exposed to injury [51,55] indicate that RWM possibly take part in the middle ear, inner ear [45,64] and RW defense system [44,65].

#### 1.4.4 Permeability and passage of substances across RWM

As the port of entry from the middle ear to the inner ear, the permeability of the RWM under normal and pathological conditions has attracted increasing interest [59]. The passages of different substances through the RWM in experimental animals have proven that the RWM behaves like a semi-permeable membrane. Extensive studies on RWM in guinea pigs [36,45,66,67] chinchillas [49,55,68,69], rhesus monkeys and cats [55,64] and mongolian gerbils [70] have revealed that the RWM is permeable to water [49] antibiotics; especially aminoglycosides, streptomycin, gentamicin and neomycin [49,71], chloramphenicol [72], tetracycline [49], antiseptics, arachidonic acid metabolites [73], bacterial endotoxins and exotoxins [41,74-77], albumin [49,78,79], tracers such as horseradish peroxidase, latex spheres (1- $\mu$ m), and cationic ferritin [44,64,67], local anesthetics [45], glucocorticoids, antioxidants [64] and gases such as oxygen [80].

Based on the animals studies, a number of factors have been described that may affect the permeability and the passage of substances through the RWM [41,44,45,55,59,81]. Some of these factors are related to the RWM such as thickness, presence of false RWM, fibro-fatty tissue plugs [38,39] and inflammation in the middle ear cavity. Other factors are related to the substance or the drug such as size, shape, molecular weight (MW), the molecular configuration, concentration, liposolubility, electrical charge, and contact with the RWM.

The permeability of substance is affected by the thickness of the RWM which can lead to variability in absorption of drugs into scala tympani [49]. Animal studies have shown that the degree of passage of substances is higher in rodents and lower in cats [55].

The severity and duration of the inflammation in the middle ear cavity affects both the permeability of the RWM and its thickness [41,60,68,69,78]. Low MW substances can traverse the RWM under normal conditions [49,50,59,64,81]. Macromolecules that do not pass under normal conditions, may do so during inflammatory process [59,78,82]. At an early phase of otitis media, there is a gradual increase in the permeability of the RWM [36,49,67-69,78] but at later stages, the changes in the membrane become more protective and result in decreased permeability [45,49,51,67-69].

Passage of different substances through the RWM is by different pathways depending on size, structure and electrical charge of the substances. Substances traverse the RWM either directly through the cytoplasm, (exotoxin), or as pinocytosis, (cationic ferritin), or through channels in between cells, (latex sphere) [45,54]. Low MW substances (<1000 MW) such as sodium ions [83], steroids, gentamicin, streptomycin, neomycin, and tetracycline are transported actively through the RWM [49]. While, high MW substances (>1000 MW) such as albumin, ferritin, and endotoxins can only be transported by pinocytosis [54]. Cationic ferritin passes easily through the RWM [64], while the anionic ferritin does not pass in rodents [36,44] and cats [55]. There are substances that can increase the permeability of RWM and passage of substances from middle ear into scala tympani. These include mediators such as histamine [84], prostaglandins and leukotrienes [49], pontocaine [45], endotoxin of *Escherichia coli* and exotoxin of *Staphylococcus aureus* [45,55,78,82,85,86]. On the other hand deposition of inflammatory products on RWM reduces the permeability [44,45]. These findings have suggested that the passage of substances through the RWM is not only a passive transport, but also an active biological passage.

## 1.5 LOCAL INNER EAR THERAPY

The use of hearing aids and, in more severe-profound hearing loss, surgical implantation of cochlear implants are the only treatment options available nowadays for the treatment or rehabilitation of sensorineural hearing loss (SNHL). New insights into the mechanisms underlying hearing impairment, offered the potential for new therapeutic approaches. Advances in the molecular and genetic processes of the auditory system have provided alternative means of biological therapy either as prevention, protection or intervention treatments for the inner ear. Local pharmacological therapy and gene therapy have been suggested as potentially effective and promising approach in the treatment of hearing disorders [87-90].

### 1.5.1 Local cochlear pharmacological therapy

Delivery of drugs to the cochlea is problematic as the cochlea is a small sized, relatively inaccessible and well isolated from surrounding tissues. Systemic drug administration is of limited therapeutic effectiveness and probably not feasible as the limited direct blood supply of the cochlea and the blood-cochlear barrier are likely to restrict transport of drugs from the serum to cellular targets in the inner ear. Moreover, the systemic application of drugs carries the risk of unwanted systemic side effects, at least for potent drugs [91]. Large inter-individual variation in responses to systemic administration, due to variation in dosage and body metabolism, further limits the use of systemic administration [92]. Thus, there is consequently an increasing interest both experimentally and clinically in local treatment of inner ear disorders. Local application of drugs into the inner ear has several key advantages such as the diseased ear is treated directly and higher concentration of drugs can be obtained in the inner ear [85,93-95]. For alternative biological therapies of cochlear diseases be clinically relevant, it is important to develop safe and reliable inner ear delivery methods for the therapeutic agents and to establish sustained and effective therapeutic levels within the cochlear fluids. Inner ear delivery methods can be divided into intratympanic and intracochlear delivery.

#### 1.5.1.1 Intratympanic drug delivery

Treatment of some well-defined inner ear disorders is becoming more dependent on inner ear delivery. As the inner ear is accessible through the round window membrane (RWM), one of the options is to apply the drugs directly to the RWM from which the active substances are expected to diffuse into the cochlear fluids and thereby to the sites of pathological changes [95-98]. This involves perfusion of the middle ear cavity with therapeutic agents through the tympanic membrane [99]. It depends on passage of the therapeutic agents through the RWM into scala tympani [100]. The intratympanic drug delivery started in 1950s for the treatment of Ménière's disease with streptomycin [31] and is still in use for delivery of different agents such as, steroids, aminoglycosides, anti-oxidants, and neurotrophins [101,102]. Experimentally, local application of drugs to the RWM has been used for a wide variety of therapeutic agents, including steroids, anti-oxidants, and neurotrophins. It has been shown that the local application resulted in significantly higher levels of substances in the inner ear fluids compared to systemic applications [91].

The administration of therapeutic agents to the middle ear cavity holds great promise in applications ranging from the treatment of Ménière's disease [103,104] to particular preventive strategies (otoprotection with antioxidants) in relation to noise exposure, intracochlear surgical trauma, head and neck radiation or treatment with an ototoxic drug such as cisplatin and aminoglycoside antibiotics. These are possible areas where local administration of antioxidants can be used to prevent the hearing loss. It also holds promise in interventional prospective in relation to treatment of sudden sensorineural hearing loss SSNHL and autoimmune hearing loss with local administration of steroids because the high systemic doses of steroids needed to reduce inflammation, cause significant side effects. Future application of local regenerative compounds may play a role for regeneration of SGN and hair cells after sensorineural hearing loss.

However, delivery of therapeutic agents in an effective manner to the inner ear is difficult and establishing an efficient and controlled administration route remains a critical issue, as there is large variability in the reported results of the pre-clinical and clinical trials [105]. This variability might result from factors such as middle ear volume, leakage, absorption and clearance of the agent from the middle ear cavity via the Eustachian tube and by the ciliated mucosa of the middle ear, condition of the RWM as well as the availability and contact of the drug with the RWM [12,106]. If the drug is placed in the middle ear, the drug can diffuse through the RWM into the inner ear but it is important that the drug remains in contact with the RWM for a sufficient period of time. Development of a method which deliver a fixed dose and ensures contact with the RWM would be of crucial importance for solving the problems relating to delivering drugs to the inner ears. Efforts to control the variability and overcome the limitations associated with local drug delivery to the inner ear, have had led to the development of passive biodegradable sustained-release vehicles and several active implantable drug delivery devices to locally deliver the therapeutic agents and achieve relatively sustained therapeutic levels in the inner ear.

Biodegradable polymers, hydrogel and nanoparticle delivery systems have emerged as promising topics for delivering substances to the cochlea in a sustained and controllable manner. Drugs may be loaded, dispersed within a matrix or contained within a reservoir encapsulated by a shell of biodegradable carrier vehicles or polymer. The release of the drug is due to either the slow degradation of the material, or drug diffusion or a combination of these. As the polymers are biodegradable, they are either removed by phagocytic cells of the immune system or drained away. The polymers can be tailored through modifications of polymer structure to control the rate of release of drugs from polymers. The vehicle can either be applied directly to the RWM or filling the whole middle ear cavity. The use of vehicles offer important benefits such as extended release of the drugs and extended contacts with the RWM so that the presence of the drug within the middle ear can be prolonged in comparison to simple middle ear perfusions. In this way the sustained diffusion into the cochlea across the RWM will be increased.

Biodegradable polymer system may consist of degradable microparticles or nanoparticles, hydrogels [107], poly lactic co-glycolic acid (PLGA) polymers [108], siloxane [95] biodegradable gelatin polymer, gelfoam, [109], alginate [63] and chitosan [105,110].

Sodium hyaluronate (HYA) enables targeting of tissues through the use of chemical (pH or ionic factors), physical (temperature or electrical potential) triggering

mechanisms which cause the hydrogel to swell and release the loaded substance in a controlled fashion [42,98,107,111-115]. The nanoparticles may be comprised of biodegradable PLGA or non-degradable materials [108,116]. Generally, it seems that the intratympanic delivery methods are most probably depend on the presence of biodegradable vehicles within the middle ear cavity for passive sustained release of drugs into the inner ear [106,117].

Implantable drug delivery systems to locally deliver the drug to the inner include the RW Microcatheter and the Silverstein MicroWick. The  $\mu$ -Cath™ and e-Cath™ microcatheters are double or triple-lumen catheters inserted through the tympanic membrane and positioned near the RWM under general anesthesia, with ability to control both the concentration and flow [7,11,95,118-121].

Silverstein MicroWick™ is a polyvinyl acetate wick (1 mm thick and 9 mm long) that absorbs drugs instilled in the external auditory canal and transports it to the RWM. The wick is inserted through a grommet which passes through the tympanic membrane under local anesthesia [95,118,122,123].

#### *1.5.1.2 Intracochlear drug delivery*

This approach involves a direct delivery into the cochlea, providing a more direct access to sensory cells and neurons. Fluid flow may augment diffusion which could provide access to more apical regions of the cochlea, allowing the drugs to reach their intended targets more directly than with systemic delivery [124]. Intracochlear drug delivery is done by cochleostomy either through the RWM, or direct injection through the otic capsule [31,125,126]. While dosage may be precisely controlled, a greater possibility of surgical trauma exists. Therefore, delicate approaches are required to avoid possible damage from the intracochlear drug delivery approach.

Advances in direct intracochlear infusions include the use of osmotic pump which offers a controlled delivery. Mini-osmotic pumps connected to a micro-cannula makes a continuous delivery to the cochlea possible. An osmotic pressure difference between a compartment within the pumps (salt sleeve) and the tissue environment in which the pump is implanted; cause the pump to work. The delivery rate is controlled by the water permeability of the outer membrane of the pump. The compressed reservoir of the pump cannot be refilled, which makes it single use only. The molecular weight of a compound has no impact on the release rate or function of the pump [127]. Alzet osmotic pumps of different reservoir volumes and flow rates are still in pre-clinical use [128].

