REVIEW

Nanoparticle based inner ear therapy

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Abstract

Synthetic nanoparticles can be used to carry drugs, genes, small interfering RNA (siRNA) and growth factors into the inner ear, to repair, restore and induce cellular regeneration. Nanoparticles (NPs) have been developed which are targetable to selected tissue, traceable *in vivo*, and equipped with controlled drug/gene release. The NPs are coated with a "stealth" layer, and decorated with targeting ligands, markers, transfection agents and endosomal escape peptides. As payloads, genes such as the *BDNF*-gene, *Math1*-gene and *Prestin*-gene have been constructed and delivered *in vitro*. Short-hairpin RNA has been used *in vitro* to silence the negative regulator of Math1, the inhibitors of differentiation and DNA binding. In order to facilitate the passage of cargo from the middle ear to the inner ear, the oval window transports gadolinium chelate more efficiently than the round window and is the key element in introducing therapeutic agents into the vestibule and cochlea. Depending upon the type of NPs, different migration and cellular internalization pathways are employed, and optimal carriers should be designed depending on the cargo. The use of NPs as drug/gene/siRNA carriers is fascinating and can also be used as an intraoperative adjunct to cochlear implantation to attract the peripheral processes of the cochlear nerve.

Key words: Synthetic vector; Gene delivery; shRNA delivery; Targeted cochlear therapy; Minimally invasive therapy

Core tip: Several novel, multifunctional nanoparticles have been developed, which are targetable to selected tissue, biodegradable, traceable *in vivo*, and equipped with controlled payload release. They can be used to transport therapeutic agents, such as drugs, genes, small interfering RNAs and growth factors into the inner ear. To visualise the targetability and accuracy of the delivery, the nanoparticles can be traced with magnetic resonance imaging. It is hoped that this technology will come to be used as an alternative carrier to viral vectors traditionally used in gene delivery, but without the severe adverse effect.

INTRODUCTION

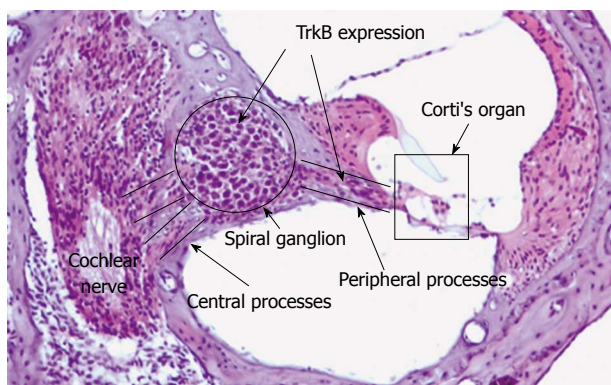Hearing loss is the leading birth defect in western societ-

Figure 1 Histology of cochlea and targets for cell specific pharmaco-/gene therapy. Three fluid filled compartments characterize the mammalian hearing organ. Nerve fibres and neurons (Spiral ganglion, peripheral processes and central processes), sensory epithelium (Corti's organ). Tyrosine kinease B receptor (TrkB) is indicated as a target for therapy.

ies affecting 2-3 children per 1000 born. In the EU more than 60 million people are affected by hearing loss and hearing loss is rated as the 9th most severe disease. The burden of the hard of hearing is considerable, in terms of physical, social and mental well-being, educational development and employment[1] In the United States people with hearing impairment represent an economic cost estimated at €222.4 billion and in the EU the costs of hearing deficit are second only to brain disorders (€ 386 billion). Moreover, because of the increasing age of population, the developed world will face an increasing number of hearing handicaps[2]. For mild to moderate forms of hearing impairment, conventional hearing aids alleviate communication problems in hearing loss. In severe hearing loss and deafness however, auditory function can only be restored by insertion of a neuro-prosthesis that functionally replaces the lost inner ear sensory cells by directly stimulating the auditory neurons. By using a cochlear implant, formerly deaf or deaf-born children can achieve functional hearing and learn to communicate with speech, and over 150000 devices are currently in use worldwide. We now know that many of these hearing impairments can be significantly alleviated with targeted drug delivery.

The inner ear has been a difficult organ to access therapeutically, due to membrane based partitioning, protected sensory cells and neural elements and its closed fluid dynamics (Figure 1). Nevertheless, the relative isolation, low immunoreactivity, availability of quantitative and objective measures of function, and the new imaging measures available for the inner ear, makes it an ideal test model by which to assess the efficacy and safety of putative thera-peutic interventions, applied locally and based on the use of nanoparticle delivery systems. Moreover, cochlear im-plant surgery offers the opportunity of direct access in a low risk (deafened) ear for the eventual clinical testing of the nanotechnology based targeted drug delivery.

The inner ear comprises the vestibular apparatus for balance control and the coiled cochlea. Sound enters the cochlea through tympanic membrane that mediates

the compression waves in the air to vibration of the os-sicular chain. The vibration enters along ossicular chain to the oval window that forces the fluid in the scala ves-tibuli to vibrate in the same phase. The scala vestibule is confluent on the apical tip with the scala tympani and contains perilymph. The vibration activates the separate membrane covered fluid space of scala media containing the organ of Corti. In the organ of Corti the sensory epithelium consists of three rows of outer hair cells and one row of inner hair cells. Isolated by the tectorial membrane and the basilar membrane the Corti's organ is bathed in the endolymph of the scala media.

The hair cells convert sound into electrical signals and wind up 32-40 mm two and a half turns around the spindle shaped modiolus that houses the spiral gan-glion cells containing the primary neurons. The sensory epithelium contains one row of inner hair cells and 3-4 rows of outer hair cells, and rests on the basilar mem-brane. This membrane vibrates when stimulated by sound in a tonotopic manner; high frequencies show maximum vibration in the basal part whilst low frequen-cies are mapped in the apical regions. The motile outer hair cells actively tune the displacement of the basilar membrane and thereby amplify and sharpen the pitch discrimination. A loss of hair cells and a retrograde de-generation of spiral ganglion cells lead to irreversible hearing loss. Cochlear implants can partly supplement the lost hair cells by direct electrical stimulation of the cochlear nerve. The inner ear is therefore an attractive model organ for treatment as it contains circumscriptive sensory elements, as well as neurons and vasculature that can be systematically analysed.

The application of a novel drug or gene therapy into the inner ear is hindered by a lack of vectors that are safe, efficacious and cell/tissue-selective[3-5]. To treat the "difficult-to-reach" tissues of the inner ear, different ef-forts have been tried such as the use of bio-implantable drug reservoirs[6], implantable catheters with a drug reservoir[4], viral carriers for DNA[7] and plasmids[8], and the recent development of synthetic vectors[4]. Viruses offer their own set of safety limitations for wider use. Implantable drug reservoirs have been of limited success and been mainly used for the delivery of corticosteroids on the round window membrane (RWM)[9]. There are also an abundance of commercially available plasmid vectors, that express the selected drug/DNA (albeit in a very transient manner) in the inner ear[10,11]. Some newer plasmids can penetrate the cell membrane, integrated in the cell genome (episomatic presentation) and remain for a longer period than conventional plasmids[12,13] but still the efficacy of this delivery mode is limited. This factor however provides an opportunity for synthetic vectors to be used as a future drug carrier to improve safety.

NANOPARTICLES: SIZE AND ADMINIS-TRATION IN INNER EAR THERAPY

The first nanoparticles were developed in the 1950s, but

Table 1 Illustration of average size of different object with reference to nanoparticles

Object	Size (nm)
Red blood cell	7000
Bacterium	1000
Virus	70-150
Nanoparticle	20-100
Gadolinium chelate	15
Width of DNA	2.5
Aspirin molecule	1

have been used for therapeutic applications only over the past two decades[14]. Drugs and genes have been recently successfully encapsulated within nanoparticles for the purpose of prolonging the circulation time of therapeutics and to protect them from enzymatic destruction[15]. Different types of nanoparticles have been developed to treat cancer, pain, and infections, as well as age-related and inherited diseases[14,16-19]. Due to an increased porosity of the blood vessels in cancer tissue, nanoparticles accumulate in cancer cells. Nanoparticle-based anticancer drugs have shown less adverse effects than non-capsulated drugs, for example in the case of doxorubicin[20]. For inner ear drug delivery, nanoparticles have been functionalized with peptides to achieve targeting.

