Nanoparticle drug delivery systems for inner ear therapy: An overview

Manuscript Details

Manuscript number	JDDST_2017_23
Title	Nanoparticle drug delivery systems for inner ear therapy: an overview
Short title	Nanoparticles for inner ear drug delivery
Article type	Review Article

Abstract

Local drug delivery based on nanoparticles (NP) represents a novel strategy to improve inner ear treatments. The intratympanic delivery of NP may be suitable to treat or prevent hearing loss originating from damage to hair cells and spiral ganglion neurons in the cochlea. Numerous experimental studies support in vitro and in vivo the biocompatibility of NP, their physical stability, target specificity, cell/tissue uptake and ability to internalize therapeutic agents. The topical use of NP helps to reduce the amount of drug required and avoid systemic side effects. This review focuses on recent findings and applications of different NP systems locally administered in the inner ear. The perspectives for clinical application of NP in inner ear drug delivery are also discussed.

Keywords	Nanoparticles; inner ear; drug delivery; intratympanic administration; local administration.

Corresponding Author	Laura Astolfi
Corresponding Author's Institution	University of Padua
Order of Authors	Filippo Valente, Laura Astolfi, Edi Simoni, Serena Danti, Valeria Franceschini, Milvia Chicca, alessandro martini

Suggested reviewers	Wei Liu, Marcelo N. Rivolta, barbara zavan, Isabel Varela Nieto

Submission Files Included in this PDF

File Name [File Type]

Cover letter 1.docx [Cover Letter]

Answer_to_Reviewers1.docx [Response to Reviewers]

MS review Revision Marked.docx [Revised Manuscript with Changes Marked]

graph abstract1.tif [Graphical Abstract]

MS review Revision1.docx [Manuscript File]

NP_Figure_Legends1.docx [Figure]

Figure 1 inner ear barrier.jpg [Figure]

Figure 2 administration routes.jpg [Figure]

Figura 3 nanoparticles.jpg [Figure]

To view all the submission files, including those not included in the PDF, click on the manuscript title on your EVISE Homepage, then click 'Download zip file'.

Dear Professor Siepmann,

thank you very much for sending me the Reviewers' comments on the manuscript "Nanoparticle drug delivery systems for inner ear therapy: an overview" (JDDST_2017_23) by Valente, Astolfi and coworkers.

One of the Reviewers mentioned that he/she did not find the Figure captions. Indeed, we had some problems in manuscript uploading: we checked on the journal site and could not find the file of Figure captions. We therefore enclose the missing Figure captions file for you and for the Reviewer.

Here enclosed you will find the detailed answers to the Reviewers. We hope that now our manuscript is suitable for publication on JDDST.

Kind regards,

Laura Astolfi, PhD

Reviewer 1

The review presented by Valente et al « Nanoparticle drug delivery systems for inner ear therapy: an overview" is well-written and interesting.

I have the following minor comments/suggestions regarding the manuscript.

1. Lines 43-44 "The NP with size between 10 and 100 nm are useful for application in biology and medicine for innovative DD systems". This range is a little bite too restrictive. The range 10 to 200 nm is commonly used.

We thank the Reviewer for his/her comment and corrected the sentence according to the suggestion.

2. Line 53, please introduce the references of the reviews

According to the Reviewer's suggestion, we introduced the references in the sentence.

3. Paragraph 2. Ear barrier

Drugs can also enter the inner ear via the oval window, another semi-permeable membrane located in the middle ear. It is important to mention in the paper the oval window as another route of penetration.

As suggested by the Reviewer, we mentioned the oval window together with the other inner ear barriers.

4. Lines 190 to 191 "Recently, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved PLGA for parenteral administration (48)". Please remove recently because PLGA microspheres and PLGA implants for the treatment of prostate cancer were marketed at the end of the eighties.

We deleted "recently" according to the Reviewer's suggestion.

5. Paragraph 5. Key aspects for nanoparticles-based delivery in the inner ear

Lines 279 to 282. Supermagnetic iron oxide NPs (SPIONs) particles are not biodegradable. Please, add a comment on the long persistence of these particles in the inner ear and their potential toxicity due to their accumulation in this compartment.

We changed the sentence according to the Reviewer's suggestion.