### **1.5.2 Cochlear gene therapy**

Gene therapy defined as targeted introduction and regulated expression of a gene that will produce certain effect in the host tissue or cell using a genetically engineered vector to achieve a biological effect. Gene therapy for the human auditory system is a potentially interesting technique for treating both genetic and acquired forms of hearing loss, but its use is still limited due to technical and ethical issues [129,130]. The inner ear constitutes a suitable and unique environment for gene therapy because the cochlea is a relatively isolated compartment, which minimizes unwanted effects of transfer vectors on other tissues. The limited direct blood supply of the cochlea should also reduce the risk of immune responses, and as the cochlea is fluid-filled, all of the functionally important cells are easily reached which facilitates

a wide transfection of the system. Furthermore, different physiological tools are available to assess the auditory function. More than 75 different genes have been identified that affect inner ear development or function, could be applicable for gene therapy for preservation and regeneration of hair cells [90,131]. Among these genes is the mammalian atonal homolog 1 (Atoh1, previously known as Math1), which is necessary for hair cell differentiation [132-134]. By re-expressing this gene, supporting cells may change cellular phenotype into a hair cell phenotype. The GJB2 is a widely expressed gene in the inner ear encoding for a gap junction protein, connexin 26 (cx26). This is essential for potassium homeostasis in the cochlea. A mutation in the GJB2 gene is a common cause of hearing loss by preventing potassium ions from being recycled by the hair cells back to the endolymph leading to potassium intoxication and apoptosis of hair cells [19,20]. Cochlear gene therapy could become useful for protection of the hearing against ototoxicity, noise exposure, and aging as it can be used for delivery of drugs and growth factors to the inner ear, to provide overexpression of antioxidants genes [133-137].

#### *1.5.2.1 Transfer vectors for cochlear gene therapy*

Generally there are two types of gene transfer vectors; viral and non-viral vectors. In vast majority of the experiments on gene delivery into the inner ear, viral vectors are used. The viral vectors are replication-defective viruses which have specific characteristics that make them useful in specific experimental and therapeutic application. For example adenovirus has the ability to infect dividing and non-dividing cells [138-140], adeno-associated virus with ability to infect non-dividing cells [139-142], retrovirus with ability to infect dividing cells only, herpes simplex virus [138,143-146], vaccinia virus [144] and lentivirus which integrates into chromosome of both dividing and non-dividing cells [135]. Viral vectors have superior gene delivery efficacy but safety concern have arisen. Thus the clinical application of gene therapy is somewhat limited by the potentially adverse effects of the virus itself, such as immunogenicity, toxicity and possible mutagenesis of the transfected cells [89,136]. Thus, using non-viral vector technology would be a safer alternative. Non-viral technology includes not only physical vehicles such as liposomes but also approaches using gene gun technology and electroporation. The advantages of non-viral vector are their ability to form a stable complex with DNA, large gene capacity, and the easiness to prepare large amounts. Liposomes can be mixed with DNA for transfection of numerous cell types, and introduced into the cochlea via injection [61,147]. Liposomes have very low risk of causing an immune response and insertional mutagenesis. But the disadvantages are inefficient gene transfer and low rate of transfection [88,135,136]. Nanoparticles have been investigated as an alternative to viral vector systems, but carry concerns about safety as well as precision of targeting of specific cells and tissues [148]. Chitosans form polyelectrolyte complexes with DNA and have been successfully used as a non-viral gene delivery system both *in vitro* and *in vivo* [149-152].

#### *1.5.2.2 Delivery routes for cochlear gene therapy*

An efficient delivery system is the corner stone for successful clinical application of gene therapy for treatment of hearing disorders. For a possible clinical use, it is not enough to identify vectors capable of transfecting inner ear cells but it is essential to identify a reliable access route to the inner ear. Since most of the vectors are not able to

diffuse through the RWM [31,61,147], most efforts in experimental delivery of gene have focused on techniques of delivery to the basal turn of the cochlea through a cochleostomy, or by direct injection through the RWM. Higher expression in cochlear tissues was achieved via the intracochlear approach when compared to the RWM diffusion approach [153]. The intracochlear delivery methods have the advantages of a controlled steady cochlear perfusion, but have the disadvantage of potentially damaging the architecture of the cochlea. Infusion directly into the scala media resulted in efficient expression of the transgene in hair cells, but also death of hair cells around the site of inoculation [154].

Therefore before moving towards clinical applications it is important to establish a method able to provide a uniform distribution throughout the cochlea, selectively deliver therapeutic genes to a sufficient number of target cells, not cause any damage, preserves cochlear integrity and ensures continued expression of the therapeutic gene over an extended period of time [89,90]. These features are crucial in a successful application of gene therapy of the inner ear.

## **1.6 BIODEGRADABLE VEHICLES USED IN THE THESIS**

### **1.6.1 Sodium hyaluronate (HYA)**

Sodium hyaluronate (HYA) is one of the major components of the extracellular matrix in skin, cartilage, synovial fluid and the vitreous humor of the eye. HYA is present in the pars flaccida, RWM, annular ligament of oval window, and muscles of middle ear [155]. It is a naturally occurring non-sulphated glycosaminoglycan polysaccharide consisting of alternating N-acetyl-D-glucosamine and monosaccharides D-glucuronic residues [156].

HYA, molecular weight ( $5 \times 10^4 - 8 \times 10^6$ ), is a biocompatible and biodegradable substance that remains at site of injection over extended period of time [156,157]. Experimentally it has been shown that most of the injected HYA into the middle ear cavities is eliminated after 28 days [158]. HYA degrades by enzymes called hyaluronidases to biocompatible materials. In humans, there are at least seven types of hyaluronidase enzymes [157]. HYA is fluid enough to be injected through a fine gauge needle into the middle ear. The viscosity of the HYA ensures that HYA remain in contact with the mucous membrane for an extended period of time. Then the gel produce a rapid burst effect and disintegrate rapidly. The combination of these properties together with the known viscoelasticity of HYA facilitates the use of HYA in the biomedical field for controlled drug delivery to the inner ear [113]. Intratympanic application of dexamethasone with HYA in guinea pigs prolonged the contact with RWM and consequently inner ear exposure to dexamethasone [159]. HYA has been used also as a drug carrier in several clinical trials for intratympanic dexamethasone injection in patients with Ménière's disease and sudden sensorineural hearing loss, SSNHL [160]. The HYA/drug preparation may provide a superior dosage form for disorders of the inner ears as HYA controls the release of the dissolved or dispersed drug. The loaded substances can be soluble or not soluble in water; they can be of low molecular weight or a polymeric substance. These properties of HYA had led us to study the effect of HYA on the passage of drugs through the intact RWM (Paper I) and on the morphology of the RWM and (Paper IV).

## 1.6.2 Chitosans

Chitosan, a glycerophosphate hydrogel, (1–4) 2-amino-2-deoxy- $\beta$ -D glucan, has structural characteristics similar to glycosaminoglycans. Chitosans are prepared by alkaline N-deacetylation of chitin, which is the main component of the exoskeleton of crustaceans. Chitosan can be degraded and metabolized by certain human enzymes, especially by lysozyme, and other glycosidases, including certain bacterial enzymes produced by intestinal flora [161,162]. The intrinsic properties of a particular chitosan, the fraction of acetylated units and the molecular weight have been shown to provide a large impact on the physicochemical and biological activities of the polymer [163,164]. Intrinsic characteristics of chitosan together with its safety profile as well as a range of possibilities for further modification [165,166] make it attractive for a variety of biomedical and pharmaceutical applications, including the drug and non-viral gene transfer delivery [164,167,168]. By being polycationic polymer, chitosans can interact and adhere with negatively charged mucosal surfaces and macromolecules [164]. This feature facilitates close contact of the therapeutic agent and sustained interaction between the mucosal surfaces and the loaded drug in chitosan [105,169,170]. It has been also reported that chitosans can open tight junctions [149,164,170]. Chitosans have been successfully used as a non-viral gene delivery system both *in vitro* and *in vivo* [149-152]. The trisaccharide substituted chitosan oligomers possess higher gene transfer efficacy compared with unsubstituted oligomers [152,171]. Combination of AAM substitution and branching of the backbone of the chitosan allow for the preparation of sterically stabilized DNA nanoparticles. The self-branched glycosylated trisaccharide-substituted chitosan oligomers (SB-TCO) is fully soluble at a neutral pH and forms physically stable DNA nanoparticles without impairing the intracellular release of DNA [152].

These interesting properties of chitosan prompt us to study the efficiency of chitosan to deliver biologically active agents into the inner ear as a delivery carrier for the inner ear therapy (Papers II and III) and its effect on the morphology of the RWM (Paper IV).



## **2 AIMS**

The objectives of this thesis were to investigate the efficiency of different delivery carriers loaded with biologically active agents applied to the cochlea with the ultimate aim to establish an efficient and reliable topical drug and gene delivery method to the inner ear through using an appropriate carrier vehicle that could be loaded with different drugs, and used both for preventive approach against the threat of injury and effective treatment of hearing loss and other inner ear disorders.

### **Paper I:**

1- To evaluate the effect of sodium hyaluronate (HYA) as drug delivering vehicle for the inner ear, and to investigate to what extent HYA loaded neomycin as a biomarker would diffuse into the inner ear and cause hair cell loss.

2- To determine the safety of intratympanic administration of sodium hyaluronate (HYA) to the middle ear by studying the effect of HYA on the cochlear hair cells and RWM.

### **Paper II:**

1- To explore the efficiency of three structurally different types of biodegradable chitosan as a delivery carrier for the inner ear.

2- To outline the suitability of intratympanic administration of chitosans into the middle ear by studying the effect of chitosans the cochlear hair cells and RWM.

### **Paper III:**

To evaluate whether expression of a reporter gene can be induced in cochlear cells using chitosan as a carrier both *in vitro* and *in vivo*.

### **Paper IV:**

To describe the structure of the guinea pig RWM and to evaluate the changes that may occur after application of sodium hyaluronate (HYA), chitosans and saline.

## 3 MATERIALS AND METHODS

### 3.1 EXPERIMENTAL ANIMALS AND TISSUE PREPARATIONS

#### 3.1.1 Experimental animals

A total of 141 healthy adult albino guinea pigs of both sexes (body weight 250-350 g; Lidköpings Kaninfarm, Lidköping, Sweden) and twenty postnatal day 2 Sprague Dawley rat pups (Harland, Netherland) were used in papers included in the thesis. Albino guinea pigs were used in the *in vivo* experiments (Papers I-IV) and the postnatal day 2 rat pups were used in the *in vitro* experiments (Paper III). The guinea pigs were free from middle ear infections as judged by otomicroscopic inspections. They were housed at animal department at the Karolinska University Hospital- Solna. They had free access to food and water and housed in temperature-controlled rooms with a 12/12 hours light/dark cycle. The animal suffering was minimized in all experiments and all animal experiments and procedures were performed in accordance with the ethical standards of Karolinska Institutet and consistent with Swedish national regulations for the care and use of animals (ethical approvals N334/05, N347/05, N32/07, N35/07, N342/07 and N13/10).

#### 3.1.2 Experimental design

The total number of animals, groups, types of treatment used and time of decapitation are summarized in table 1.