Usually, a batch of nanoparticles has a wide and non-uniform size distribution and this size pattern is usually described as a dispersity index. Some nanoparticles can be very tiny and the largest may exceed a micrometre in size. Particles below 1 nm may be excluded in order to avoid mixing them with the terms of clusters of atoms (Table 1). Biologically however, if the particle size is less than 5 nm then they tend to pass through cell membranes and structures without any obstruction. When the particle size increases, different uptake mechanisms are involved in cellular entry and intracellular trafficking and these play an important role. There is no accepted international definition of a nanoparticle size, but one offered in the new PAS71 document developed in the United Kingdom is: "A particle having one or more dimensions of the order of 100 nm or less" (PAS 71:2011 Vocabulary-Nanoparticles). The ASTM standard also defines that two or three dimensions must be between 1-100 nm (ASTM 2456-06). The current agreement amongst the standards groups is that a scale from 1-100 nm defines the size range of a nanoparticle (ISO ISO/TS 27687:2008). This provides for example for carbon nanotubes to be included as a nanostructure. Their diameters are less than 100 nm but the length may vary from 200 to 2000 nm. Due to their diameter however, they biologically act as nanostructure, therefore a loose definition of size is more applicable to nanoparticles.

Choosing an appropriate delivery method is necessary for the efficient distribution of therapeutic agents in the inner ear while minimizing any adverse effects. The low delivery efficacy and the associated adverse effects of systemic administration however, do not make this method an ideal treatment for inner ear diseases[21,22]. Topical RWM surface delivery has proved moderately efficient, and systemic delivery has the lowest efficiency[23,24]. Local drug RMW delivery is now used extensively in the clinic for treating and diagnosing diseases[22,25]. At present, intracochlear delivery through cochleostomy appears to be the most effective method for delivering nanoparticles to cochlear cells[24], however cochleostomy can be performed only under certain circumstances as it can potentially result in inner ear damage.

BASIC CONSTRUCTS OF NANOPARTICLES FOR INNER EAR THERAPY

The characteristics of nanoparticles are determined by specific chemical compositions (Figure 2). Nanoparticles can be produced by a variety of techniques including interfacial deposition, emulsion or sonication. Polymer matrices currently used for drug delivery include poly-lactic-co-glycolic acid (PLGA), polylactic acid (PLA), polyε-caprolactone (PCL), polyethylene glycol (PEG), silica and chitosan. PLGA, PCL, PEG and PLA are biodegradable and FDA approved polymers, but typically suffer from low drug incorporation efficacy and rapid drug release rates for organic molecules with low molecular weight. Other materials such as silica based materials and chitosan may be manufactured with better incorporation and slower release rates, but may suffer from poorer biodegradability or biocompatibility[6]. Composite nanoparticles are composed of two block copolymers, *e.g.*, AB-type PLLA-PEG and ABA-type PLLA-PEG-PLLA. These nanostructures may be constructed as "shell-in-shell' nanospheres using, *e.g.*, PLLA-PEG@PNIPAAm-PDLA, containing the drug inside PNIPAAm-PDLA nanospheres which can be released at a specific temperature. Combinations of different isomers are used in order to manipulate the degree of porosity and rate of degradability of the matrix. Besides containing the drug, the composite nanoparticles can also incorporate environment-sensitive components and visualization agents (either as MRI-contrast agents or fluorescent materials).

Liposomes
Liposomes (termed as lipoplexes when containing the payload) are the most common nanoparticles. They are composed of a dual lipid layer with a hydrophilic component outside and inside and a hydrophobic component in the middle[26]. The core or outer surface of liposomes can carry hydrophilic drugs and genes whereas the bipolar lipid layer can carry hydrophobic drugs. In designing the liposomes, appropriate amounts of the desired lipid stock solution and the indicated lipid-peptide conjugate are mixed to obtain the desired composition. For lipoplexes used in inner ear drug delivery, the lipid films are composed of Sph, eggPC, DSPE-PEG-2000, peptide-PEG-lipid conjugate, and DPPRho. The solvents are thereafter removed and mixed with the cargo
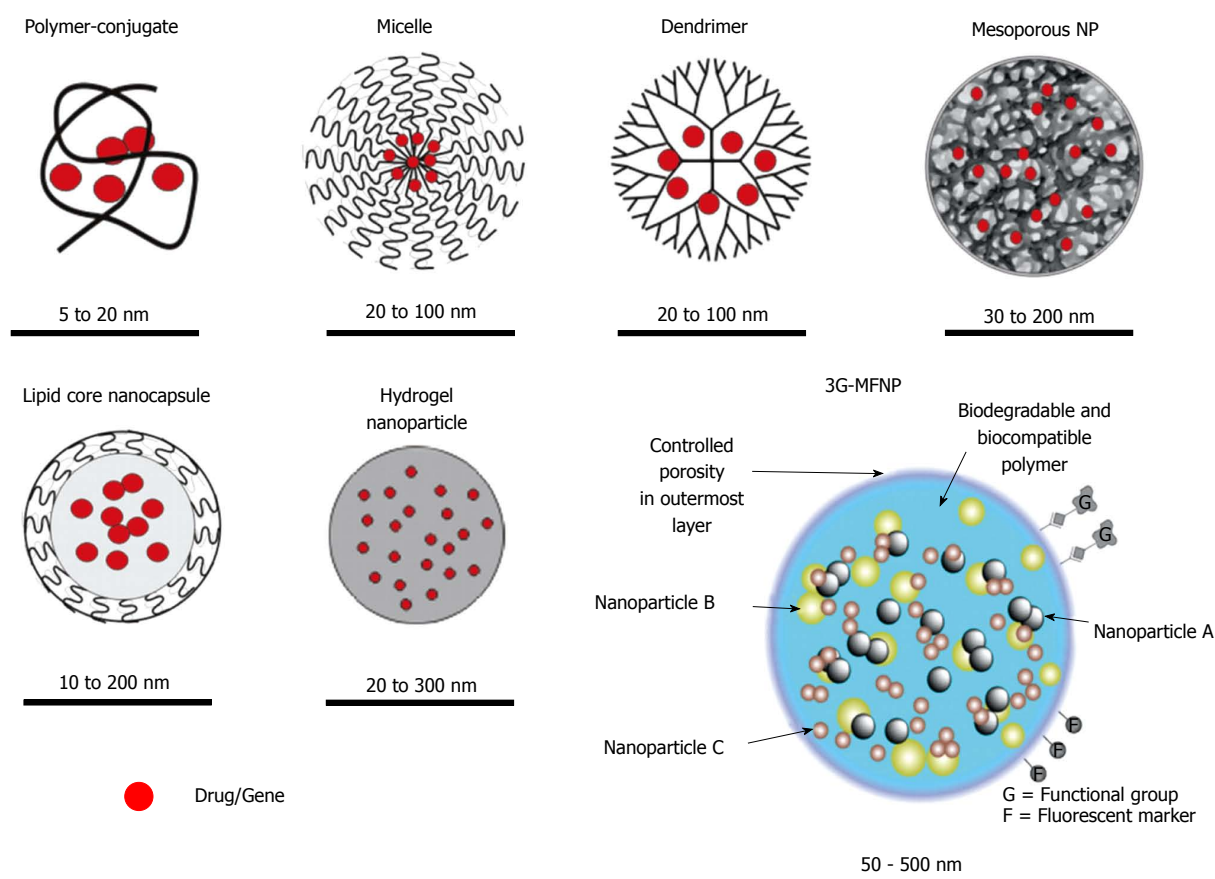
Figure 2 Examples of nanoparticles. The 3G-multifunctional nanoparticle (MNFP) indicates the functionalized third generation multifunctional nanoparticle. The red dots indicate drug/gene incorporation. The respective nanoparticle sizes are shown for each nanoparticle (NP).

by ultrasound[10,27].

Amorphous silica

Amorphous silica based nanoparticles are biocompatible and biodegradable. These nanoparticles represent a new class of mesoporous materials, which exhibit a high surface area and pore volume, in combination with a tuneable pore size in the range of 2-15 nm. Furthermore, the particle sizes can also be tuned and it is possible to synthesize nanostructures with a size range of 30-1000 nm particles in diameter, with a narrow particle size distribution. Both direct synthesis methods (wet processing) and spray-drying for the synthesis of mesoporous silica nanoparticles is used[28].

Polymerosomes

Polymerosomes may have various structures: diblock[29], triblock, graft[30] and dendritic copolymers[31]. Polymerosomes used in inner ear delivery consist of di-block copolymers and have hydrophobic and a hydrophilic components[32,33]. Polymerosomes have a quite stable structure compared to liposomes; they are stable for several months at 4 ℃ and release their loaded drugs for up to 5 wk at 37 ℃[34]. Unlike liposomes which self-assemble from low-molecular weight lipids, polymerosomes self-assemble from amphiphilic block copolymers, which consist of linked hydrophilic and hydrophobic polymer chains. Amphiphiles can self-assemble into spherical micelles, rod micelles[35] and network phases[36]. By manipulating the molecular weight of the hydrophobic chain, the size of the polymerosomes and the thickness of the lamella can be modified[37]. By manipulating the weight fraction of the hydrophilic component in the amphiphilic block copolymer, the morphology of the formations can be modulated[36,38]. The lamella of the polymerosome is thicker than that of the liposomes, and the aqueous core of the polymerosome can be larger than that of the liposome. The polymerosome lamella can load up to 10 mol% of a hydrophobic substance, and is quite stable at room temperature[39]. Polymerosomes can be easily loaded with hydrophilic and hydrophobic molecules in their internal cavity and their membrane respectively[39,40].