Lines 298 to 300 "The incorporation of loaded NP into hydrogels could increase their residence time in the middle ear, thus enhancing drug release in the perilymph». Please, add the reference El Kechai et al., "Hyaluronic acid liposomal gel sustains delivery of a corticoid to the inner ear, Journal of Controlled release, 2016, 226, 248-257". This paper presents in vivo results on pegylated liposomes dispersed in hyaluronic hydrogel following injection in the middle ear. This recent paper completes examples on liposomes.

- A prolonged residence time at the site of injection as well as in the round window were achieved without any negative effect on the hearing thresholds of the animals.

- The presence of liposomes in the formulation resulted in sustained release of the drug in the perilymph for 30 days and promoted the conversion of the prodrug loaded within the liposomes (dexamethasone phosphate) into its active form (dexamethasone).

- A small amount of intact liposomes was visualized in the perilymph, whereas the main proportion of liposomes seemed to be trapped in the round window resulting in a reservoir effect.

We thank the Reviewer for his/her comment and added the reference as suggested.

6. The quality of the figure entitled “Inner ear nanoparticles delivery” is not good. It is difficult to read the legend.

We thank the Reviewer for his/her comment and improved the quality of the figure legends.

Reviewer 2

The manuscript focuses on recent findings and applications of different nanoparticle-based systems locally administered in the inner ear. This review is extensive and interesting, thus is worth be publication. There are several issues to be addressed:

It might be worthwhile to at least briefly review the disadvantage of local delivery to the inner ear in the first paragraph of “Introduction”.

We modified the sentence as suggested by the Reviewer.

The hierarchy of the manuscript should be reconstructed to be quite clearer, i.e. PLGA NPs don’t belong to the polymersomes and should be separated from “4.3. Polymersomes”.

We agree with the Reviewer that PLGA NPs are not polymersomes *sensu stricto*, therefore we corrected the title of the paragraph as “4.3 Polymersomes and copolymers” and specified their classification as copolymers in the text of the paragraph.

Keywords: “intratympanic” should be “intratympanic administration”.

We corrected the keyword according to the Reviewer’s suggestion

Reviewer 3

The review submitted by Filippo Valente et al. deals with different nanoparticles (NPs) used to administer drugs to the inner ear for the prevention of hearing loss.

After a short introduction into inner ear physiology, the different administration routes have been discussed. Since a minimally invasive technique seems to be promising, the review is focused on intratympanic administration of NPs. Subsequently, seven types of NPs are discussed giving examples from recent findings. Finally, a discussion about key aspects of inner ear drug delivery with NPs is presented.

The article gives a good overview on recent findings providing an interesting insight into new research. Also, the article is within the scope of the journal’s topics and written according to the journal’s standards pointed out in the guidelines for submission.

Minor remarks:

I did not find the legends of the figures. Please add them in the final version. Since the legend is missing, I cannot give comments on the figures.

We thank the Reviewer for his/her remark. We checked on the journal site and could not find the file containing the figure captions, probably because of problems in uploading manuscript files. We therefore enclose a copy of the file containing the figure captions, as reference for the Reviewer and for the Editor.

Page 2, line 29: “…administered in the inner ear.”: I would replace “inner” by “middle” ear or “to deliver drugs to the inner ear” because with intratympanic administration normally the drug is administered in the middle ear cavity.

We corrected the sentence according to the Reviewer’s suggestion.

Page 3, line 50: Could you precise what do you mean by “perilymph rheology” and why it is “facilitating the distribution”?

Research from Stefan Plontke and Alec N. Salt indicate the opposite: distribution of a drug inside the cochlea is even hindered so that a large concentration gradient is observed when the drug is administered intratympanically.

The term “rheology” indicates the flow of the matter in the liquid state, thus it can be applied to perilymph. We accordingly modified the sentence on drug distribution in the perilymph.

Page 3, line 51: The term used frequently is “Noise Induced Hearing Loss” or only “Hearing Loss”.

We corrected the sentence according to the Reviewer’s suggestion.

Page 4, line 91: “…induced side effects are developed.” Delete “induced”.

We deleted the word “induced” from the sentence.

Page 6, line 131: Change the term “middle ear barrier”. Before, you have written “blood inner ear barrier (BB)” (page 3, line 62) and in my opinion it might be confusing if you name the RWM “middle ear barrier”.

We corrected the sentence according to the Reviewer’s suggestion.