##### 3.1.2.1 Paper I

The animals (n=65) were treated with a single injection, into the auditory bulla, of either neomycin solution (Neo), or neomycin loaded in 0.5% sodium hyaluronate (HYA/Neo), or 0.5% sodium hyaluronate alone (HYA). The ototoxic drug neomycin was used as biological marker at a concentration of 15%, 5% and 2%. The animals were divided into groups according to the type of treatment and time of collecting the cochleae either after 7 or 28 days.

##### 3.1.2.2 Paper II

Paper II consisted of two experimental series, in the first experimental series, guinea pigs (n=21) were used to test the efficiency of three types of chitosans (Ch1, Ch2, and Ch3) as drug carrier for the inner ear. The animals were divided into 7 groups. The ototoxic drug neomycin was used as biological marker at a concentration of 2%. The chitosans and neomycin 2% were used either alone or mixed. The design of the second series of experiment was based on the results of the previous experiments. Ch3 was chosen for further evaluation of chitosans as a drug carrier into the inner ear. Animals (n=14) were divided into 3 groups and Ch3 was used either alone or when loaded with neomycin, 2% and 5%.

### 3.1.2.3 Paper III

In the *in vitro* experiments, postnatal day 2 rat pups were used for preparation of organotypic culture of organ of Corti. These cultures were transfected with chitosan/pDNA polyplexes and were processed for immunohistochemistry. For the *in vivo* experiments, adult albino guinea pigs (n=41) were divided into 3 groups; chitosan/pDNA polyplexes group (n=15), pDNA group (n=14), and chitosan group (n=12). The left cochleae were infused and right cochleae used as contra-lateral control. The samples (cochleae) were processed either for immunohistochemistry or PCR.

### 3.1.2.4 Paper IV

Right and left cochleae (n=9) of albino guinea pigs used in previous two papers, I and II were processed for structural analysis of the round window membrane (RWM) using transmission electron microscopy. Normal RWM compared with the membranes treated for 7 days with sodium hyaluronate (HYA), Ch1, Ch 2, Ch 3 and saline. RWM treated with HYA for 7 days were compared with RWM treated with HYA for 28 days.

**Table 1 Summary of the groups, total number of animals and time of decapitation.**

PAPERS	GROUPS	GUINEA PIG'S DECAPITATION TIME		
		1 day	7 days	28 days
Paper I	HYA/Neo15%		8	4
	HYA/Neo5%		5	0
	HYA/Neo2%		5	5
	Neo15%		8	0
	Neo5%		5	0
	Neo2%		5	0
	HYA alone		9	5
	Saline		6	0
Paper II	1 <sup>st</sup> part			
	Ch1		3	0
	Ch2		3	0
	Ch3		3	0
	Neo2%		3	0
	Ch1/Neo2%		3	0
	Ch2/Neo2%		3	0
	Ch3/Neo2%		3	0
	2 <sup>nd</sup> part			
	Ch3/Neo5%		6	0
	Ch3/Neo2%		4	0
	Ch3		4	0
Paper III	Chitosan/pDNA	4	11	
	pDNA	4	10	
	Chitosan	4	8	

### 3.1.3 Surgical procedures

Animals were deeply anaesthetized (45-60 minutes) by an intramuscular injection of a mixture of Ketalar (ketamine, 40 mg/kg) and Rompun (xylazine, 5mg/kg). The head, neck and post-auricular area were shaved and the skin of the areas to be incised and over the mid-line of the back (Paper III) was infiltrated s.c. with 0.25% bupivacaine. The surgical area was cleaned thoroughly using 10% povidone-iodine solution and 70% ethanol and draped in sterile fashion. Doxycycline (Doxyferol® 1 mg/kg i.p) was administered as prophylactic antibiotic prior to surgery and on the following day. Lubricant eye ointment was applied to prevent corneal ulcers as the blinking reflex disappears during anesthesia. The animals kept on a heating pad (Kanthal, type 3654M) to maintain a constant core body temperature. At the end of surgery, the animals received 5 ml of body warm saline s.c. to prevent dehydration, and buprenorphine hydrochloride (Temgesic® 0.05mg/kg) s.c. as long acting analgesic agent. A sterile environment was maintained throughout the experiments. The well-being of the animals was kept under control and their weight was carefully monitored for a few days after surgery. At one, seven or 28 days after surgery, the guinea pigs were anesthetized with sodium pentobarbital (90 mg/kg, i.p.) and sacrificed.

#### 3.1.3.1 Middle ear injection procedure (Papers I, II and IV)

Under an operating microscope, a paracentesis (small opening) was made in the tympanic membrane to prevent rupture of the RWM during intratympanic injection as the middle ear cavity is an air filled space that might exert pressure on the RWM during middle ear filling. Then the middle ear cavity was completely filled (0.15 ml) through a fine needle (BD Microlance™ 30G; external diameter 0.3 mm) injected into the auditory bulla through the skin of the auricle, with either sodium hyaluronate (HYA), chitosans or saline. The injections were delivered steadily in order not to cause rupture of the tympanic membrane and RWM as well as damaging adjacent structures. The surgical procedure was performed on both left and right sides. Left ear was first injected and 20 minutes later the right side was injected.

#### 3.1.3.2 Pump insertion procedure (Paper III)

A post-auricular skin incision was made down to periosteum, which was elevated over the bulla then a pocket under the skin on the animals back was made for hosting the pump. Under an operating microscope, muscles covering the temporal bone were separated and the bulla was exposed. Then a hole in the bulla was made to visualize the middle ear cavity, the basal turn of the cochlea, and round window membrane (RWM). A small hole was drilled in the basal turn (cochleostomy) 2 mm above the RWM. The tip of the prefilled cannula (Scientific Commodities Inc.) with either chitosan, pDNA or chitosan/pDNA polyplexes was inserted and secured at the bulla with tissue adhesive (Histoacryl, Aesculap AG, Germany). Dental cement (Dentalon plus Heraeus Kuzler, Inc. NY, USA) was used to seal the hole in the bulla and secure the cannula in place. Miniosmotic pumps (Alzet® mini-osmotic pump, either 1007D; flow rate 0.5µl/h or 2001D; flow rate 8µl/h;) was filled with 100-200 µl (depending on the duration of treatment and model of the pump) of either chitosan, pDNA or chitosan/pDNA polyplexes and connected to the cannula and placed in the pocket under the skin on the back of the animal between the scapulae. Then the skin was closed in layers. Only the left cochleae were infused whereas the right cochleae were used as a control.

### 3.1.4 Tissue preparations

#### 3.1.4.1 *In vivo experiments (Papers I-IV)*

The guinea pigs were deeply anesthetized with an overdose of sodium pentobarbital and decapitated. After decapitation, the temporal bones were removed immediately, trimmed of excess bone and tissue. The cochleae were dissected out and depending on the subsequent procedures the cochleae were either dissected fresh or fixed before dissection. For cochlear hair cell counting and immunohistochemistry, the left cochleae were immersed in 4% paraformaldehyde. Under the microscope, a small opening was made in the apex, and the round window membrane (RWM) was perforated to allow the fixative to reach the entire organ of Corti. One hour later the samples were transferred to 0.5% paraformaldehyde and stored at 4°C until further processing. For the immunohistochemistry, the samples were then decalcified in EDTA solution (0.1 M ethylenediaminetetraacetate) made in 0.1 M phosphate buffer for 2 weeks or until the bony capsules were soft enough for the subsequent processing.

For morphological evaluation of the RWM with light and transmission electron microscopy, the right cochleae immersed and fixed immediately after dissection in the 2.5% gluteraldehyde overnight at 4°C. Local perfusion through the RWM step was omitted.

For the PCR analysis, the temporal bones (n=26 samples) were opened and immersed in RNA later solution (Qiagen; GmbH, Hilden, Germany) for further dissection. The RWM and apical turn were fenestrated. Soft parts of the cochlea including the organ of Corti, lateral wall and modiolus were isolated under the microscope and transferred to a tube containing 1ml of ice-cold TRIzol (Invitrogen, Carlsbad, CA, USA) and homogenized thoroughly with a pestle, and samples were stored overnight at -20°C.

#### 3.1.4.2 *In vitro experiments: Organotypic cultures of organ of Corti (Paper III)*

Rat pups were sacrificed and the cochleae were carefully removed from the skull under microscope (Zeiss). The cochleae were then placed in phosphate-buffered saline (PBS) supplemented with 5.5 µl/ ml of 30% glucose. The stria vascularis, spiral ligament and spiral ganglion were carefully pulled away from the organ of Corti. Then 4-5 explants per group were transferred to culture inserts (Millicell- CM 0.4 µM 30 mm diameter; Millipore) placed in 6-well plates and cultured in 750 µl DMEM, (Gibco, Invitrogen) supplemented with 10 µl/ ml of N1 supplement (Sigma); 5.5 µl/ ml of 30% glucose (Sigma) and 100 units/ml penicillin (Gibco, Invitrogen). After 24 hours of incubation at 37°C and 5% CO<sub>2</sub>, the cultures were exposed to chitosan/pDNA polyplexes (750 µl) for 24 or 48 hours. Following 24 hours incubation with chitosan/pDNA polyplexes, the media was replaced by 750 µl of fresh culture medium in the 24 hours exposure group and were further incubated for another 24 hours but in the 48 hours exposure group the cultures were left all 48 hours with chitosan/pDNA polyplexes. The cultures were grouped as follows: (1) cultures exposed to chitosan/pDNA polyplexes for 24 hours, and (2) cultures exposed to chitosan/pDNA polyplexes for 48 hours. Control cultures, containing only fresh culture media, were run concurrently with the experimental cultures. The green fluorescent protein (GFP) expression was measured by recording of fluorescence intensity using fluorescence microscopy. The cultures were rinsed in PBS and fixed for 1 hour in 4% paraformaldehyde while still on the culture membranes. The culture explants were rinsed in PBS, removed from the membranes and processed for immunohistochemistry.

## **3.2 HISTOLOGICAL ANALYSIS**

### **3.2.1 Cochlear hair cell loss counting (Papers I and II)**

The organ of Corti was incubated with phalloidin-TRITC (Sigma-Aldrich) in order to stain the filamentous actin and identify the hair cells. After rinsing in PBS, the organ of Corti was dissected into approximately 3-mm long pieces and placed on an 8-well slide. The hair cell loss was counted using a fluorescence microscope (Zeiss Axioplan). The disappearance of stereociliary bundles and scars formation allowed for the appropriate quantification of missing hair cells. The percentage of hair cell loss for each 0.25-mm segment of the cochlea was calculated.

### **3.2.2 Light and electron microscopy (Papers I, II and IV)**

The right cochleae were rinsed several times with phosphate buffer (PB); osmicated in 1% osmium tetroxide, and after several rinses with PB, dehydrated with increasing concentrations of ethanol. The cochleae were then transferred to propylene oxide, and embedded in Agar 100 (Agar 100 Resin kit, Agar Scientific Limited, Essex, England). The cochleae were oriented and hardened at 40°C for 24 hours then at 60°C for 48 hours. After polymerization, the area of the round window niche with the RWM was cut out and re-embedded on a blank block of Agar 100. The specimens were sectioned at 2.5- $\mu$ m thickness with an ultratome (LKB Ultramicrotome system 2128 Ultratome). The sections were mounted on glass slides, stained with toluidine blue and examined under a light microscope (Zeiss). Ultra-thin sections (1- $\mu$ m) were obtained for transmission electron microscopy (TEM) with an ultratome (LKB Cryo Nova), mounted on copper grids and stained with alcoholic uranyl acetate and lead citrate and then examined with a transmission electron microscope (JEOL 1230) and photographed. Images were imported into Adobe Photoshop CS3, and the standard methods were used to optimize contrast and brightness.