Chitosan

Chitosan is a natural polymer consisting of a biodegradable polysaccharide primarily consisting of chains formed of *N*-glucosamine units. Due to its polycationic nature, chitosan has been found especially useful in condensating plasmid DNA into particles[41]. Chitosan nanoparticles are prepared by mixing a solution containing chitosan dissolved in lactic acid and trisodium-polyphosphate solutions, and the variation of the ratio

of the two solutions allows the formation of nanoparticles ranging from 20 to several hundred nanometers, with a narrow size distribution. The fabrication method does not include harsh chemical conditions which may be harmful to the drugs or genes to be incorporated[41].

Hyperbranched polylycine

Hyperbranched polylycine is based on the dendritic and hyperbranched systems containing poly (α-hydroxy acids) or amino acids. These highly branched polymers are formed by iterative chemical reactions. Such polymers are of a fractal nature, containing a maximum 2D surface area within a minimum 3D space and are below 20 nm in diameter. Poly L-lycine acid forms the polymeric building block as it is possible to produce it with an accurate control of molecular weight, molecular weight distribution and end-group functionality[42]. The amino acid based hyperbranched polymers can complex with DNA for gene delivery and the manufacturing parameters and composition can be tailored for the desired dendrimer/hyperbranched nanostructure[42].

Lipidic-core nano-capsules

Lipidic-core nano-capsules (LNCs) are a novel class of very stable nanoparticles, dispersed in aqueous solutions. They are biocompatible and biodegradable molecules and can be fabricated without the use of organic solvents[43]. The size of LNC nanoparticles can be tailored by the modification of the composition, to the range of 10-200 nm, with a very narrow particle size distribution. The synthesis of LNCs is made possible through the self-organization of poly ethylene glycol like surfactants[44]. LNCs are constructed as a lipidic core (triglycerides, mineral oils, vitamin A, *etc.*) surrounded by a surfactant shell (stearate of PEG and lecithin), where lecithin is located in the inner part of the shell. The properties of their self PEGylated surface can be tuned simply by the modification of the PEG chain length used. These nanocapsules are particularly suitable for the encapsulation and release of hydrophobic drugs dispersed in the oily core, which makes them excellent candidates for the delivery of drugs and DNA[45].

FUNCTIONALIZATION OF NANOPARTICLES

Immunoshielding

Although the inner ear is an immune-privileged site, a delayed immune response may occur in the inner ear and lead to an inflammation of the cochlea and vestibule, caused by the nanoparticles. To reduce the opsonization and immune defence, nanoparticles are often coated, commonly with PEG or polyethylene oxide (PEO)[46]. Coatings can be created in two main ways; by the addition of a PEG containing surfactant at nanoparticle production, or if the nanoparticles are reasonably resistant to aggregation, coating can be performed after the nanoparticle manufacture[47]. An alternative method is through the covalent conjugation of PEG to the polymer during particle manufacture[48]. PEO and PEG tend to repel macromolecules from the coating and reduce contact on the nanoparticle surfaces. The molecular mass and surface density of PEO and PEG molecules[49,50] must be tailored to achieve a protein repelling effect. Nanoparticle coatings should also inhibit aggregation and reduce non-specific uptake by non-targeted cells[47]. Unless particles demonstrate significant charge stabilization, they will tend to aggregate, due to their hydrophobicity. Hydrophobic particles and positively charged complexes (for example uncoated polyplexes) will also tend to bind to cell surfaces, which in turn will lead to a non-specific uptake. PEGylated "stealth-nanoparticles" have prolonged circulation times, but can still activate the complement system[51]. A single intravenous injection of PEGylated liposomes elicited an anti-PEG immunoglobulin M response[52], similar to that of PEGylated liposomes[53]. In cancer therapy however, nanoparticles can cause anaphylactic shock by complement activation[54].

Targeting of nanoparticles

The concept of the specific targeting of nanoparticles to inner ear has been recently introduced[4]. The surface of nanoparticles is functionalized with targeting moieties, specific to the selected cell population. Previously, antibodies and proteins have been conjugated to nanoparticles to induce a targeted delivery of drugs and genes[55-57]. To reduce the size of nanoparticles, short peptides have been developed and these suit different targeting purposes[58]. Cell-penetrating peptides have also been designed to enhance the internalization and cellular penetration of nanoparticles[59]. The use of short peptides as a targeting moiety is favoured as the immune response is minimal and the increase of the nanoparticles size is a few nanometers. However, the discovery of targeting peptides is both time consuming and laborious work and therefore monoclonal antibodies can provide a more practical choice.

Bitsche *et al*[60] demonstrated that at the onset of hearing, TrkB-immunopositive staining occurred in inner hair cells and in cell bodies of the spiral ganglion neurons. TrkC was detected in nerve endings beneath inner and outer hair cells and in supporting cells. Root cells within the spiral ligament and the spiral ganglion neurons in the Rosenthal's canal showed a high level of TrkC expression. The p75 neurotrophin receptor (p75NTR) was found in the organ of Corti similar high rate of expression to TrkC, and scattered neurons showed strong immunoreactivity in the Rosenthal's canal[61]. Neurotrophic receptors are therefore, attractive targets for inner ear therapy[44,62-64]. Peptide-functionalized PMs were shown to be capable of targeting specific inner ear cell populations. For example, nerve growth factor-derived peptide-functionalized PEG-*b*-PCL PMs can specifically target the cochlear nerve and SGCs[44].

Ranjan *et al*[27] targeted TrkB positive SH-SY5Y cells using a TrkB ligand (18-mer peptides)-conjugated liposome. Specific binding and augmented uptake were confirmed for TrkB positive SH-SY5Y cells, with the targeting liposome appearing in the cytoplasm after 20 min of incubation. Ranjan *et al*[27] demonstrated the feasibility of targeting TrkB-expressing cells with a TrkB ligand conjugated-liposome and the promotion of cellular uptake (entering at least partly into endosomes) *via* receptor-mediated pathways.

Potential targetability with TrkB affinity peptide-functionalized liposome nanoparticles was observed in the adult rat cochlea[44]. Zou *et al*[61] evaluated the internalisation of liposomes functionalized to target TrkB receptors. Roy *et al*[44] investigated the utility of nerve growth factor-derived peptide (hNgf-EE) functionalized polymerosomes to target the cells of the inner ear. The nanoparticles were introduced to organotypic explant cultures of the mouse inner ear and to rat pheochromocytoma cells (PC-12). Specific targeting and a higher binding affinity to spiral ganglion neurons, Schwann cells and nerve fibres of the explant cultures were achieved through the ligand mediated multivalent binding to TrkB and p75NTR. Nonspecific uptake of nanoparticles was investigated using nanoparticles conjugated with a scrambled hNgf-EE peptide. The results indicated a selective cochlear cell targeting.

Zhang *et al*[65] evaluated the targeting ability of polymersomes to the cochlear nerve by functionalizing the polymersome surface with a tetanin peptide (Tet1) sequence. Tet1 peptide specifically binds to the trisialoganglioside clostridialtoxin receptors abundantly present in the cochlear nerve. Tet1 functionalized PEG-*b*-PCL polymersomes were administered using transtympanic injection and cochleostomy. The delivery *via* cochleostomy of Tet1 functionalized polymersomes resulted in a cochlear nerve targeting which in contrast was not seen after transtympanic injection.

Surovtseva *et al*[10] evaluated prestin binding ligands to target nanoparticles to the outer hair cells. In biopanning, two 12-mer peptides (A665 and A666) with an affinity to prestin were identified. The binding properties of the A665 and A666 ligands shown by flow cytometry demonstrated a selectivity to prestin expressing Chinese hamster ovary cells. PEG6K-b-PCL19K polymersomes covalently labelled with these peptides demonstrated an effective targeting to the outer hair cells of a rat cochlear explant.

Internalization and transfection

Previous efforts have mostly relied upon diffusional, non-targeted methods to deliver the nucleic acid into the cell and nucleus. The use of dynamic chemical processes have provided improved directionality and functionality to carry, for example small interfering RNA (siRNA) and DNA to target cells and into target locations by developing better nanoparticles. Factors that increase internalization include the smaller size of the nanoparticles, a posi-

tive charge, higher nanoparticle concentrations, longer incubation times, higher incubation temperatures and the use of internalization enhancing peptides[66-69]. The surface charge of the delivery vehicle is an important property of nanoparticles that affects their interaction with cells. A charge promotes cellular uptake but also promotes non-specific interactions with non-target cells and extracellular components such as serum proteins and extracellular matrices[70].

Clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis are the primary internalization pathways of nanoparticles. The pathway by which the carrier enters the cell depends on the carrier type, surface charge, and surface modifications[67,68,71]. In polymerosomes it was demonstrated that the core/corona structure (determined by the composition of the hydrophobic and hydrophilic fractions) affects the internalization pathway, whereas the polymerosomes size does not[72]. Peptide-modified nanoparticles are most likely to be internalized *via* caveolae-mediated endocytosis or macropinocytosis[71,73,74]. When clathrin and caveolae-mediated endocytotic pathways are inhibited, the internalization *via* the macropinocytosis pathway increases[67]. The internalization of the block copolymer micelles endocytotic pathways seems to involve pinocytosis, caveolae, clathrin, and caveolae-clathrin independent processes[75-77].

Soo *et al*[78] investigated the endocytic trafficking of silica nanoparticles within the immortalized inner ear cell line (HEI-OC1 cells). They used different sizes of silica nanoparticles of 50, 70 and 100 nm. The 50 nm NPs were the easiest to be internalized and macropinocytosis was the dominant mechanism. During intracellular trafficking, silica nanoparticles were localized in the early endosomes. The trafficking to lysosomes was delayed, however the particles could not escape the endosomes. Similar to these findings is that fluorescent-labelled micelles do not enter the nucleus but can also be found in endosomes/lysosomes, as can most other nanoparticles.

An enhancement of internalization can be achieved through the incorporation of specific ligands to the surface of the nanoparticle. Transferrin has been used as a cell penetrating peptide as most cells have transferrin receptors[79]. The transferrin targeting ligands theoretically have a disadvantage as transferrin is recycled in the cells and the nanoparticles may be transported outside of the cell. The transcriptional transactivator peptide, tat-peptide, or a modified sequence of the human immunodeficiency virus surface peptide, is commonly used to enhance nanoparticle delivery efficacy. Tat-peptide usage also improves the transfection, *i.e.*, the delivered DNA reaching into, and reading within the nucleus. Internalization peptides are usually covalently bound on the PEG layer. The targeting peptides, internalization and transfection peptides cover about 1%-2% of the surface of the PEGylated nanoparticle[65,80]. Functionalized nanoparticles offer opportunities to achieve many desired characteristics. Figure 3 shows an example of
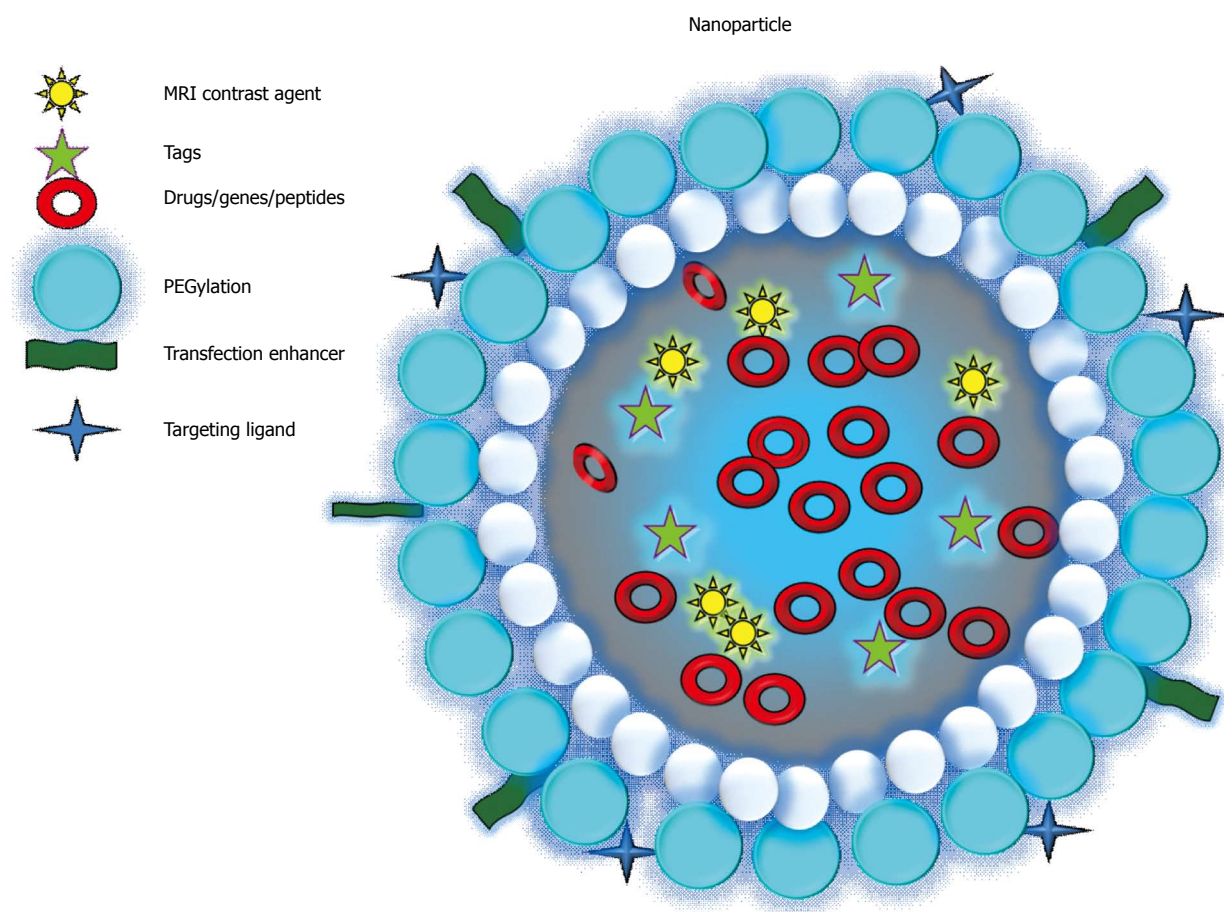
Figure 3 Composition of a targeted nanoparticle. MRI: Magnetic resonance imaging.

ideal nanoparticle for inner ear therapy. It contains nerve stimulating growth factor (BDNF)[61], the *Atoh-1* gene for transformation of supporting cells to outer hair cells[11] , siRNA for removing the inhibition of E-Box of *Atoh-1* gene action[61], dexamethasone to diminish tissue trauma during surgery[81], SPION to allow visualization of the nanoparticle payload in MRI[82], tat-peptide to improve internalization[83] and TrkB receptor-peptide for targeting[4,44].

Endosomal escape

Depending on internalization pathways, nanoparticles usually enter the early endosomes, continue to the late endosome and finally are stored and destroyed by lysosomes (Figure 4). A promising method to escape the late endosomes and lysosomes is to create nanoparticles that are chemically dynamic, and are able to cleave the chemical bonds of lysosomes. An example of this approach is the use of endosomal escape mediating proteins that improve the release of nucleic acids from the endosomes, a key step during the transport of genetic material[76].

Several peptides have been shown to disrupt the endosomal membrane and allow pathogens, therapeutics and plasmids transit to the cytoplasm[84]. These fusogenic, pH sensitive peptides are mostly derived from bacterial pathogens. This conceptual approach was first revealed using the cationic peptide melittin and cyclic anhydrides[85]. Melittin is an extremely cytotoxic, membrane lytic peptide that contains several critical lysine residues. To mask melittin's membrane activity, its lysine epsilon amino groups were reversibly modified using cyclic anhydrides. The key to this strategy is the choice of the labile bond. When the endosomal escape mediating peptides (EEMPs) enter the acidic environment of the endosome, a pH-labile bond is broken, so releasing the agent's endosomolytic capability[86]. The use of labile bonds to mask membrane activity provides a critical design feature of EEMPs, because it enables efficient *in vivo* delivery without sacrificing endosomolytic function for release into the cytoplasm. The HA2/INF peptide has been used by several researchers[87-89]. The fusogenic pH sensitive peptide GALA provides a different strategy to combat endosome trapping[84,90,91]. Some of these peptides form pores in the membrane. Recently, an endosomolytic agent used within dynamic polyconjugates, enabled the efficient delivery of siRNA into hepatocytes *in vivo*[92]. In gene transfer, *in vitro* luciferase gene expression was increased 1800-fold when melittin was modified with dimethylmaleic anhydride and covalently coupled to poly-*L*-lysine (PLL), when compared to unmodified PLL[93].