Page 8, line 191: “PLGA” is singular, if you want to use plural in the following paragraph replace by “PLGA nanoparticles” or something similar. Please consider this remark for the whole paragraph until page 9, line 207.

According to the Reviewer’s suggestion, we added “NP” (nanoparticles) where required.

Page 11, line 242: Please write out “FITC” at least once.

Following the Reviewer’s suggestion, we substituted “FITC” with “fluorescein isothiocyanate”

Please correct the references:

17, 70 – Author names

26 – Points after author names

65, 70 – abbreviation of the journal

We corrected all references according to the Reviewer's suggestion

Answer to Reviewers

Reviewer 1

The review presented by Valente et al « Nanoparticle drug delivery systems for inner ear therapy: an overview" is well-written and interesting.

I have the following minor comments/suggestions regarding the manuscript.

1. Lines 43-44 "The NP with size between 10 and 100 nm are useful for application in biology and medicine for innovative DD systems". This range is a little bite too restrictive. The range 10 to 200 nm is commonly used.

We thank the Reviewer for his/her comment and corrected the sentence according to the suggestion.

2. Line 53, please introduce the references of the reviews

According to the Reviewer's suggestion, we introduced the references in the sentence.

3. Paragraph 2. Ear barrier

Drugs can also enter the inner ear via the oval window, another semi-permeable membrane located in the middle ear. It is important to mention in the paper the oval window as another route of penetration.

As suggested by the Reviewer, we mentioned the oval window together with the other inner ear barriers.

4. Lines 190 to 191 "Recently, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved PLGA for parenteral administration (48)". Please remove recently because PLGA microspheres and PLGA implants for the treatment of prostate cancer were marketed at the end of the eighties.

We deleted "recently" according to the Reviewer's suggestion.

5. Paragraph 5. Key aspects for nanoparticles-based delivery in the inner ear

Lines 279 to 282. Supermagnetic iron oxide NPs (SPIONs) particles are not biodegradable. Please, add a comment on the long persistence of these particles in the inner ear and their potential toxicity due to their accumulation in this compartment.

We changed the sentence according to the Reviewer's suggestion.

Lines 298 to 300 "The incorporation of loaded NP into hydrogels could increase their residence time in the middle ear, thus enhancing drug release in the perilymph». Please, add the reference El Kechai et al., "Hyaluronic acid liposomal gel sustains delivery of a corticoid to the inner ear, Journal of Controlled release, 2016, 226, 248-257". This paper presents in vivo results on pegylated liposomes dispersed in hyaluronic hydrogel following injection in the middle ear. This recent paper completes examples on liposomes.

- A prolonged residence time at the site of injection as well as in the round window were achieved without any negative effect on the hearing thresholds of the animals.

- The presence of liposomes in the formulation resulted in sustained release of the drug in the perilymph for 30 days and promoted the conversion of the prodrug loaded within the liposomes (dexamethasone phosphate) into its active form (dexamethasone).

- A small amount of intact liposomes was visualized in the perilymph, whereas the main proportion of liposomes seemed to be trapped in the round window resulting in a reservoir effect.

We thank the Reviewer for his/her comment and added the reference as suggested.

6. The quality of the figure entitled "Inner ear nanoparticles delivery" is not good. It is difficult to read the legend.

We thank the Reviewer for his/her comment and improved the quality of the figure legends.

Reviewer 2

The manuscript focuses on recent findings and applications of different nanoparticle-based systems locally administered in the inner ear. This review is extensive and interesting, thus is worth be publication. There are several issues to be addressed:

It might be worthwhile to at least briefly review the disadvantage of local delivery to the inner ear in the first paragraph of "Introduction".

We modified the sentence as suggested by the Reviewer.

The hierarchy of the manuscript should be reconstructed to be quite clearer, i.e. PLGA NPs don't belong to the polymersomes and should be separated from "4.3. Polymersomes".

We agree with the Reviewer that PLGA NPs are not polymersomes *sensu stricto*, therefore we corrected the title of the paragraph as "4.3 Polymersomes and copolymers" and specified their classification as copolymers in the text of the paragraph.

Keywords: "intratympanic" should be "intratympanic administration".

We corrected the keyword according to the Reviewer's suggestion

Reviewer 3

The review submitted by Filippo Valente et al. deals with different nanoparticles (NPs) used to administer drugs to the inner ear for the prevention of hearing loss.