### **3.2.3 Immunohistochemistry (Papers I and III)**

In the *in vivo* experiment, decalcified cochleae were washed with 0.1 M PBS, cryoprotected in a 10% and 30% sucrose solution (each for 24 hours) at 4°C, embedded in OCT (Tissue-Tek, CA, USA), frozen in super cooled isopentane and stored in the freezer. The samples were sectioned (14  $\mu$ m) using a cryostat (Leica CM 3050S) and mounted onto SuperFrost glass slides (Thermo Scientific). The sections were re-hydrated in PBS and permeabilized with 0.2% Triton X-100 for 10 min. The sections were incubated in PBS containing 10% normal goat serum, 5% bovine serum albumin (BSA) and 0.3% Triton-X, for one hour at room temperature. The sections were then incubated over night with primary antibodies at 4°C (table 2) and after washes with PBS; the sections were incubated with secondary antibody for one hour. Finally, sections were washed in PBS before mounting in water soluble mounting media Mowiol (Calbiochem) and cover slipped. The sections were examined using fluorescent microscopy (Zeiss).

In the *in vitro* experiment (Paper III), organotypic cultures of organ of Corti were blocked for 1 hour with 10% normal goat serum and 5% BSA with 0.3% Triton X-100 to block unspecific binding sites, permeabilized with 0.3% Triton X-100 in PBS for 10-20 min at room temperature. Afterwards cultures were incubated overnight with primary antibodies at 4°C, table 2. On the following day, cultures were washed and incubated with secondary antibodies. Finally the cultures were stained with DAPI and mounted in Mowiol (Calbiochem). The cultures were examined using fluorescent microscopy (Zeiss).

**Table 2 Primary and secondary antibodies used in the papers I and III.**

Antibody	Host	Concentration	Producer	Paper
Primary Anti CD45	Rat	1:100, 500, 1000, 2000	Chemicon	I
Primary Anti PKC $\beta$ II	Rabbit	1:100, 500, 1000, 2000	Sigma-Aldrich	I
Primary Anti myosinVIIa	Rabbit	1:1000	Proteus Bioscience	III
Primary Anti GFP	Goat	1:500	Abcam	III
Primary Anti GFP	Chicken	1:1000	Chemicon	III
2ndry FITC anti rat IgG	Goat	1:1000	Sigma-Aldrich	I
2ndry TRITC anti rabbit IgG	Goat	1:1000	Sigma-Aldrich	I
2ndry Alexa 488 anti goat	Donkey	1:400	Molecular Probes	III
2ndry Cy3 anti rabbit	Goat	1:400	Jackson ImmunoResearch	III
2ndry FITC anti chicken IgY	Goat	1:200	Aveslab	III

### 3.3 MOLECULAR BIOLOGY TECHNIQUES

#### 3.3.1 Total RNA isolation (Paper III)

The Total RNA was isolated using TRIzol reagent and RNeasy® MinElute Cleanup kit (Qiagen). To eliminate possible DNA contamination, samples were subjected to on-column DNase digestion with DNase I (Qiagen). Final RNA concentration was measured using Invitrogen Qubit.

#### 3.3.2 RT-PCR analysis (Paper III)

Due to its convenience and high reproducibility, one-step RT-PCR was performed on all samples using SuperScript III One-Step Invitrogen kit. The contents of a 10- $\mu$ l RT-PCR reaction were 5  $\mu$ l of 2X Reaction mixture buffer, 1 $\mu$ l template RNA, 0.4  $\mu$ l of sense and anti-sense primers (10  $\mu$ M), 2.8  $\mu$ l RNase free water and 0.4  $\mu$ l of SuperScriptIII RT/ Plat TaqMix. Optimal annealing temperature and PCR cycle number for the primers were determined with a gradient thermocycler (PTC-200, MJ research). The reverse transcription (cDNA) synthesis was performed in one cycle of 50°C for 30min. The PCR amplification consisted of 30 cycles of 15 seconds denaturation at 94°C, 30 seconds at 59°C annealing temperature and one minute of extension at 68°C. A final single extension cycle of 68°C for 5 minutes was added at the end. No-RT controls were run for each sample by replacing the RT/ TaqMix with a DNA polymerase (Invitrogen). RT-PCR products were run on 2% agarose gels containing ethidium bromide (0.5 $\mu$ g/ml) and visualized in ultraviolet light. A One-Step RT-PCR with GAPDH specific primers was performed to verify RNA integrity. The cDNA synthesis was performed in one cycle of 55°C for 30 min. The PCR amplification consisted of 33 cycles at of 15 seconds denaturation at 94°C, 30 seconds at 58°C annealing temperature and one minute of extension at 68°C. A final single extension cycle of 68°C for 5 minutes was added at the end. The content in the reaction tubes was as described above. The primers used were designed using online free software Primer3 [172] and were as follows:

GFP forward primer (GCCCGAAGGTTATGTACAGC) and reverse primer (GTCCCAGAATGTTGCCATCT).

GAPDH forward primer (GCCAACATCAAGTGGGGTGATG) and reverse primer (GTCTTCTGGGTGGCAGTGATG).

## 3.4 MATERIALS

### 3.4.1 Neomycin (Papers I and II)

The ototoxic drug neomycin trisulfate salt hydrate (Sigma-Aldrich) was used as an indicator for the RWM passage.

### 3.4.2 Sodium hyaluronate, HYA (Papers I and IV)

Sodium hyaluronate, HYA 0.5% was a kind gift from Advanced Medical Optics, Uppsala, Sweden.

#### 3.4.2.1 Preparation of HYA/neomycin formulation (Paper I)

Three concentrations of neomycin were used with 0.5% HYA. A stock solution of 1% (w/w) HYA was prepared in PBS and was then autoclaved. Parallel to this, a 30% (w/w) neomycin stock solution was prepared. This solution was not autoclaved, as the heat would degrade the neomycin. However, as neomycin is antibacterial in itself bacterial growth was efficiently prevented. The final mixtures were then composed from 50% HYA solution together with 50%, 16.67% or 6.67% neomycin stock solution to give final neomycin concentrations of 150 mg/ml, 50 mg/ml, and 20 mg/ml, respectively. The samples were adjusted to final volume with PBS. Additionally, a 0.5% reference HYA solution was prepared from 50% HYA stock solution and 50% PBS. Preparation of the neomycin solutions was performed in a similar manner. A 30% neomycin stock solution was prepared, which was then used for proportional mixing with PBS to yield neomycin solutions at final concentrations of 15%, 5%, and 2%.

### 3.4.3 Chitosans (Papers II and IV)

In paper II and IV we used three different types of chitosans and in paper III a self-branched and trisaccharide-substituted chitosan oligomer (SB-TCO) was used (table 3). Chitosan 1 (Ch1) was a completely deacetylated polymer with high charge density and low molecular weight. Chitosan 2 (Ch2) represents a conventional polymer with middle molecular weight. Chitosan 3 (Ch3) was an oligomeric glycosylated chitosan which was completely soluble at neutral pH. All chitosans were prepared by heterogeneous de-N-acetylation of chitin and converted to hydrochloride (HCl) salts. Ch1 was prepared by nitrous acid depolymerization of fully de-N-acetylated chitosan. Ch2 was a direct product of the de-N-acetylation. The glycosylated Ch3 and SB-TCO were prepared by controlled nitrous acid depolymerization of fully de-N-acetylated chitosan followed by self branching and substitution with the trimer 2 acetamido-2-deoxy-D-glucopyranosyl- $\beta$ -(1-4) 2-acetamido-2-deoxy-D-glucopyranosyl- $\beta$ -(1-4)-2,5-anhydro-D-mannofuranose (A-A-M) as described previously [152]. All samples were dissolved in Milli-Q water (5-7 mg/ml) and filtered through 0.22  $\mu$ m syringe filter (Millipore). The column used was TSK 3000, and sample was eluted with 0.2 M ammonium acetate (pH 4.5) at a flow rate of 0.5 ml/min. The molecular weight (Mw) and molecular weight distribution (Mn) were analyzed by Size-Exclusion Chromatography with refractive index (RI) and a Multi-Angle Laser Light Scattering detector (SEC-MALLS). The degree of substitution of A-A-M was determined by nuclear magnetic resonance spectroscopy <sup>1</sup>H NMR.



**Table 3 Characteristics of the chitosans used in the papers II, III and IV.**

Sample	F <sub>A</sub> <sup>a</sup>	M <sub>n</sub> <sup>b</sup>	M <sub>w</sub> <sup>c</sup>	PDI <sup>d</sup>	d.s. A-A-M <sup>e</sup>
Chitosan 1 (Ch1)	<0.002	17,600	32,900	1.87	0
Chitosan 2 (Ch2)	0.15	64,000	148,000	2.31	0
Chitosan 3 (Ch3)	<0.002	8600	11,800	1.37	7.3
SB-TCO	<0.002	13 700	22 000	1.61	7.3

(a) Fraction of acetylated units. (b) Molecular weight. (c) Molecular weight distribution. (d) Polydispersity index. (e) Degree of substitution.

#### 3.4.3.1 Preparation of chitosan/neomycin formulation (Paper II)

Chitosan-HCl salts were dissolved in Milli-Q grade water and the ionic strength was adjusted to 0.15 M by addition of NaCl. The pH of the chitosan solution (5 mg/ml) was carefully adjusted to 6.0 and the solutions were filtered through 0.22- $\mu$ m filter. Four percent and 10% w/v stock neomycin solutions were prepared in 20 mM N-morpholino-ethanesulfonic acid (MES, gma M3058) buffer pH 6.2, supplemented by 270 mM mannitol to retain osmolarity. Both stock solutions were filtered through a 0.22- $\mu$ m filter (Millipore). The final formulations were then prepared by mixing equal amounts of chitosan solutions and neomycin stock solutions (4 and 10%) so that the final concentration of chitosan was 2.5 mg/ml and the concentrations of loaded neomycin were 20 mg/ml (2%) and 50 mg/ml (5%).

#### 3.4.3.2 Preparation of chitosan/DNA polyplexes (Paper III)

Polyplexes of chitosan with amino/phosphate (A/P) ratios of 30:1 and pDNA were prepared by the self-assembly method. For the *in vitro* experiments, the polyplexes were prepared as described above with a pDNA concentration of 13.3  $\mu$ g/ml. The polyplexes were further diluted 1:2 in OptiMEM I (Gibco, Invitrogen) supplemented with 270 mM mannitol and 20 mM HEPES to adjust the osmolarity and pH of the formulation. For the *in vivo* experiments, an aliquot of 0.264 ml of pDNA (0.5 mg/ml) was diluted in deionized water (MQ-Millipore) and a required amount of sterile filtered chitosan stock solution in MQ-grade water (4 mg/ml) was added during intense stirring on a vortex mixer (1200 rpm, Heidolph REAX 2000, Kebo Lab, Sweden), yielding a pDNA concentration of 110  $\mu$ g/ml. The size of chitosan/pDNA polyplexes, expressed as mean hydrodynamic diameter (z-average), was  $128 \pm 7$  nm as determined by dynamic light scattering on a Nanosizer ZS (Malvern Instr., Malvern, UK).