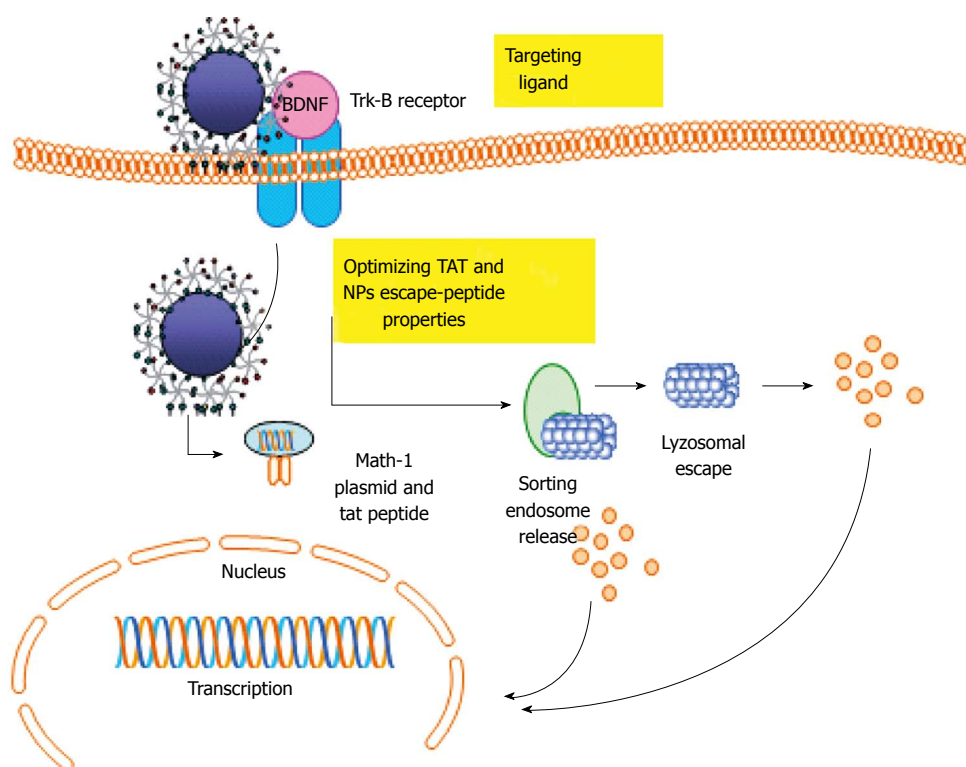In liposomes, citraconic anhydride has been used to

Figure 4 Internalization and cellular trafficking of nanoparticles. TrkB: Tyrosine kinease B receptor; BDNF: Brain derived neurotrophic factor; TAT: Tactical arrival time.

reversibly modify the primary amine of dioleoyl phos-phatidylethanolamine (DOPE), to form citraconyl-DOPE[94]. In an acidic environment, the citraconyl group is cleaved reforming DOPE. In nanoparticles, pDNA was complexed with poly-lysine, which was then mixed with negatively charged liposomes containing citraconyl-DOPE, DOPE and DOPE-PEG-folic acid. This ternary complex showed a higher transfection activity than those liposomes containing cholesterol hemisuccinate instead of citraconyl-DOPE[95]. An extensive review on the use of escape peptides has been provided elsewhere[93].

Nuclear entry and DNA integrating in the genome

DNA introduced into cells by synthetic carriers is usu-ally trafficked to the cell interior along the endocytic pathway. Once released from the endosomes, the DNA or nanoparticle with DNA must travel through the cyto-plasm in order to reach the nuclear envelope. However, the diffusion of large (> 500 kDa) molecules through the molecularly crowded and sterically obstructed en-vironment of the cytoplasm is highly restricted. The results of non-viral gene transfer have shown surpris-ingly low levels of overall transfection, with < 1% of the DNA, which is taken up by a cell, ever being ex-pressed[96,97]. The unique structural properties of DNA further attenuate its diffusion. However, when plasmids with different sizes were injected into the mice tail vein, the efficiency of delivery was independent of the vectors size[98]. Even if a DNA vector reaches the nuclear enve-lope it must then translocate across nuclear pore com-plexes in order to reach its destination[99]. Nuclear pore

complexes (NPC), which operate as selective conduits for nucleo-cytoplasmic exchange in eukaryotes, support two modes of transport. For non-dividing, differenti-ated target cells, the nuclear entry is highly dependent on transport *via* NPCs. Particles smaller than 8-9 nm may enter by diffusion, while larger particles are ushered selectively by soluble receptors of the karyopherin/im-portin β family, which recognise the specific nuclear im-port signal (NLS) or export signal peptides displayed by the cargo[100,101]. This mode is used to transport objects below 40 nm in diameter. Given its large hydrodynamic radius and the lack of nuclear targeting signals, DNA is expected to translocate very poorly through nuclear pore complexes. Unless assisted, the translocation of DNA through nuclear pores is limited. Only very short segments, in the order of approximately 100-500 bp can pass the NPC[100]. Obstruction of these two important cellular processes therefore render transfection efficien-cies by synthetic carriers as unacceptably low, particularly in the case of non-dividing cells and localised gene deliv-ery.

Extensive effort has been placed on enhancing the nuclear import of delivered DNA by direct or indirect association with NLS moieties[100,102]. This effort has largely been in vain, because the size and chemical prop-erties of the DNA dramatically attenuate its transport into the cell cytoplasm and through the nuclear pore complex channel, so resulting in poor nuclear transfer. The use of nano-carrier DNA complexes, functionalized by moieties with nuclear-targeting activity, offers a highly promising means to overcome the nuclear barrier and in-
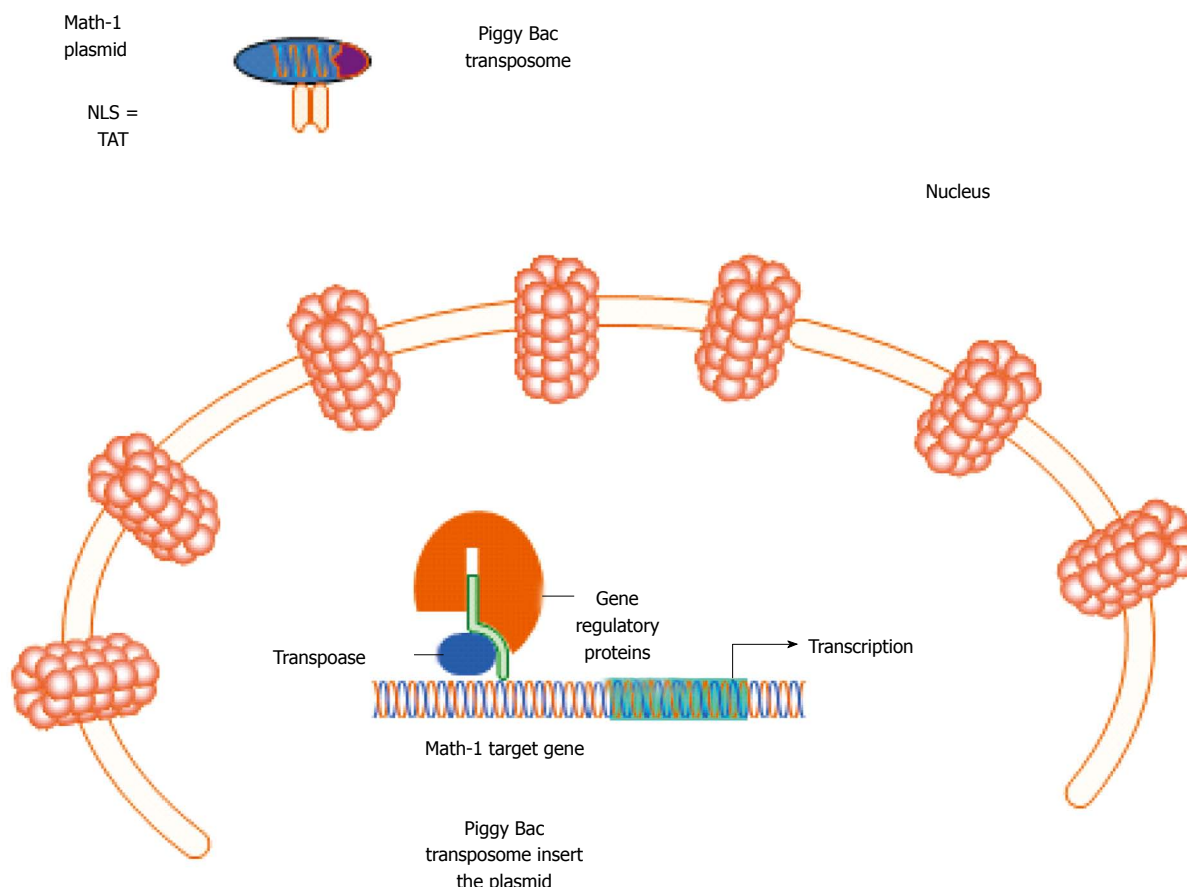
Figure 5 Example showing nuclear pore complexes targeted Tat-coated nanoparticle containing Math-1 plasmid and PiggyBac transposome. NLS: Nuclear import signal; TAT: Tactical arrival time.

duce the efficient nuclear import of DNA[103]. To maximize nuclear targeting, various nucleus targeting moieties (NLSs) chosen from aptamer libraries and sulfhydryl-reactive linker molecules, have been utilized[104]. With the goal of identifying the most efficient NLSs, the nuclear accumulation and kinetics of the nuclear import of various quantum dots-NLS complexes have been used[105]. Despite present knowledge of the interaction of NLSs with their cellular partners, it remains impossible to predict their effectiveness in nuclear penetration.