After a short introduction into inner ear physiology, the different administration routes have been discussed. Since a minimally invasive technique seems to be promising, the review is focused on intratympanic administration of NPs. Subsequently, seven types of NPs are discussed giving examples from recent findings. Finally, a discussion about key aspects of inner ear drug delivery with NPs is presented.

The article gives a good overview on recent findings providing an interesting insight into new research. Also, the article is within the scope of the journal's topics and written according to the journal's standards pointed out in the guidelines for submission.

Minor remarks:

I did not find the legends of the figures. Please add them in the final version. Since the legend is missing, I cannot give comments on the figures.

We thank the Reviewer for his/her remark. We checked on the journal site and could not find the file containing the figure captions, probably because of problems in uploading manuscript files. We therefore enclose a copy of the file containing the figure captions, as reference for the Reviewer and for the Editor.

Page 2, line 29: "…administered in the inner ear.": I would replace "inner" by "middle" ear or "to deliver drugs to the inner ear" because with intratympanic administration normally the drug is administered in the middle ear cavity.

We corrected the sentence according to the Reviewer's suggestion.

Page 3, line 50: Could you precise what do you mean by "perilymph rheology" and why it is "facilitating the distribution"?

Research from Stefan Plontke and Alec N. Salt indicate the opposite: distribution of a drug inside the cochlea is even hindered so that a large concentration gradient is observed when the drug is administered intratympanically.

The term "rheology" indicates the flow of the matter in the liquid state, thus it can be applied to perilymph. We accordingly modified the sentence on drug distribution in the perilymph.

Page 3, line 51: The term used frequently is "Noise Induced Hearing Loss" or only "Hearing Loss".

We corrected the sentence according to the Reviewer's suggestion.

Page 4, line 91: "…induced side effects are developed." Delete "induced".

We deleted the word "induced" from the sentence.

Page 6, line 131: Change the term “middle ear barrier”. Before, you have written “blood inner ear barrier (BB)” (page 3, line 62) and in my opinion it might be confusing if you name the RWM “middle ear barrier”.

We corrected the sentence according to the Reviewer’s suggestion.

Page 8, line 191: “PLGA” is singular, if you want to use plural in the following paragraph replace by “PLGA nanoparticles” or something similar. Please consider this remark for the whole paragraph until page 9, line 207.

According to the Reviewer’s suggestion, we added “NP” (nanoparticles) where required.

Page 11, line 242: Please write out “FITC” at least once.

Following the Reviewer’s suggestion, we substituted “FITC” with “fluorescein isothiocyanate”

Please correct the references:

17, 70 – Author names

26 – Points after author names

65, 70 – abbreviation of the journal

We corrected all references according to the Reviewer’s suggestion

Nanoparticle drug delivery systems for inner ear therapy: an overview

Filippo Valente[1*], Laura Astolfi[1,2*], Edi Simoni[1], Serena Danti[3], Valeria Franceschini[2,4], Milvia Chicca[5], Alessandro Martini[1,2]

[1]Bioacoustics Research Laboratory, Department of Neurosciences, University of Padua, 35129 Padua, Italy; filippo.valente@studenti.unipd.it; laura.astolfi@unipd.it; edi.simoni@unipd.it; alessandromartini@unipd.it.

[2]Foundation Onlus 'Staminali e Vita', Padua, Italy; valeria.franceschini@unibo.it.

[3]Department. of Civil and Industrial Engineering, University of Pisa, Pisa, Italy; s.danti@med.unipi.it.

[4]Department of Biological, Geological and Environmental Sciences, University of Bologna, Bologna, Italy.

[5]Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy. milvia.chicca@unife.it.

*Both authors contributed equally to this work

Corresponding author

Dr. Laura Astolfi,

Bioacoustics Research Laboratory,

Department of Neurosciences,

University of Padua, 35129 Padua, Italy.

E-mail: laura.astolfi@unipd.it

Abstract

Local drug delivery based on nanoparticles (NP) represents a novel strategy to improve inner ear treatments. The intratympanic delivery of NP may be suitable to treat or prevent hearing loss originating from damage to hair cells and spiral ganglion neurons in the cochlea. Numerous experimental studies support *in vitro* and *in vivo* the biocompatibility of NP, their physical stability, target specificity, cell/tissue uptake and ability to internalize therapeutic agents. The topical use of NP helps to reduce the amount of drug required and avoid systemic side effects. This review focuses on recent findings and applications of different NP systems locally delivered to the inner ear. The perspectives for clinical application of NP in inner ear drug delivery are also discussed.