#### 3.4.4 Plasmid DNA (Paper III)

Reporter plasmid (gWiz<sup>TM</sup>GFP) containing a cytomegalovirus promoter (CMV) and green fluorescent protein (GFP) was purchased from Aldevron, Fargo, ND, USA.

#### 3.4.5 Mini-osmotic pumps (Paper III)

Alzet® mini-osmotic pump 1007D; flow rate 0.5  $\mu$ l/h; release duration of one week and pump 2001D; flow rate 8  $\mu$ l/h; release duration of one day (ALZET Osmotic Pumps, Cupertino, CA and Scanbur AB, Stockholm-Sweden) were used.

#### 3.4.6 Micro-cannula (Paper III)

A 7.5 cm vinyl tubing (Sscientific Commodities Inc., AZ, USA) was used to make handmade cannula. A 7 mm piece of polyimide tubing (MicroLumen, FL, USA), was

placed 1-2 mm into the end of the vinyl tubing. The two component silicone glue (MDX 4-4210, Dow Corning Corp., MI, USA) ten parts base and one part of curing agent were mixed thoroughly. With a fine probe the silicone was put at the opening and allowed to flow into the vinyl tube. Approximately 3.75 mm of the polyamide protruded out of vinyl tube. On the part outside the polyamide tubing, a silicone ball was placed leaving a 0.5 mm tip on the micro-cannula to be inserted to the scala tympani. This silicone ball is important to close the cochleostomy and prevent leakage of perilymph. Then the cannulae were allowed to dry overnight and their patency was checked. Prior to use, the cannulae sterilized by soaking overnight in 70% ethanol [128,173].

### **3.4.7 Statistical analysis**

Statistical analysis was made with the software SigmaStat 9.0 for Windows. The data were analyzed with the Wilcoxon paired 2-sample test, Mann-Whitney U test, and Kruskal Wallis variance. Descriptive statistics representing mean and standard deviation have been calculated for the hair cell loss, and the RWM thickness. The level of significant statistical difference was set at  $p < 0.05$ .

## **4 RESULTS**

### **4.1 COCHLEAR HAIR CELL LOSS (PAPERS I AND II)**

The ototoxic drug neomycin, well known to cause rapid degeneration of the cochlear hair cells, was used as a biological trace marker for RWM passage. It was expected that once released from the loading carriers (sodium hyaluronate and chitosans) and passed across the RWM, the neomycin would cause loss of outer hair cells (OHCs) and inner hair cells (IHCs). It was assumed that the amount of neomycin that passed through the RWM was correlated to the resulting loss of hair cells i.e. the hair cell loss was dependent on the concentration of neomycin released and the amount that diffused across the RWM. Sodium hyaluronate (HYA) was loaded with three different concentrations of neomycin, 2%, 5% and 15% and three structurally different chitosans were loaded with neomycin 2% and 5% in order to observe a dynamic range of effect throughout the length of cochlea. As illustrated by the ototoxic effect on the cochlear hair cells, neomycin was readily delivered from the HYA and chitosans, and transported into the cochlea. In contrast, the HYA and chitosans themselves caused no damage to the hair cells.

#### **4.1.1 Hair cell loss following HYA/neomycin exposure**

Loss of OHCs in all three rows as well as IHC loss was seen 7 days after treatment, even at the lowest concentration of neomycin tested (see Fig.1 and 2 in Paper I). In general, for comparable dose groups there were small differences in the toxic effect. However, the differences in hair cell loss among the basal regions of Neo15% and HYA/Neo15% groups and apical regions of Neo2% and HYA/Neo2% groups were statistically significant ( $p < 0.05$ ).

#### **4.1.2 Hair cell loss following chitosans/neomycin exposure**

In the first series of experiments, neomycin at a 2% concentration was loaded in three different chitosan formulations. Although the differences between the three types of chitosan in releasing neomycin were not statistically significant ( $P \geq 0.05$ ), Ch3 seemed slightly more efficient in releasing neomycin (see Fig.1 in Paper II). To facilitate comparisons, only data from the counts of the second row OHC 2 were shown. In the groups treated with neomycin 2% whether alone or when loaded in chitosans, the hair cell loss was seen in the basal region sparing the apical regions. In contrast, the chitosans alone had no damaging effect on the hair cells.

The glycosylated chitosan, Ch3 loaded with Neo2% and Neo5% in order to see if an increase in the neomycin concentration would produce more hair cell loss along the length of organ of Corti, i.e. to increase the dynamic range of the model. The differences in cochlear hair cells loss between the treatment groups Ch3/Neo5% and Ch3 (see Fig. 4 in Paper II) were statistically significant ( $P \leq 0.05$ ). In the Ch3/Neo5% group there was more cochlear hair cells loss along the length of organ of Corti than the Ch3/Neo2% group; however, the differences were not statistically significant ( $P \geq 0.05$ ).

## **4.2 RWM THICKNESS (PAPERS I AND II)**

To study the effects of HYA and chitosans with neomycin on the RWM, the exposed tissue was sectioned and the RWM thickness was measured. The thickness of the RWM in normal, unexposed control ears were close to 10  $\mu\text{m}$  (see Fig. 5 in Paper I).

### **4.2.1 RWM thickness following HYA/neomycin exposure**

There was a swelling of the RWM at day 7 after administration in all groups receiving either neomycin solution or HYA with neomycin (see Fig. 5 and 6 in Paper I). In the HYA/Neo15% group the thickness of the RWM had increased significantly to (25.2  $\mu\text{m} \pm 11.7$ ). At 28 days the thickness returned to normal (13.3  $\mu\text{m} \pm 1.9$ ). In the HYA/Neo2% group the RWM increased to (16.1  $\mu\text{m} \pm 2.7$ ) but, again the thickness was normalized at day 28 (12.1  $\mu\text{m} \pm 0.5$ ). In the Neo15% group at 7 days post treatment, the thickness of the RWM had increased to (16.6  $\mu\text{m} \pm 0.1$ ). Interestingly, also when the HYA alone was administered, the thickness of the RWM showed a significant increase to (19.7  $\mu\text{m} \pm 4.5$ ). At day 28, the thickness of the RWM had returned to normal values (11.8  $\mu\text{m} \pm 0.9$ ).

#### *4.2.1.1 Inflammatory marker*

At the light microscopic level there was no sign of any inflammatory processes in any of the animals. To further investigate whether the surgical procedures and the HYA administration caused any signs of inflammatory processes, sections from the RWM of two guinea pigs receiving the HYA alone were processed for immunohistochemistry, table 2. However, no positive immunostaining signals were detected from the exposed (HYA) and the non-exposed RWM.

### **4.2.2 RWM thickness following chitosans/neomycin exposure**

At day 7 after administering the neomycin solution, chitosan/neomycin, and interestingly also the free chitosans, the RWM showed an increase in thickness (see Fig. 2 and 5 in Paper II). The Ch2/Neo2% group (24.2  $\mu\text{m} \pm 4.9$ ) and Ch2 group (23.2  $\mu\text{m} \pm 5.1$ ) were associated with the greatest increase in RWM thickness. The difference between the treatment groups Ch2/Neo2% and Ch3/Neo2% (17.4  $\mu\text{m} \pm 2.3$ ) was statistically significant ( $P \leq 0.05$ ). The difference between the Ch3/Neo5% (15.9  $\mu\text{m} \pm 1.5$ ) and Ch3/Neo2% (17.4  $\mu\text{m} \pm 2.3$ ) group was not significant.

### **4.2.3 RWM thickness following saline exposure**

These membranes showed a slight thickening, (12.0  $\mu\text{m} \pm 0.6$ ) when compared with unexposed RWM (see Fig. 5 in Paper I).

## **4.3 RWM MORPHOLOGY (PAPER IV)**

To study the effects of HYA and chitosans (Ch1-3) on the ultrastructure of the RWM, nine cochleae were prepared for transmission electron microscopy (TEM). Sections of non-exposed membranes, membranes exposed to HYA for 7 and 28 days, membranes exposed to Ch1-3 and saline for 7 days were analyzed with TEM.

### **4.3.1 Normal RWM morphology**

The RWM consists of three layers (see Fig 2 in Paper IV): an outer epithelium, connective tissue, and an inner epithelium.

*Outer Epithelium* is a single layer of epithelial cells. The epithelial cells have rounded nuclei located centrally within the cytoplasm. On the free surfaces of the cells there are short microvilli. The cytoplasm contains mitochondria and Golgi apparatus. On their lateral walls, the epithelial cells are interdigitating with each other and there are tight junctions.

*Connective Tissue layer* constitutes bulk of the RWM. Collagen fibers together with fibroblasts, elastic fibers and ground substance make up the connective tissue core. It contains blood vessels which run from the edges to the center beneath the epithelial layer.

*Inner Epithelium* consists of a single layer of epithelial cells with long lateral extensions. The cells junctions are loose and the space between the cells is wide. Unlike the outer epithelial cells, the nuclei are elongated; the cytoplasms contain pinocytotic vesicles and few organelles, and have no microvilli.

### **4.3.2 RWM morphology following HYA and chitosan exposure**

At day 7 after middle ear injection of HYA, chitosans and saline, the membranes were thickened. HYA, Ch1 and Ch3 had comparable effects on the thickness of the RWM while Ch2 induced greater thickening. RWM exposed to saline showed a slight increase in RWM thickness (see Fig. 3 in Paper IV). The thickening was confined to the connective tissue layer due to edema which resulted in widening of the extracellular spaces.

In the HYA and chitosans exposed RWM, the epithelial cells of the outer epithelial layer became larger in size and swollen. Their nuclei appeared swollen and the cytoplasm showed numerous intracellular vacuoles and enlarged intercellular spaces. Twenty-eight days after RWM administration, the RWM started to regain its normal characteristic structure and thickness. However, the epithelial cells looked larger than the normal ones and the connective tissue layer contained more collagen fibers and elastic fibers that were more pronounced close to the inner layer (see Fig. 4 in Paper IV). As an interesting finding, the membrane exposed to HYA for 28 days, showed opening up of the tight junction between the epithelial cells (see Fig. 5 in Paper IV).

#### **4.4 TRANSFECTION OF THE INNER EAR (PAPER III)**

The feasibility of chitosan mediated gene delivery tested both *in vitro* and *in vivo* and the samples were processed for immunohistochemistry and PCR.

##### **4.4.1 *In vitro* results**

Organotypic cultures of organ of Corti, prepared from postnatal day 2 rats, were incubated with chitosan/pDNA polyplexes for 24 or 48 hours (see Fig. 2 in Paper III). Immunostaining of the cultures revealed GFP expression in cultures treated with chitosan/pDNA polyplexes which was not observed in control cultures. The cells were not transfected equally and expression was observed in some regions of the cultures. Additionally, more transfected cells were observed in the cultures incubated for 48 hours compared to cultures incubated for 24 hours.

##### **4.4.2 *In vivo* results**

We tested the feasibility of chitosan mediated gene delivery in the guinea pig following cochlear infusion using osmotic pumps. The spiral ganglion and organ of Corti were examined for signs of GFP expression in treated samples and compared with corresponding areas in control samples with and without using primary antibodies. There were no differences between sections treated with chitosan/pDNA polyplexes, pDNA, and chitosan alone (see Fig. 3 in Paper III).