This limitation of biological activity is exacerbated by the fact that in most cases, DNA remains epi-chromosomal, so necessitating repeated administrations (Figure 5). Kim *et al*[106] reviewed the possible use of transposons to integrate DNA in the genome with nanoparticles. Transposons have proved promising elements for gene integration and the Sleeping Beauty system has been predominant for many years, although there have been several other transposon systems available, for example, Tol2[107]. Recently however, another system known as PiggyBac has been introduced and developed for fulfilling the same purposes (for example, mutagenesis, transgenesis and gene therapy). In some cases there has been improved transposition efficiency and advantages over the Sleeping Beauty transposon system have been demonstrated. The improved hyperactive transposase

in PiggyBac has increased the transposition efficacy of Sleeping Beauty[108]. PiggyBac is still to be tested however in the context of different functionalized nanoparticles. A further hindrance in the development of successful gene therapy has been the detrimental response of the immune system. Understanding the sequence of inflammatory responses which occur after PiggyBac delivery, may though enable the identification of points at which immune modulation could dramatically improve the efficacy of gene therapy of the inner ear[106].

PAYLOAD

Delivering growth factor to non-targeted cells may cause adverse effects. This has been recently well illustrated in the innovative treatment of Parkinson's disease with glial nerve growth factor (GDNF), in that the drug was delivered *via* catheters locally into the CNS. However, the therapy was partly unsuccessful and caused serious adverse effects as the drug could not be sufficiently targeted to specific cells[109]. This issue may be managed with targeted delivery[44,65,110].

Nanoparticles loaded with tracers, drug, genes and siRNA have been examined in the context of the inner ear. When compared to pDNA, the much smaller size of the siRNA and a need to achieve only a cytoplasmic
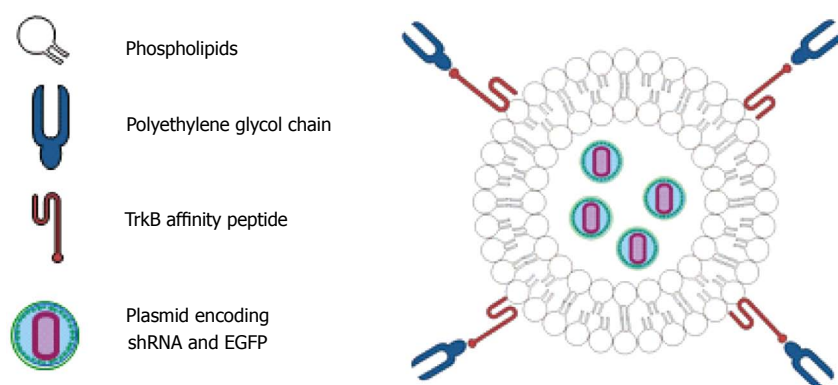
Figure 6 Illustration of the tyrosine kinease B receptor-targeting liposome nanoparticles that express short hairpin RNA to silence inhibitor of differentiation and DNA binding-2 and a reporter enhanced green fluorescent protein. shRNA: Short hairpin RNA; EGFP: Enhanced green fluorescent protein; TrkB: Tyrosine kinease B receptor.

delivery, renders the delivery of siRNA more tractable. McCall *et al*[8] review the use of siRNA in silencing dominant mutations of genes. Although RNAi techniques are still in the early stages of development, their potential to dramatically alter human inner ear disease is enormous in nanoparticle based therapy[4]. RNAi technology seems particularly well-suited for treating dominant-negative forms of hearing loss by reducing the amount of aberrant mRNA available for translation. It has been successfully applied in a mouse model for GJB2-related hearing loss caused by an autosomal dominant nonsyndromic form of hearing loss caused by a dominant-negative mutation[111]. RNAi has also been used to reduce cisplatin-related hearing loss in a rat model[112]. Tamura *et al*[23] investigated the efficacy of PLGA nanoparticles to deliver therapeutic molecules to the cochlea. After a systemic application of rhodamine labelled nanoparticles, fluorescence was identified in the liver, kidney, and cochlea. A sustained delivery of rhodamine however, only took place in the liver and not in the kidney or cochlea. Rhodamine nanoparticles placed on the RWM were identified in the scala tympani, indicating that PLGA nanoparticles can permeate through the RWM. Furthermore, the local application of rhodamine nanoparticles to the RWM was more effective in targeted delivery to the cochlea than in systemic application. In a successive study, PLGA nanoparticles encapsulating lidocaine were placed on the RMW of guinea pigs, and resulted in reasonable concentrations of lidocaine being seen in the cochlea without serious adverse effects[110,113]. The authors of the study postulated its use against tinnitus. They also applied insulin-like growth factor-1 in combination with a gelatine hydrogel on the RWM to improve hearing after sudden deafness[114] and the phase I / II a clinical trial of these experiments has now been commenced.

Gene therapy offers new treatment possibilities for a large number of commonly acquired and inherited human inner ear diseases where conventional therapy has proven ineffective. Effective methods to stimulate new functional hair cell regeneration in the inner ear would be of therapeutic value for treatment of hearing and balance deficits. One potential strategy to regenerate hair cells is to induce a phenotypic trans-differentiation of the non-sensory cells that reside in the inner ear by using *Math-1* gene, or to remove the inhibition exerted by id-proteins[45,115].

A TrkB affinity peptide was synthesized and coupled to liposome nanoparticles carrying the plasmid pGene-Clip™ hMGFP encoding shRNA, to transiently silence the inhibitor of differentiation and DNA binding-2 (Id2) along with the reporter EGFP[45] (Figure 6). Internalization and targetability were analysed in primary cochlear cell culture, cochlear explants, and live rats. There was efficient internalization in primary cochlear cell culture for both peptide-functionalized liposome nanoparticles and blank liposome nanoparticles in a concentration-dependant manner. Both particles showed uptake in the spiral ganglion cells and adjacent nerve fibres in live rats. A more efficient gene expression was seen for the peptide-functionalized liposome nanoparticles, and the function of shRNA was demonstrated in cochlear explants and adult rat cochleae[45,61]. It was concluded that the functionalization of liposome nanoparticles with a TrkB ligand did not change cellular internalization, but did enhance gene expression.

Zhang *et al*[45] studied transduction of the Math1 gene using nanoparticle based gene delivery. After constructing the pCDNA6.2/C-EmGFP-Math1 expression plasmid, the plasmid was transfected into different cell lines and primary cochlear cells. Liposome nanoparticles containing the Math1 plasmid expressed the gene with variable efficiencies, depending on the particle size, surface charge and PEGylation status. The unique intracellular trafficking of Math1 with dynamics from the cytoplasm to the nucleus was demonstrated. The modification of mesenchymal stem cells by *Math1* gene delivery together with BDNF and GDNF treatments can potentially be applied to cell replacement for the treatment of cochlear spiral ganglion cell loss in deafness.

DELIVERY ROUTES OF NANOPARTICLES INTO THE INNER EAR

Therapeutic agents for the treatment of inner ear dis-

Schematic display of human RWM

Str. cuboidal epithelium

Pseudostr. sq. epith.

Fibrocyte

Collagen I and III
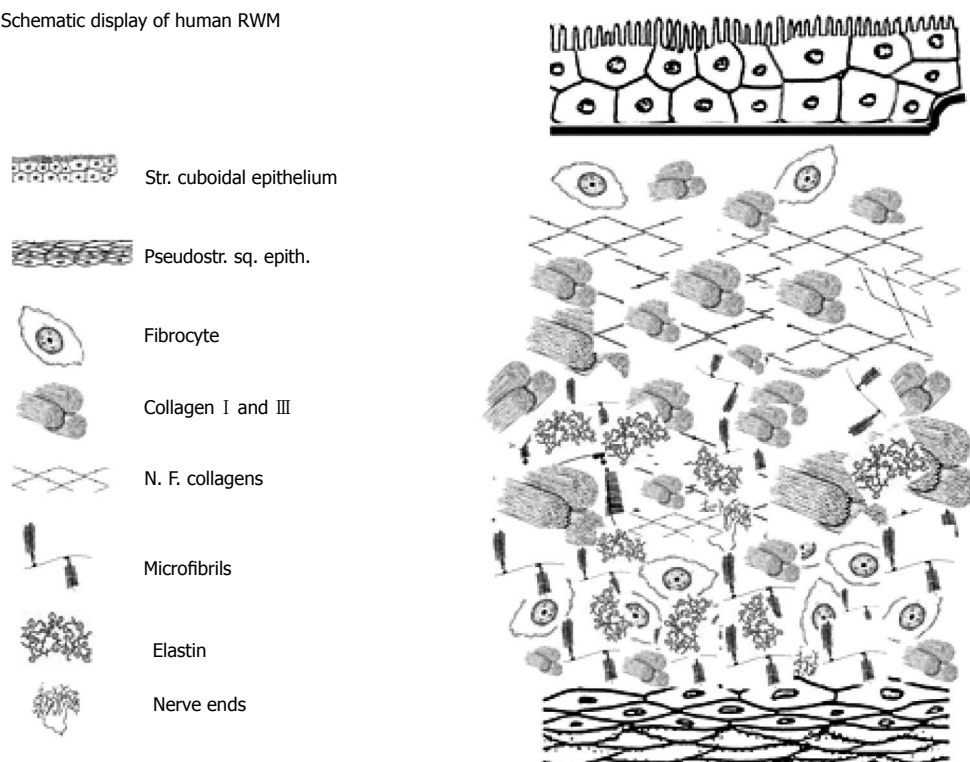
N. F. collagens

Microfibrils

Elastin

Nerve ends

Figure 7 Human round window membrane and inner ear with different layers. RWM: Round window membrane.