Keywords

Nanoparticles, inner ear, drug delivery, intratympanic administration, local administration

1. Introduction

The treatment of inner ear diseases through drug delivery (DD) faces numerous challenges (1), among which the limited blood flow to the inner ear (2), the presence of physical barriers acting as a selective filter for drug transportation to the inner ear from the circulatory system (3), the small size of the cochlea and its isolated location in the petrous bone. As a result, research in local drug applications and medications has recently attracted interest because it is a more effective and preferable treatment than the systemic one. Case studies involving steroids (4) and gentamicin treatment for Meniere's disease (5) have been documented, but these approaches could be improved for clinical protocols by the development of controlled and targeted delivery systems.

Nanoparticles (NP) are a possible option to improve existing therapeutic strategies (6). The NP with size between 10 and 200 nm are useful for application in biology and medicine for innovative DD systems. NP-based strategy could be more efficient and reduce drug-associated side effects because

of the ability to deliver the therapeutic agent to the target site. Moreover, the controlled release of compounds conjugated to NP results in a lower dose of drug required to achieve the therapeutic effects (1, 7).

The cochlea is a good model for studying the NP-based DD due to its isolated structure and the perilymph rheology. The intratympanic delivery of NP could be suitable to treat the hearing loss and prevent its progression when hair cells and spiral ganglion neurons are damaged (8).

Several works and reviews have been published in the past decade, focusing on NP type, pathology involved, delivery approach or a combination of these topics (1-4, 6-8). The goal of the present review is to provide an updated general overview of NP-based strategies and their advantages and disadvantages for local DD into the inner ear.

2. Ear barriers

The human inner ear consists of two main parts, the auditory system (the cochlea) and the vestibular system. The cochlea is a bony spiral canal, about 30 mm long and divided in three fluid-filled compartments: the scala tympani, the scala media and the scala vestibuli. The round window membrane (RWM), the blood inner ear barrier (BB) and the oval window are physical barriers that isolate the cochlea from the middle ear and from the circulatory system (Figure 1). The RWM is a three-layer semi-permeable membrane, composed of an outer epithelial cell layer, a middle connection layer and an inner connection layer facing the perilymph of the scala tympani (9). In humans, the variable thickness of RWM affects the response of patients to DD treatments. In animal models, its thickness is different among species but its composition is similar (10).

Both the RWM and the oval window membranes have been investigated for DD, as connections between the middle ear cavity and the cochlear perilymph. The DD strategies for the inner ear currently rely mostly on RWM (11). The passage of molecules across this membrane is not only

influenced by thickness, but also by its morphological integrity, inflammation and weight, concentration, liposolubility and external charge of the therapeutic compound (12). The drugs deposited topically in the middle ear cavity are internalized by pinocytosis and transported to the perilymph through blood vessels or by diffusion. Thus the direct application of drugs in the proximity of RWM is a suitable approach for treatment of inner ear pathologies (13).

The BB is a major barrier in the stria vascularis separating the cochlear tissues from the circulatory system (14). Its role is to maintain the homeostasis of cochlear fluids and protect the inner ear integrity. Its main components are principally the endothelial capillaries whose cells are connected by tight junctions, which lay over a basement membrane. However numerous accessory cells have recently been observed in the complex structure of the barrier, such as perycites and perivascular resident macrophage-like (11). The BB has been described to act as a physical and biochemical barrier through an efflux pump, the P-glycoprotein 1 (P-gp) (15). The BB is therefore considered a rate-limiting barrier in the passage of therapeutic agents from the circulatory system to the inner ear. However, the current knowledge about drug transportation processes through BB is still limited (16).

3. Administration routes

The clinical protocols for inner ear therapies mostly rely on systemic and local DD routes. The systemic administration represents a classical route for DD, but in the inner ear only few drugs may reach the target site at therapeutic concentrations. If high doses of systemic drugs are employed, often side effects are developed (17, 18). Systemic applications of NP in inner ear have been recently investigated: poly(lactic-co-glycolic acid) NP conjugated with rhodamine B and applied systemically were detected in the liver, but not in the cochlea (19). The limited bioavailability of NP after systemic administration could be due to the rapid clearance from the circulation in liver and spleen (20).