Several runs of RT-PCR were performed on the samples treated with chitosan /pDNA polyplexes, pDNA and chitosan alone (see Fig. 4 in Paper III). The results showed varying degree of GFP expression as four out of 9 samples treated with the chitosan/pDNA polyplexes showed positive transfection. The sample that showed the most intense band originated from an animal receiving chitosan/pDNA polyplexes for 7 days (see Fig.4 A, lane 6 in Paper III). However, the overall transfection pattern was inconsistent, as the replicates in the chitosan/pDNA polyplexes groups and pDNA groups for either one or seven days showed varying degree of transfection.

## 5 DISCUSSION

One of the clinically most interesting and relatively non-invasive potential pathways for the inner ear drug administration is the round window membrane (RWM). The RWM is surgically accessible and offers, at least theoretically where the drug (loaded in a polymer or a gel) applied to the RWM, direct access to the perilymphatic compartment of the cochlea and thus to the cells of the hearing organ without damaging the cochlea [174]. Intratympanic drug application is a method of introducing substances into the inner ear without damaging the cochlea by perfusion of the middle ear cavity with therapeutic agents through the tympanic membrane [99]. The intratympanic drug application depends on passage of the therapeutic agents through the RWM into scala tympani. The RWM approach for drug delivery is known to lead to much higher intracochlear concentrations than systemic drug delivery [91] hence promoting optimal outcomes in the cochlea while avoiding side effects in other organs. A variety of compounds of interest have been demonstrated to diffuse across the RWM in animals, for example antibiotics, dexamethasone, liposomal and adenoviral gene transfer vectors, and neurotrophins [1-4,45,61-63,98,175]. Since it was started for treatment of Meniere's disease with streptomycin by Schuknecht in 1956 [31], intratympanic pharmacotherapy is still in use for delivery of gentamicin for the treatment of Ménière's disease and steroids for the treatment of sudden sensorineural hearing loss and tinnitus [7-11]. In the future, cochlear gene therapy and stem cell transplants will likely require delivery of drugs and growth factors into the inner ear [135-137].

To test the suitability of the RWM as a site for inner ear drug delivery, we used two different types of drug vehicles, sodium hyaluronate (HYA) and chitosans. The vehicles were loaded with the ototoxic drug neomycin as a pharmacological “tracer” in guinea pigs model to show whether the drug carrier can release the drug and cause cochlear hair cell loss and how the vehicles affect the morphology of the RWM. The pattern of ototoxicity in the guinea pig resembles that in humans [176]. The use of a slow-releasing vehicle injected into to the middle ear cavity is considered as a promising candidate for clinical pharmacological therapy of inner ear disorders. This type of drug administration may be especially important in searching for strategies to prevent hearing loss in patients receiving systemic treatment with a highly ototoxic drug. The advantages in such cases are to avoid drug interaction at the systemic level. In addition to pharmacological substances, it is of interest to explore the use of gene transfection for inner ear treatment. Again, for a possible clinical use, RWM administration seems most feasible.

The literature shows few studies comparing an i.v. route for drug administration with intratympanic administration. However, paper I and II were the first studies comparing two different modes of local administration (fluid-based and vehicle-based) to the inner ear and reflections concerning differences between the two modes of topical administration. In paper I and II, the ototoxic effects of neomycin loaded in HYA and chitosans were quantified as the percentage of hair cell loss along the length of the cochlea and compared with that induced by free neomycin solution. In both types of topical administration, neomycin exerted varying damage to the cochlear hair cells with greatest hair cell loss in the basal regions of the organ of Corti. Neomycin was successfully released from both HYA and chitosans which then diffused into the inner

ear as evidenced by loss of hair cell in a degree depending on the concentrations of neomycin loaded in the HYA and chitosans.

Main limitation of intratympanic injection is that many variables exist that may alter the amount of the drug that comes into contact with RWM and concentration in the perilymph. This mode of entry depends on simple diffusion to pass drug from the middle ear cavity to the inner ear, and once in the scala tympani, the only mode of distribution is simple diffusion in a fluid space. Therefore, access to the apical regions of the scala tympani can be limited. RWM drug delivery often leads to a severe base-apex concentration gradient, making it difficult to predict the amount of drug that will be available for cells in different turns of cochlea. The assumption that drugs applied to the RWM may have access to all turns of the cochlea may be valid for some rodents [63,177-180] but is not likely to be true for the human. The membrane is a major absorption site but blood vessels, lymphatics and annular ligament of the oval window also play a role. Beside the presence of fibro-fatty tissue in the RW niche which gives variation in the rate of absorption. Uncontrolled duration of the contact with RWM and clearance of the drug from middle ear either by drainage through Eustachian tube or by absorption through the middle ear mucosa need to be considered when extrapolating results from preclinical studies for planning clinical studies with intratympanic drug delivery to the inner ear.

## **5.1 COCHLEAR HAIR CELL LOSS (PAPERS I AND II)**

In paper I we tested the effect of three different concentrations of neomycin in a sodium hyaluronate (HYA) vehicle versus neomycin (see Fig. 1 and 2 in Paper I). Controls consisted of HYA alone or saline. Our aim was to evaluate HYA as a carrier to aid in drug delivery to the inner ear. Seven days after middle ear injection, cochlear hair cell loss was seen in a dose-dependent way, strongly indicating that neomycin was released from the HYA and transported to the inner ear and thereby inducing loss of cochlear hair cells. It is speculated that HYA can modulate the RWM permeability [181] but our findings (Paper I) provides no indication that this occurs to a significant degree and we have not found the support for a change of RWM permeability induced by HYA. Similarly, in paper II three different types of chitosans (see. Fig. 1 and 4 in Paper II) were loaded with neomycin, the data showed that neomycin applied with or without chitosans caused very similar effects and gave no indication that drug entered perilymph more easily with chitosans present. One question is whether treatment of the RWM with HYA and chitosan increases permeability or not. Neomycin applied with HYA and chitosans was not delivered more easily to the inner ear than neomycin in solution. There was neither a difference in total hair cell loss nor a difference in the apical versus basal regions for the same neomycin concentration. However, the main points are that HYA and chitosans indeed cause no detrimental effects and that they do release the test drug. Hair cell loss was as an endpoint for toxic effect of neomycin on the inner ear. We used the ototoxic drug neomycin as a “bio-indicator” for drug release to show whether the drug carrier can release the drug to pass through the RWM and cause cochlear hair cell loss. The effect of the tracer drug per se was not one of objectives to assess as it is well known that neomycin is a potent ototoxic drug once in the inner ear fluids. One cannot exclude that the situation may be different with other compounds. The HYA and chitosans themselves exerted no toxic effect on hair cells.



In animals treated with Neo15% and HYA/Neo15%, the ototoxic effect was severe and seen throughout the whole length of the organ of Corti (see Fig. 1 in Paper I). At the highest concentration of neomycin tested (15% in paper I) the ototoxic effect was slightly greater in the neomycin solution group as compared to the HYA group. At this dose level the variability of hair cell loss was greatest in the HYA group.

Provided that there was a correlation between hair cell loss and amount of neomycin reaching the inner ear compartments, this finding reflects that the solution vehicle was more efficient to transport neomycin across the RWM at a very high concentration of neomycin. In contrast, by lowering the dose of neomycin no enhanced ototoxic effect was seen in the solution groups. In guinea pigs, the bone of the cochlear apex is thinner than that in the basal turn [182]. Thus, the high apical concentrations might be consistent with the substance entering the perilymph through the thin bony otic capsule. As the bone surrounding the human cochlea is much thicker; it is expected that high drug levels exist in the basal turn and very low levels in apical regions [182]. However, it is also possible that drug permeability through the bone may be changed in pathologic lesions affecting the otic capsule, such as otosclerosis [183].

By lowering the concentration of neomycin no enhanced ototoxic effect was seen in the apical region. Neomycin destroys the OHC as well as IHC and the supporting cells of the organ of Corti in a dose dependent manner [176]. The toxicity may be a dose dependent phenomenon in the inner ear with higher levels resulting in profound hair cell loss. HYA/Neo5%, HYA/Neo2%, Ch1/Neo2%, Ch2/Neo2%, Ch3/Neo2%, Ch3/Neo5%, Neo5% and Neo2% produced a stepladder effect with a greater loss of OHCs in the basal turn of the cochlea compared to the apical parts.

Despite different properties, the three tested chitosans showed comparable effects on the delivery of neomycin (see Fig. 1 and 4 in Paper II). The differences in cochlear hair cells loss, between the treatment groups Ch3/Neo5% and Ch3 were statistically significant. In the group received Ch3/Neo5%, there were more cochlear hair cells loss along the length of organ of Corti than the group received Ch3/Neo2%, showing that increased neomycin concentration, changed the dynamic range of ototoxic effect of neomycin. However, the differences in cochlear hair cells loss between Ch3/Neo5% and Ch3/Neo2% groups were not statistically significant. The difference between loss of OHCs in the basal parts and the apical parts, suggests a gradient for neomycin along the cochlear compartments after RWM administration. This demonstrates that substances entering the perilymph only move slowly along the perilymphatic spaces and a higher absorption rate would present the basal turn of the cochlea with a higher level of ototoxic drug. Longitudinal perilymph flow has a marked influence on the amount of a substance reaching the second turn [184]. It cannot be excluded that the pharmacokinetics of two types of topical administration might differ and thereby the induction of cell death pathways. A number of studies investigating the entry, distribution and quantification of tracers and drugs into the inner ear have determined that a substantial concentration gradient is established with highest concentrations at the basal turn and low concentrations at the apex in a way the concentration near the RWM remained higher than that of apical turns. This has obviously important implications for the clinical use of a RWM approach for drug delivery. [100,184-189]. Noushi and co-workers suggest suitability of the RWM as a delivery site for the hair cells and SGN protection despite the concentration gradients [63].

## 5.2 RWM THICKNESS (PAPERS I AND II)

The importance of the RWM in drug delivery into the inner ear led us to investigate the RWM thickness after application of sodium hyaluronate (HYA) and chitosans. The thickness of the midportion of the RWM was calculated 7 or 28 (Paper I only) days after topical administration. All groups showed an increase in RWM thickness (see Fig. 3-5 in Paper I and Fig. 2, 3 and 5 in Paper II). Even saline 0.9%, HYA and chitosan without any loaded neomycin caused an increase in the thickness of the RWM. The change in thickness was mostly due to edema in the middle connective tissue layer. The thickness of RWM exposed to both the HYA and chitosan loading neomycin, was found to be depending on the concentration of neomycin and duration of the follow-up period.

Greatest effects on the thickness occurred in the HYA/Neo15% group, increasing from normal 10  $\mu\text{m}$  to 25  $\mu\text{m}$  and at 28 days the RWM thickness decreased to 13 $\mu\text{m}$  (see Fig.6 in Paper I). One might question if the RWM react to any solution or vehicle instilled into the middle ear. The question arises because the swelling of the RWM appears related to the injection of "anything" into the middle ear. A single injection of HYA alone and chitosans alone caused the RWM to swell. The thickening of the RWM caused by the HYA alone and HYA/Neo, disappeared within 28 days so it was concluded that HYA causes no permanent structural damage to the RWM and it is a safe vehicle for drugs aimed to pass into the inner ear through the RWM. This was further supported by the apparent lack of inflammatory processes. This finding is in accordance with a number of earlier studies showing that HYA instilled into the middle ear do not produce signs of cochlear toxicity [181,190-193]. Biodegradability of HYA depends on the viscosity and concentration of the HYA used. Engström et al. have experimentally shown that most of the injected HYA into the middle ear cavity will be eliminated after 28 days [158].