eases are being rapidly developed, although the delivery methods are still undergoing improvement. Nanoparticle based delivery into the inner ear has been investigated using either systemic or local delivery[11,12,81,116-118]. There are two different approaches to deliver the nanoparticles locally: (1) Through transtympanic delivery (*via* an intact RWM or oval window); and (2) intracochlear delivery[3,119-121]. Tamura *et al*[23] compared PLGA nanoparticles delivered *via* systemic injection, administered either locally onto an intact RWM or injected into the vestibulum of the inner ear. The results indicated that an injection into the vestibulum was the most efficient method, with intact RWM delivery being moderately efficient and systemic delivery exhibiting the least efficiency[23]. In another study, the systemic delivery of viral vectors resulted in no cochlear gene expression[122]. The low delivery efficiency and obvious adverse effects render systemic administration as a non-optimal method with which to treat inner ear diseases[21,22].

A comparison of topical RWM surface delivery, transtympanic injection and cochleostomy provided information regarding the optimization of inner ear drug delivery using polymerosome nanoparticles[65]. Although transtympanic injection was not superior to topical RWM surface delivery (using a gelatin sponge) of nanoparticles into the cochlea, it was more efficient in delivering nanoparticles into the vestibulum. However, both methods failed to introduce nanoparticles into the spiral ganglion or into Corti's organ *in vivo*. Among the evaluated delivery approaches, cochleostomy is the most promising method with which to deliver polymerosomes

into these locations[45,65]. For RWM delivery, Zhang *et al*[45] concluded the passage of nanoparticles as taking the following steps: polymerosomes were first taken up by the endothelial cells, then diffused into the connective tissue core through the loose intercellular gaps of the endothelium. They were finally internalized by the epithelial cells when they were delivered into the cochlear perilymph. However, most of the polymerosomes were unable to cross the tight junction of the epithelial layer of the RWM when they were administered either *via* topical RWM surface delivery or transtympanic injection (Figure 7). The topical RWM surface administration of gadolinium chelate results in the efficient loading of the contrast agent in the rat inner ear as shown by *in vivo* magnetic resonance imaging (MRI)[123]. Transtympanic injection of PEGylated liposome nanoparticles encapsulating gadolinium-tetra-azacyclododecane-tetra-acetic acid (Gd-DOTA) passed through the RWM efficiently *in vivo* as determined by the Gd-DOTA MRI signal in the inner ear[124]. The variable efficacies of different substances in crossing the middle-inner ear barriers may be dependent upon the surface characteristics of the nanostructures.

Permeability of the RWM is influenced by several factors, including the thickness of the membrane and the size, configuration, concentration, liposolubility, and electrical charge of the delivered substance[125]. The thickness of the RWM is variable depending on the species studied. In mice this thickness is 6-10 μm[126], in rats 15 μm[127], in chinchillas 10-14 μm[125], in guinea pigs 10 μm[128], and in humans 70 μm[129]. Humans have the thickest RWM, which is thicker at the rim than in the

Figure 8 Porous annular ligament between the stapes footplate and vestibular window. Source: Reference[141], with permission. VW: Vestibular window; S: Stapes.

central region. The epithelial layer of the RWM has tight junctions[125], whereas the connective tissue layer and the endothelial layer are loosely arranged. The results showing that polymerosomes were detected only in the epithelial layer of the RWM following topical RWM surface delivery and transtympanic injection, but detected in all three RWM layers when administered through cochleostomy, suggest that the epithelial layer is the flow limiting structure for polymerosomes in their transport from the middle ear to the cochlea[11]. Nanoparticles, such as liposomes, polymers and polymer-encapsulated SPIONs were shown to cross the RWM[12,25,130]. However, the penetration efficacy of nanoparticles is not as high as that of viral vectors. The chosen delivery method also influences the passing of substances through the RWM. For example, BDNF administrated by a single transtympanic injection or by sustained RWM application in rats resulted in 0.0001% and 0.002% of the original concentration in the perilymph after 3 d, respectively[131] .

PEG-*b*-PCL polymerosomes, LNCs and silica nanoparticles loaded with fluorophores can pass through the RWM and the oval window, and are detected in the cochlea and the vestibulum[12,13,132]. PEG-PLA and PLGA nanoparticles can deliver drugs to the inner ear *via* either local RWM or systemic application[81,113]. Contrast agents, such as Gd-DOTA and superparamagnetic iron oxide nanoparticles (SPIONs), encapsulated in liposomes, polymers and copolymers were visualized in the inner ear using MRI, light microscopy and electrical microscopy. The penetration of the SPIONs was enhanced through the use of an external magnetic field[116,117,133-135].

Several efforts have been made to improve the permeability of the RWM for delivery. For example, a disruption of the RWM epithelial layer was attempted with silver nitrate, trichloroacetic acid and phenol, but these methods did not achieve satisfactory results[136,137]. A partial digestion of the RWM using a collagenase solution increased the RWM permeability to recombinant adeno-associated virus (rAAV) vectors and enhanced the transfection efficacy[138]. Cell-penetrating peptides were also used to improve the passage of nanoparticles through

model membranes[139,140].

Oval window delivery

The oval window (OW) is the barrier between the perilymph in the vestibulum and the middle ear cavity. A histological study in rats demonstrated that the annular ligament across the stapedio-vestibular joint is a porous structure composed of fibrillin, 36-kDa microfibril-associated glycoprotein (MAGP-36), and hyaluronic acid[141] (Figure 8). The distance from the oval window to the saccule is 1.66 mm in humans, and the distance to the utricle is 2.25 mm[142]. *In vivo* MRI studies of guinea pigs, rats, and humans indicate that the oval window is more permeable to gadolinium than the RWM[134,135,143]. Selective vestibular delivery *via* the oval window pathway was demonstrated in a recent MRI study performed by Zou *et al*[118] in which the contrast agent was injected into the epitympanum. This novel method was translated into practice in the ear clinic to treat Meniere's disease, and we have therefore changed our own clinical practice and currently use a 50 μL injection into the upper posterior tympanic cavity to allow the OW penetration of gentamicin. This seems to be more effective than filling the whole tympanic cavity and so far we have not experienced severe hearing loss (personal information). Therefore, for nanoparticle delivery, the OW provides an alternative pathway especially to the vestibulum and cochlea.

Intracochlear delivery

At present, intracochlear delivery is the most efficient method for gene delivery to the inner ear[65,120,144]. Although hydrogel-administered BDNF to the topical RWM surface with sustained release was reported to partially protect SGCs from degeneration, an intracochlear application of BDNF resulted in full preservation[131,145]. During cochlear implantation, protective therapeutic agents such as neurotrophins and dexamethasone, can also be administered with an electrode array[146,147]. However, intracochlear application of a given substance is not an optimal method except in cochlear implants, as it has the potential to induce deafness and inflammation[130].
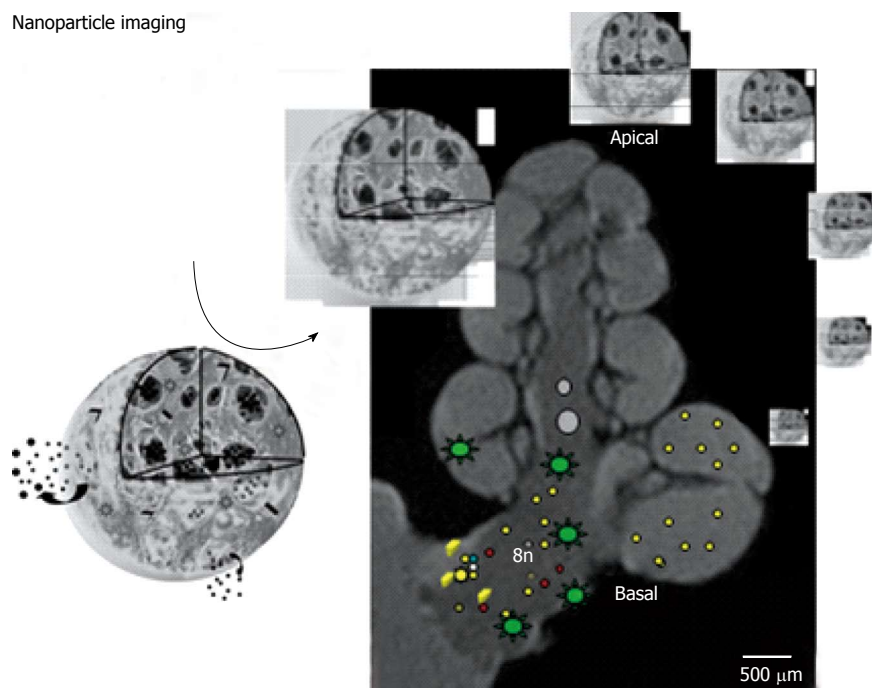
Nanoparticle imaging

Figure 9 Schematic representation of imaging of nanoparticles in the inner ear of guinea pig with magnetic resonance imaging. The cochlear nerve (8n) and cochlear basal turn (basal) are indicated. As nanoparticle transmission electron microscopy of multifunctional poly-lactic-co-glycolic acid -nanoparticle is shown. The star-like dots in the fluid spaces of the cochlea and the cochlear nerve demonstrate the distribution of gadolinium chelate used for visualization. The small dots indicate nanoparticles, their ingredients and dye for histological confirmation of targeting.