Local administration appears more suitable for inner ear DD (19). This approach allows a quick distribution of the drug inside the cochlea, improving their delivery to the target site; it also requires lower drug doses, avoiding side effects (21) (Figure 2). Two main routes are presently used for this purpose, the intratympanic (IT) or the intracochlear administration, but the second one is rarely performed because it is highly invasive and limited to surgery cases (22). On the contrary, the IT injection is minimally invasive and relies on passive diffusion of the active molecules through RWM to access the inner ear. This review focuses on development of these methods for DD with minimal trauma for the cochlea. However, local delivery trials show a high variability in results (23) because of some key factors: 1) the drug clearance within the middle ear through the Eustachian tube; 2) the permeability of RWM; and 3) the residence time of the drug in contact with RWM (24). A method to reduce variability of results and increase the drug concentration in the perilymph could be to better control the residence time of the drug at close range with RWM, using specific delivery systems based on NP (25).

4. Nanoparticle-based systems

The NP (also called nanocarriers or nanovectors) are artificial compounds with size at the nanoscale, which aim to compensate for adverse drug properties such as low solubility, degradation and short half-life (26). The NP may also be adapted to target a specific tissue of the inner ear. However, when injected in the middle ear as a liquid suspension, NP will undergo clearance through the Eustachian tube (27), thus significantly reducing their residence time near RWM. The NP suitable for DD systems should therefore increase the residence time, together with the ability to cross RWM and their biocompatibility (Figure 3). A detailed description of physico-chemical characteristics of NP and their applications is reported.

4.1. Lipid Core NP

Lipid Core NP (LCN) possess a lipid core matrix (usually triglycerides) with a surrounding shell of lecithin, polyethylene glycol or poloxamers as stabilizing agents. The LCN structure can be changed to include different drugs and control the kinetics of drug release (28). It has been shown to be stable up to six months in aerosol dispersion (29). These NPs did not induce toxicological effects *in vivo* in mice after systemic applications (12 mg/kg intravenously for five days) (30) and their cell uptake and cell viability was *in vitro* verified on fibroblasts by confocal scanner laser microscopy (31). In rat animal models LCN were able to cross RWM and reach inner ear targets after middle ear application *in vivo*, while not affecting hearing capacity (32). Their preferred pathway to diffuse inside the cells was also investigated: they followed a "nerve pathway", diffusing from the perilymph in the scala tympani to the spiral ganglion, nerve fibres and later approaching the inner and the outer hair cells (33). Their variability in diffusion and ability to cross RWM depends on their lipid composition, size and external charge. The ability to cross the RWM has been shown to be size-dependent, because the percentage of particle diffusion was inversely proportional to their size (31). Surface charge may also affect the uptake and biodistribution of LNC. Some NP candidates based on glycerol mono-oleate were studied under different external charges: after an *in vivo* application to RWM, LCN expressing stronger positive charges were detected in the deeper turns of the cochlea (34). The LCN were also tested as a drug carrier, delivering dexamethasone in the inner ear through IT injection and comparing the results with a systemic application of the same LCN. The amount of dexamethasone detected in cochlear fluid after local LCN application was significantly higher compared to the systemic application, also increasing the half-life and the average residence time of the drug in the perilymph by 1.9 folds (35). All these results indicate a great potential for LCN for sustained drug release and targeting of inner ear tissues after local administration.