In paper II, all tested chitosans caused the RWM to swell but Ch2 had the greatest effect (see Fig. 2 and 3 in Paper II). Compared to Ch3, Ch1 and Ch2 possessed higher molecular weights and lower solubility at physiological conditions which may have delayed the degradation. The saline 0.9% exposed membranes showed a non-significant thickening compared with the unexposed control membranes. There are earlier studies showing that even an injection of saline induces a swelling of the RWM when unexposed and saline exposed membranes were compared. Any local application initiates a certain swelling of the RWM and as the middle ear is normally an air-filled cavity, exposure to saline or any other vehicle is unnatural [58,65]. HYA is not permeable through the RWM and therefore most likely acts through an osmotic effect on the perilymph [194]. There is possibility that some uncontrolled aspect of the injected solution might have influenced the RWM thickness. So we cannot fully exclude the possibility that an ingredient in the HYA and chitosans could influence on RWM thickness or cause a difference in thickness as an osmotic effect but exploring this issue further was beyond the scope of this thesis. The implication of swelling to the passage of drugs is not clear. It could be speculated that the swelling, despite making the membrane thicker, actually makes the tissue more loose and permeable thus facilitating drug entry into the inner ear fluids.

### 5.3 RWM MORPHOLOGY (PAPER IV)

In paper IV we wanted to investigate how the carriers influence the structural components of the RWM in guinea pigs using transmission electron microscopy after instillation of sodium hyaluronate (HYA), three different chitosans and saline into the middle ear cavity. In general, the RWM of the human and other species are similar in that they are three-layered membrane [41,195]. But the RWM of the rodents is much thinner than RWM in the human [41,44,55]. Our findings (see Fig. 2 in Paper IV) have confirmed the normal structure of the RWM described in the literatures [43-45]. The presence of microvilli is interesting in that they increase the surface area of the RWM and should promote drug uptake. Toward the inner cell layer, the number of fibroblasts, collagen fibers and elastic fiber increase [51,60]. It may be assumed that the area of connective tissue layer close to the inner epithelia layer could be associated with the perilymph wave and thus involve elasticity. The high density of elastic fibers toward the inner epithelial layer suggested that they may participate in the barrier function of the RWM [60]. The discontinuity in the inner cell layer suggests that the perilymph may communicate directly with the connective tissue layer [44,45,60].

The HYA, chitosan, and saline-exposed membranes were thickened when compared with non-exposed membranes (see Fig.3 in Paper IV) but there were no signs of pathological and inflammatory process which are associated with exposure of RWM to a notorious agent [58,85]. The difference in the HYA, chitosans and saline exposed RWM thickness was due to the increase in the connective tissue layer. Changes were about the same following single exposures of HYA and Ch1 and Ch3, except for membranes exposed to Ch2 which had the greatest effect on the RWM thickness. The high molecular weight of Ch2 might have role in this effect. Although it is reported that chitosans can open the tight junctions between the epithelial cells [149,164,170] we could not observe such finding in the chitosans treated RWM. This finding was seen in the membrane exposed to HYA for 28 days as an odd finding (see Fig.5 in Paper IV).

The saline exposed RWM slightly increased in the thickness if compared to normal membrane. This might be due to effect of the physical manipulation of the RWM and/or osmotic effect of the ionic composition of the saline.

The important finding was that the increase in the thickness of the RWM exposed to HYA after 7 days restored to normal level after 28 days (see Fig. 4 in Paper IV). This finding implies that the HYA caused no permanent changes in the structure of the RWM. Since the HYA and chitosans have comparable effect on the RWM, it is expected that the thickness of the RWM exposed to chitosans also return to normal values after 28 days of exposure. Earlier reports suggested that no histologic cochlear damages and no permanent structural damage to the RWM were seen after administration of high concentrations of HYA and chitosan into the middle ear [105,181,193,194,196-199]. In the early stages of an experimentally induced serous otitis media in guinea pigs, Nomura and Sahni and co-workers have reported features of inflammatory reaction in the RWM such as edema of the connective layer with dissociated collagen fibers, elastic fibers and fibroblasts. In the later stages of the inflammation there was more edema in such way that the collagen fibers, elastic fibers, and fibroblasts were existed independently and it was not possible to identify different cell organelles. Twenty eight days after application of HYA, the collagen fibers, elastic fibers and fibroblasts did not show the pathological changes seen after inflammatory reaction in the RWM [36,51]. However, since it has been reported earlier

[44,45,59,60,200] that during inflammatory conditions of the middle ear cavity and manipulations of the RWM initially there will be an increase in RWM permeability due to increase in its thickness, later thickening and scarring might affect the permeability of the RWM, this is an important point to investigate since it affects the passage of drug in the subsequent drug applications. Thus further studies should investigate the impact of the structural changes of RWM on the passage of drug loaded in the HYA or chitosans.

#### **5.4 CHITOSAN AS A GENE CARRIER INTO THE COCHLEA (PAPER III)**

In paper III, we investigated the efficiency of chitosan as a vehicle for gene delivery into the cells of the inner ear both *in vitro* (see Fig. 2 in Paper III) and *in vivo* (see Fig. 3 in Paper III). In the *in vitro* experiments, both the IHCs and the OHCs displayed expression confirming successful pDNA delivery *in vitro*. However, not all cells were transfected equally and they showed different GFP intensity in cultures incubated with the transfection medium for 48 hours compared to cultures exposed for 24 hours. In the 24- hour group, the chitosan/pDNA polyplexes was replaced by fresh culture medium after 24 hours and the tissue was further incubated for another 24 hours whereas in the 48-hour exposure group the cultures were incubated with the chitosan/pDNA polyplexes for the entire time period. Thus the growing time was the same in all cultures (48 hours). The results suggest that the prolonged incubation time led to increased cellular uptake of chitosan/pDNA polyplexes. However, it is possible that with the prolonged incubation time (48 hours) more time had elapsed for protein translation leading to increased expression.

The *in vivo* transfection in the inner ear did not show good correlation with the *in vitro* transfection. The immunostaining, showed no specific expression of GFP in sections treated with chitosan/pDNA polyplexes, indicating no transfection of GFP.

With RT-PCR analyses, the results showed varying degree of GFP expression in replicates of same treatment group. The absence of a band in some samples most probably was due to absence of transfection as all samples were tested for RNA integrity. Despite inconsistent results of the *in vivo* experiments, the results suggest that the chitosan/pDNA polyplexes are capable of transfecting cell in the cochlea and organ of Corti *in vitro* and to some extent *in vivo*.

The low correlation of the *in vivo* experiments with those performed *in vitro* could be partially due to direct application of the chitosan/pDNA polyplexes in the transfection medium to the cultures in the *in vitro* experiments. The differences in transfection between *in vivo* and *in vitro* experiments might also be due to the exposure sites (scala tympani in the *in vivo* experiments and the perilymphatic and endolymphatic spaces in the *in vitro* experiments) and the administered amount of chitosan/pDNA polyplexes. The pDNA concentrations were 13.3 $\mu$ g/ml and 110 $\mu$ g/ml in the *in vitro* and *in vivo* experiments respectively. The transfection media were 750  $\mu$ l in the *in vitro* experiments and 100-200  $\mu$ l in the *in vivo* experiments depending on the type of the osmotic pumps used. Preparation of higher concentration of DNA nanoparticles would have resulted in an immediate aggregation. Another possible cause for the inconsistent transfection might be the aggregation of nanoparticles *in vivo*. It is very typical for all cationic nanoparticles to aggregate partially at physiological condition, and these aggregate are too large to be taken up. This could further reduce the amount of available pDNA

The absence of transfection of the GFP in the inner ear *in vivo* might be related to some of the factors which have been previously described by (Borchard, 2001) that hinder the transfection such as ability of the delivery means of targeting the target cells, ability to cross the cell membrane and entering the nucleus [201]. The route of gene delivery to the cochlea is complicated by its structural complexity, isolated endolymphatic and perilymphatic compartments, and sensitivity of the hair cells to trauma [202]. The variability of the results; no expression with the immunostaining and inconsistent expression levels with PCR analyses may be due to the pDNA not reaching the target cells facing the endolymphatic fluid within scala media. Tan and co-worker suggested that there is no dissemination of transfecting agent from the perilymphatic to the endolymphatic fluid space especially when using cationic polymer-DNA complexes [203]. Contact between the transfecting agent and regions of transfection are important to get successful transfection. Since most of the vectors are not able to diffuse through the RWM [31,61,147] most efforts in experimental delivery of gene have focused on techniques of delivery to the basal turn of the cochlea with the disadvantage of potentially damaging the cochlea. Liposomes and some viral vectors can diffuse across the RWM to the perilymph although there is no control over the rate of the delivery and similar to the delivery of other therapeutic agents to the inner ear, diffusion of vectors through the RWM shows a gradient of transfection [61], but for a possible clinical use, RWM application seems most feasible and viable route of cochlear gene delivery in humans as it is an easy technique, less invasive and less destructive.

## **5.5 DELIVERY VEHICLES: HYA AND CHITOSANS (PAPERS I-IV)**

In papers I-IV we used sodium hyaluronate (HYA) and chitosans as biodegradable vehicles. The method of delivering HYA and chitosans was through a transbullar injection using a fine needle to fill the middle ear cavity (Papers I and II). By this route of administration we assume that the mixture would be in direct contact with the RWM. It was also our intention to test this topical administration which has the advantage that the middle ear is kept intact and not injured by any surgery. With the intratympanic drug application in experimental animals filling the auditory bulla with drug solution [63,177-180,204] drugs do not come into contact with the RWM only. The drugs might be absorbed through alternative routes into the inner ear including the annular ligament of the oval window, blood and lymph vessels [84,93] thin bone [182] or evacuated from the middle ear space by the Eustachian tube[105]. Therefore, the concentration of drug in the inner ear is most probably related to the amount of drug coming into contact with the RWM. Contact between the RWM and the vehicle carrier is essential.

Searching for inner ear drug delivery strategies to control the variability associated with intratympanic infusions led to the development of sustained release vehicles to maintain therapeutic levels of drug on the RWM for a longer time [11,205-207]. Use of a biodegradable carrier vehicle is one of the modifications which offer benefits such as extended release of the drugs and extended contacts with the RWM so that the presence of the drug within the middle ear can be prolonged in comparison to simple middle ear perfusions of solutions [106,117]. This in turn can overcome some of the variability associated with perfusion into the middle ear cavity. When a drug is dissolved or dispersed in the biocompatible polymer, its diffusion is substantially slower than when in solution and this contributes to the delivering properties of the vehicle. In this way the sustained diffusion into the cochlea across the RWM will be

increased and achieve a passive sustained therapeutic agent delivery to the cochlea [177,208]. Both the HYA and chitosans displayed no damaging effect on the cochlear tissue and were thus considered safe. An ideal biodegradable biomaterial should in order to be useful for topical delivery system, degrade in a controlled manner and disappear from the site of implant. It should neither cause morphological alterations nor elicit any inflammatory reactions [209].