Trans-cochlear pathways

In vivo MRI studies on small animals, including guinea pigs, rats, and mice have demonstrated novel routes of gadolinium through the modiolus and lateral wall[124,134,143,148-150]. Increased evidence for this novel communication route was obtained through combined SEM and light microscopy in the human cochlea, so demonstrating that the modiolar wall of the SV and ST in the first and second turn is porous, forming a perilymphatic communication route to the peri-vascular and peri-neural spaces in the modiolus. A "peri-modiolar lymph" or fluid space can be identified in the modiolar periphery[151]. The openings on the modiolar wall may be used therapeutically, in that nanoparticles migrate to SGCs from the perilymph of the ST[135,143,149,151]. Migration can also take place from the ST to the SV *via* either the SL or the modiolus[123,143,149,152]. When substances enter the perilymph of the ST through the RWM or intracochlearly, they perfuse through fibrocytes of the SL *via* openings in the mesothelial sheet facing the perilymph[151-155]. A fourth pathway is through Corti's organ which has a loose structure, and drugs applied to the perilymph of the ST can access nearly all of the cochlear cell populations and the nerve fibres of the peripheral processes of the SGCs[156]. Lastly, perfusion can also occur between the basal SV and the vestibulum[152].

IMAGING OF THE NANOPARTICLE *IN VIVO*

Tracking the distribution of nanoparticles within the inner ear is an important parameter that can be determined with expert imaging tools[25,123]. In experimental studies, several investigations have been carried out with 4.7 T magnetic resonance imaging (MRI) to evaluate the dynamics of nanoparticles (Figure 9). When injected into the middle ear, the nanoparticles containing contrast agent allow the determination of the dynamic distribution rate of nanomaterial in a living organism. Both SPION nanoparticles and gadolinium chelate containing nanoparticles have been used. The resolution obtained *in vivo* by the system is at best 78 μm × 78 μm × 78 μm. The two contrast agents are complementary to each other and can be applied to human studies to describe accurately the pathway and destiny of the nanoparticles in the inner ear.

Zou *et al*[157] developed MRI traceable tracking lipoplexes by encapsulating gadolinium-tetra-azacyclo-dodecane-tetra-acetic acid (LPS + Gd-DOTA) within the liposome nanoparticles. The functionalized nanoparticles could be traced after either intratympanic or intracochlear administration and their distribution in the inner ear could be visualized. LPS + Gd-DOTA with 130 nm size were efficiently taken up by the inner ear 3 h after transtympanic injection and disappeared after 24 h[118]. With intracochlear injection, LPS + Gd-DOTA were rapidly distributed throughout the inner ear, including the cochlea and vestibule. The transportation efficacy of LPS + Gd-DOTA across the middle-inner ear barriers depends on the size of the liposome[118]. The disadvantage of using chelated gadolinium is that the concentration of gadolinium must be high enough to be detected:
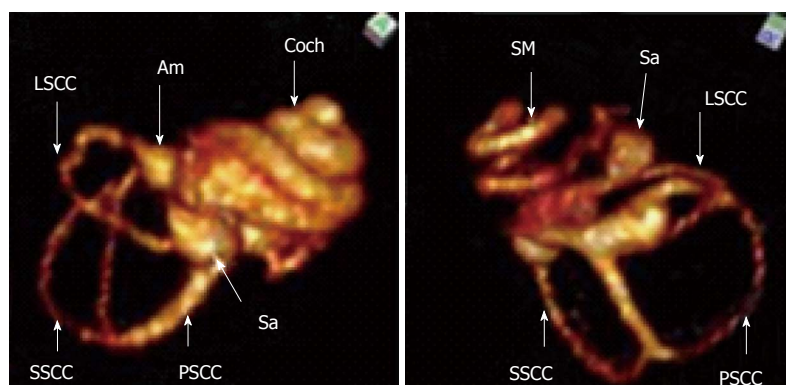
Figure 10 Magnetic resonance imaging of the inner ear of rat using SPION as contrast agent. The upper figure shows T1 weighted imaging where all cochlear fluid spaces are visible. The lower figure shows T2 weighted image where SPION injected into the perilymphatic space will reduce the T2 signal and the perilymphatic space signal is extinguished. Only the fluid in scala media (endolymph) is visible. Am: Ampulla; Coch: Cochlea; Sa: Saccus; LSCC: Lateral semicircular canal; SSCC: Superior semicircular cananal; PSCC: Posterior semicicrcular canal; SM: Scala media.

when compared for example to using SPION, the detectable amount of gadolinium (Gd) must be 100-500 times greater.

Poe *et al*[82] compared SPION with Gd in MRI in the visualization of the inner ear in a rat model (Figure 10). While Gd demonstrated enhancement of the perilymph, SPION attenuates the proton signal in perilymph and in MRI was devoid of a signal, yet the sensitivity of SPION was much greater than Gd. Zou *et al*[124] used SPION in the visualization of the cochlear compartments with a 4.7 T MRI scanner. POA@SPION was administered through intracochlear, intratympanic and intravenous routes. It passed through the middle-inner ear barriers in only small amounts, but stayed in the perilymph for 3 d. There was no traverse of the blood-perilymph barrier or blood-endolymph barrier. The inner ear distribution of POA@SPION was confirmed by histology. POA@SPION is a promising T2 negative contrast agent. The authors of the study demonstrated that POA@SPION can be introduced into the perilymph space, after which it becomes widely distributed. As such, SPIONs can be used to demonstrate the integrity of the perilymph-endolymph barrier.

Labelling cells with SPIONs has potential advantages over gadolinium. These are owing to the high spatial resolution of MRI and the fact that SPION labels are non-toxic to the cells since the iron oxide nanoparticles are biodegradable and metabolized by the same cells[158,159]. SPION labelling of cells may provide researchers with a tool to understand the role or contribution of a specific cell population in normal and abnormal development or in pathological processes. *In vitro* labelling of cells with SPIONs allows for the detection of single labelled cells within target tissues using MRI, following either direct implantation or intravenous injection[160]. *In vitro* experiments have shown that SPION labelled cells can move towards an external magnetic field and magnetically labelled cells can be delivered and retained at a site of interest by applying an external magnetic field[160]. The magnetic targeting of genetically altered cells or cells serving as delivery vehicles for genes may be feasible in

the future by infusing SPION labelled cells during angiography or invasive procedures and by placing an external magnet over a predetermined region, using image guidance to maximize the delivery and retention of cells in a target tissue[158].

It has been suggested that *in vivo* imaging will provide demonstration of biocompatibility, membrane penetration, and targetability of the MFNP within the cochlea. With the development of novel magnetic nanoparticles, MRI has led to wider biomedical applications in "theragnostic" (therapeutic and diagnostic) applications such as in hyperthermia[161], stem cell tracking[162], gene expression[163], cancer detection[164], and inflammation[165].

CONCLUSION

Hearing loss is a large burden affecting around 13% of the European population. Novel techniques to downsize drug carriers to nano-sizes and attach them with targeting moieties demonstrate new opportunities for successful drug/gene delivery into the inner ear. Nanoparticle-mediated gene transfer is an attractive methodology because of its simplicity and reduced toxicity, and it holds promise in providing a major break-through for future therapy. In spite of the modest efficacy of gene transfection, several disease models have been successfully modulated by use of the synthetic vector system. To enhance the utility of these vectors it is necessary to increase their packing capacity and the level of gene expression of which they are capable. It is also important to prolong gene expression, as well as to increase their target cell specificity. For protection of hostile DNAse from hostile enzymes, PEGylation and covalently attaching targeting moieties, peptides enhancing internalization and peptides that mediate the nuclear localization signal, may provide a solution for a drug delivery system for organs difficult to reach, such as the inner ear.

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