4.2. Liposomes

144 Liposomes are artificial phospholipid bilayers, similar to those found in the cell membrane, but
145 surrounding an aqueous core. They exhibit a wide size range (between 50 nm and 5 μm) and
146 morphology, depending on the phospholipid used and the preparation method (36). Liposomes can
147 encapsulate either hydrophobic molecules in the phospholipid bilayer or hydrophilic molecules in
148 their aqueous core (37). The uptake of these NP *in vitro* or *in vivo* usually relies on the passive
149 diffusion inside the cells, but their surface can be modified with polyethylene glycol, antibodies,
150 peptides, carbohydrates, hyaluronic acid and folic acid (35). Such modified liposomes successfully
151 targeted cells expressing tropomyosin receptor-B (TrkB) by using 18-mer peptides to promote
152 cellular uptake (38). Liposomes labelled with fluorescent markers applied *in vivo* to a mouse model
153 with a single IT injection were identified in all cochlear turns, with a concentration gradient
154 decreasing from the base to the apex and, to a lesser extent, in the lateral wall and in the organ of
155 Corti. No morphological or functional damages to the inner ear were detected 24 hours after the
156 application (8). Disulfiram, a neurotoxic agent, was used as model payload for DD analysis: NP
157 loaded with Disulfiram damaged the spiral ganglion 48 hours after application, with an associated
158 threshold shift reaching 35 dB. No significant effects were observed with a similar application of a
159 pure Disulfiram solution (8). To test the drug delivery efficiency of liposome nanocarriers, NP of
160 different size (95, 130, 240 nm) encapsulating the contrast agent gadolinium-tetra-azacyclo-
161 dodecane-tetra-acetic acid (Gd-DOTA) were applied in the middle ear and analyzed with MRI: the
162 results showed that the liposome carrier efficiency was inversely proportional to NP size (39, 40).

163 **4.3. Polymersomes and copolymers**

164 The polymersomes (also called multifunctional NP) are a wide class of amphiphilic copolymers,
165 consisting of a self-assembled membrane of hydrophobic units, surrounding an aqueous core, and of
166 a hydrophilic corona (41). Structurally they are similar to liposomes, with the advantages that the
167 membrane thickness can be controlled by the molecular weight of the hydrophobic block of
168 copolymer to achieve stronger, thicker and more stable membranes. The hydrophilic corona can be

169 modified to regulate the biodistribution of polymersomes and induce specific cellular uptake (42).
170 Hydrophilic drugs can be loaded in the core, while hydrophobic ones in the membrane (43).

171 Different multifunctional polymersomes were studied for inner ear DD targeting specific tissue or
172 conjugated with ferromagnetic materials.

173 In a mouse model, poly(ethylene glycol)-*b*-poly (ε-caprolactone) NP (PEG-*b*-PCL) labeled with
174 fluorescent markers were detected in the spiral ganglion, in the organ of Corti and in the lateral wall
175 after 24 hours from RWM application *in vivo* (8). Tissue specificity was also investigated: PEG-*b*-
176 PCL were conjugated with a nerve growth factor derived peptide and tested *ex-vivo* on explanted
177 mouse cochleae and *in vitro* on PC12 cells. No significant toxic effect was observed and a specific
178 targeting to spiral ganglion neurons, Schwann cells and nerve fibres was achieved by conjugating
179 the NP with tyrosin kinase and p75 neurotrophin receptors (44).

180 Poly(2-hydroxyethyl aspartamide) NP (PHEA) were observed to enter *in vitro* the immortalized
181 mouse organ of Corti cell line (HEI-OC1) and the human middle ear cell line (HMEEC). When
182 applied *in vivo* near the RWM in a mouse model, PHEA were also detected in the inner ear tissue
183 (45). In order to improve NP uptake, PHEA were modified with oligoarginine peptide, a positively
184 charged copolymer, and conjugated with fluorescent Nile red as a hydrophobic model drug (46). In
185 these conditions the NP uptake *in vitro* on HEI-OC1 and HMEEC cells was significantly improved
186 after 15 and 24 hours, compared to pure Nile red solution. Modified PHEA were detected after 24
187 hours from application in the inner hair cells and supporting cells (47).

188 Poly(lactic-co-glycolic acid) (PLGA) NP are copolymers among the novel carrier developed for
189 DD. The Food and Drug Administration (FDA) and the European Medicines Agency (EMA)
190 approved PLGA NP for parenteral administration (47). PLGA NP are interesting because of their
191 hydrophilicity, biocompatibility and easy derivatization by functional groups on the surface or
192 inside the polymer. Their surface may be modified for target specificity by PEGylation, chitosan

193 absorption and binding of antibodies and oligopeptides (48), and different molecules (proteins,
194 steroids, antibiotics and nucleic acids) have been successfully encapsulated and delivered by PLGA
195 NP (49). Programmed degradation of the polymer may therefore yield quantitative delivery of
196 drugs, plasmids or other bioactive molecules. The PLGA NP tested in the inner ear were first
197 conjugated with rhodamine B, a red fluorescent dye, and applied via IT injection: they were
198 identified in the scala tympani, showing that PLGA NP are able to cross RWM by diffusion and
199 their clearance depends on the perilymph flow rate (19). A quantitative pharmacokinetic study
200 recently showed that PLGA NP applied locally *in vivo* in Guinea pigs significantly improved the
201 drug distribution within the inner ear (52). When PLGA NP were loaded with the fluorescent dye
202 coumarin-6 and applied through IT injection, the concentration of the compound after 96 hours
203 from treatment was 10.9-fold higher in the perilymph than when administered in pure solution.
204 Similar results were obtained for other therapeutic payloads such as antioxidants and antiapoptotic
205 drugs (50). Thus PLGA NP are an useful DD system for inner ear because of their high versatility
206 in adaptation to drug properties and tissue targets (51).