While delivery of a vehicle can prolong the stay of the drug within the middle ear cavity, it does not guarantee control of the rate and duration of drug delivery, or direct contact between the drug vehicle and the RWM. In Paper I and II, the left side was first injected and 20 minutes later the right side was injected. The left cochleae were used to estimate the hair cell loss whereas the right cochleae were used for RWM morphology. Thus, the same side was always used for comparisons within the same “experiment”. The first ear operated on would probably have a shorter exposure time to the drug especially for the less viscous preparation. Also the average length of anaesthesia time could have influence on the length of time the liquid neomycin had contact with the RWM. The average length of anesthesia time with i.m. ketamine and xylazine was 45-60 minutes. However the surgical procedure on both sides was finished within 10-15 minutes in addition to the 20 minutes waiting time after injecting the first side. A possible cause of variability is the contact between the drug and the RWM and this is a major problem in case of intratympanic injection [42,106]. Due to the increased adhesion time of the HYA and chitosans in the middle ear cavity one can expect a higher concentration of neomycin transported into the inner ear. However, the HYA and chitosans loaded with neomycin seemed not offer any prolonged drug release.

We used in paper II three different chitosans that gives important insights in the field of inner ear drug delivery including *in vivo* data. Due to the bio-adhesive properties of chitosan, it was hypothesized that injected chitosans to fill the middle ear by a single transbullar injection, will form an adhesive layer in the middle ear cavity and on the RWM. Possibly, adhered chitosan may affect the tight junctions present in the epithelial cell layer of RWM thus increasing the permeability of the RWM. Furthermore, drug loaded in the chitosan solution may remain longer in contact with RWM due to biological properties of chitosan which can promote increased contact and duration of exposure with the RWM and eliminate thus some of the variability. Seven days after application, the chitosan Ch1 and especially Ch2 were clearly seen macroscopically as a gel layer wrapping the cochlea and surrounding structures. In contrast, no depositions were observed in the cochleae treated with Ch3. Compared to Ch3, Ch1 and Ch2 possessed higher molecular weights and lower solubility at physiological conditions which may have delayed the degradation. These differences indicate that Ch1 and Ch2 would probably stay for a longer period of time within the middle ear cavity. Chitosan degradation could be delayed after administration without eliciting any inflammatory reactions or gross pathological changes or inducement of adhesions in the middle ear of mice [105].

## 5.6 VARIABILITY OF THE OUTCOMES

There was an individual variation in terms of the effect on the cochlear hair cells and thickness of RWM. A number of different factors may contribute to the variability such as variations in RWM membrane permeability, attachment or contact of the vehicle and solution to the RWM as well as an inter-animal variation in susceptibility to the ototoxic effect of neomycin. If air bubbles formed on the RWM, it cannot be guaranteed that drugs will enter the cochlea at all [101]

The delivery of drug across the RWM into the cochlea is dependent also on the composition of the vehicle solution. Presence of histamine, prostaglandins, leukotrienes and bacterial toxins can increase the permeability of RWM and subsequently the passage of substances from middle ear into the inner ear. On other hand deposition of inflammatory product on RWM reduces the permeability [44,45,49,55,78,82,84-86].

The RWM permeability may vary between individual animals within the same species [210]. Additional variability arises due to variability of RWM permeability between species. One can speculate that permeability of the RWM varies depending on whether there are inflammatory conditions in the middle ear and the degree, stage and severity of these conditions. These initially increase permeability; subsequently result in thickening and scarring so that the membrane become more protective [36,44,45,49,60,67-69,78] which decrease the permeability for the subsequent trials of inner ear drug delivery. Identification of a carrying agent, which decreases inter-animal or interspecies variability, would have significant clinical application.

Variability may be a result of differences in RWM thickness [51]. The application of a drug and its vehicle changes the morphology of the RWM [58] and this will possibly affect the permeability of the RWM to drug application later on. Some anatomic variations of the round window niche could explain some of the variability of the results of the clinical trials for inner ear disorders [38,42,211]. In contrast to the human temporal bone, false RWM membrane and tissues covering the RM niche have not been described or reported to exist in other species specially the guinea pigs. The guinea pig RW niche is shallow, making almost the entire membrane visible and uncovered [40-42].

Once a drug enters the perilymph; its transport is most probably affected by some variables. However, the distribution of a drug from the perilymph to deeper compartments is not fully known. In addition to diffusion along the length of the cochlea, transport through the tissue of the cochlea from one scala to another must be considered as well.

Clearance of the drug from the middle ear cavity is an important issue that is relevant and important for the field of local drug delivery to the cochlea. Several mechanisms are involved in the clearance of fluids from the middle ear; pumping action through the Eustachian tube, contractions of the tensor veli palatini muscle, mucociliary system mechanism [212] and osmotic outflow of water from the middle ear as a consequence of osmotic pressure gradients [213]. Pumping clearance and mucociliary mechanism are suppressed by presence of large volume of fluid in the middle ear cavity [212] and this was the case in papers I and II where the middle ear cavity was completely filled with the vehicles solution. The muscle pumping mechanism of the tensor veli palatini muscle is prevented during anesthesia, which will leads to occlusion of the Eustachian tube [214].

## 6 CONCLUSIONS

The following conclusions were drawn based on the main findings of the thesis:

- 1- The main finding in paper I is that sodium hyaluronate (HYA) is a safe vehicle for medications aimed to pass into the inner ear through the RWM. No damaging effects on the cochlear hair cells were shown after local administration of HYA. However, the HYA did not provide any benefit by increasing RWM permeability to the drug.
- 2- The use of chitosan as carrier is promising and all the three types of chitosans were able to carry and release neomycin, which was then transported into the inner ear. Chitosans per se were free from any detectable noxious effect on the cochlear tissue. However they caused the RWM to swell and the chitosans did not provide any benefit by increasing RWM permeability to the drug. The chitosans, especially the glycosylated derivative (Ch3 and SB-TCO) are safe and effective carriers for drug and gene delivery through RWM into the cochlea.
- 3- The middle and inner ears are not two isolated cavities but that they interact as a combined unit. Thus, the RWM can be seen as a door to the inner ear. However, it is not necessarily the only route for passage of substances from the middle to the inner ear.
- 4- The morphological characteristics of the RWM can be changed under the influence of carriers and drugs. After exposure to HYA, chitosans and even saline, all the layers of the RWM were affected and thickened. The major changes were limited to the middle connective tissue layer. HYA and chitosans cause no permanent structural damage to the RWM and the RWM retained its normal thickness after 28 days exposure to HYA.
- 5- Despite the inconsistent results, the chitosan/pDNA polyplexes are capable of transfecting cell in the cochlea and organ of Corti *in vitro* and to some extent *in vivo*. With continued research into this field, gene therapy directed towards hair cells and SGN may become a clinical reality.



## 7 ACKNOWLEDGEMENTS

On the completion of this thesis, I thank first and foremost with all sincerity the Most Gracious and the Most Merciful God (**ALLAH**).

The present work was carried out at the Center for Hearing and Communication research, Department of Clinical Neuroscience, Karolinska Institute and Karolinska University Hospital in Stockholm during the years 2006-2010. I would like to express my deepest and sincere gratitude to all who have helped me, gave me their support during PhD time and who have contributed to my work with this thesis in different ways, and who I worked, interacted and collaborated with. Without their support, understanding and help, this work would not have been completed. In particular I would like to give my thanks to:

**Professor Mats Ulfendahl**, my supervisor for providing me the opportunity to be a PhD student in your lab and work on such an attracting research project, for leading me into the auditory research field, for being encouraging and sharing both your extensive scientific knowledge and limited time with me, for supporting me to attend those exciting ARO conferences. Thank you for making the thesis possible, and having faith in me. Being the director of the hearing center, your way of thinking, non-bossing type of attitude, generosity and unexhausted energy deeply impressed me, so thank you for creating such an excellent unique environment in the lab. Without yours always friendly and patient leadership, thoughtfulness, generosity and trust in me I would not been able to accomplish this work, so thanks for everything.

**Professor Göran Laurell**, my co-supervisor for your optimism and endless support, for being an outstanding scientist, for introducing me to inner ear local therapy and giving me new aspects in my research work from time to time.

**Associate Professor MaoLi Duan**, my co-supervisor, for your encouragement and support to my work, for your advice whenever needed.

**Associate Professor Beston Nore**, my external mentor, for introducing me into the field of research, for recommending me to study in Sweden, for coordinating the exchange program, for your enthusiastic and valuable advices on my daily life, future career and for keeping an eye on the progress of my work. Your encouragement had great effect on the completion of this work. I will remain grateful for your continuous encouragement and support.

**Associate Professor Anders Fridberger**, for sharing extensive knowledge of neuroscience, hearing physiology and hair cells, for support and advice whenever needed.

**Professor Allen Counter**, for your innovative, joyful, wonderful humor, and for always believing in me.

My co-authors: **Sabina P. Strand**, for sharing tremendous knowledge about chitosan and for extensive contribution to the manuscripts; **Katarina Edsman** and **Tobias Bramer**, for good scientific discussions and successful collaboration in the sodium hyaluronate project; **Mette Kirkegaard**, for valuable instructions, comments and discussion about PCR and immunohistochemistry; **Paula Manström**, for excellent smooth co-operation from tissue embedding to TEM imaging, for skilled technical support, for helping me whenever and wherever needed in the lab and for always on time ordering of lab stuff; **Suvarna Dash-Wagh**, for friendly attitude and great help with the *in vitro* experiments; **Cecilia Engmér**, for teaching me the intratympanic injection; and **Cecilia Gont**, for the help with the PCR analysis.

My roommates **Shaden, Stefan Cecilia Johanson, Sara and Ekaterina**, and former roommates **Igor, Miriam, Åsa, Yen Fu and Palash**, thanks for being there, for the friendly attitude, for all the nice discussions and for all the nice time we have passed as roommates.

**Anette Fransson**, for teaching the histological techniques and animal surgery, for always on time ordering of animals, and of course for creating a well organized atmosphere in the lab.

**Louise von Essen**, for administrative assistance and for organizing the cake lists!

All present and former colleagues at the Center for Hearing and Communication Research and ENT lab especially **Anna, Eric, Beata, Yu (Oliver), Pierre, Henrik, Andreas, Masato, Ali, Per, Martin, Zhe, Sri, Futoshi, Sachi, Schuichi, Poki and Aleksandra** for being always helpful, for a friendly environment, and for interesting scientific discussions. Thanks also to the ones I have forgotten to mention their names here.

**College of Medicine, Hawler Medical University-Iraq**, for continuous support and for the scholarship.

My uncle **Khalid**, for hosting me when arrived in Sweden and for endless support.

My ever supporting and understanding family: My parents, my brother, my sisters and my beloved wife, who constantly and without end encouraged and supported me during the past years. Thanks for helping me out with everything one can imagine, for unlimited love, always encouraging me, always believing in me and always being supportive without condition. Last, but for sure not the least, my son **Dara**, for bringing me joy, love and happiness.

Financial support for the included studies in this thesis was provided by grants from the European Commission (FP6 Integrated Project EUROHEAR; contract grant number: LSHG-CT-20054-512063), the Swedish Council for Working Life and Social Research through the FAS Center program Hearing Disabilities in Working Life and Society, the Swedish Research Council, the Tysta Skolan Foundation, the Petrus and Augusta Hedlund Foundation, AFA Insurance and a fellowship from Hawler Medical University-Iraq (HMU).

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