207

208 **4.5. Silica NP**

209 Silica NP are modified colloidal silica particles (52) used to transfect *in vitro* plasmid DNA (53) but
210 also as a DD system (54). A pilot study in mice tested the efficacy of diffusion of Cy3-labeled silica
211 NP administered near the RWM: these NP were found inside the inner hair cells, the vestibular hair
212 cells, the spiral ganglion neurons and the supporting cells, without any hearing impairment. Since
213 the NP also reached the dorsal cochlear nucleus and the superior olivary complex, the authors
214 suggested a retrograde axonal transport and concluded that silica NP could be applied for safe drug
215 deliver in the auditory system (55).

216 **4.6. Supermagnetic iron oxide NPs (SPIONs)**

Magnetic NP are synthetic Fe_3O_4 (magnetite) particles, with a core diameter around 15 nm, that can be widely applied for magnetic targeting of cells (56). Unlike large ferromagnetic materials, the smaller supermagnetic iron oxide NP (SPION) are characterized by the absence of residual magnetic interactions when the magnetic field is not active, thus they are more suitable for biomedical applications (57). The SPION derivatized to increase biocompatibility and cell interactions could be guided by an external magnetic field to a specific biological target, but they cannot encapsulate any drug (58). For *in vivo* applications, to prevent particle aggregation and favour dispersion SPION were coated by organic compounds (59). In inner ear drug delivery, SPION have been encapsulated in PLGA (60), silica (58) and dextran (61) and their biocompatibility was tested and verified *in vitro* and *in vivo* (59). The mobility of SPION induced by a magnetic field was also quantified and the results of flux density, gradients and NP properties were compared between *in vitro* and *in vivo* models (62). The magnetic force required for SPION to cross RWM *in vivo* in Guinea pigs was significantly lower than that of the *in vitro* RWM model (63). Another study *in vivo* in Guinea pigs revealed that the concentration of coated SPION inside the cochlea significantly increased (330% above control) when a magnetic field was active (64). Recently, SPION coated with PGLA NP were tested as drug carriers with dexamethasone-acetate (Dex-Ac) as a payload: the levels of Dex-Ac detected in the inner ear fluids after 1 hour from treatment were significantly higher compared with those in absence of a magnetic field (65). All these results support the application of SPION for inner ear drug delivery protocols.

4.7. Hyperbranched poly-L-lysine NP

Hyperbranched poly-L-lysine (HBPL) are high cationic charged dendrimers widely used for non-viral gene transfer (67, 68). The HBPL were applied *in vivo* in Guinea pig inner ears without any sign of cell toxicity or permanent hearing loss (31): they were detected in the stria vascularis and hair cells (31). Nanoparticles based on HBPL and conjugated with fluorescein isothiocyanate were

242 tested *ex-vivo* on freshly frozen human temporal bones, placing them near the intact RWM: HBPL
243 were detected in hair cells, nerve fibres and other cochlear tissues (66).

244

245 **5. Key aspects for nanoparticle-based drug delivery in the inner ear**

246 There are several key parameters to consider for NP-based local DD in the inner ear: the RWM
247 permeability; the NP cochlear targeting; their payload ability and the controlled drug release; their
248 biocompatibility and their stability in cochlear fluids and tissues. All these aspects were evaluated
249 with different NP systems *in vitro, ex-vivo* or *in vivo* in animal models. However, studies on their
250 therapeutic efficacy are still in progress (26).

251 The RWM is considered the main access to the inner ear after the administration in the middle ear
252 (67). NP with different composition and size between 10 and 640 nm were able to cross RWM. The
253 size and the surface charge are determinant factors that affect NP diffusion through RWM. The
254 number of NP crossing from middle ear to inner ear was inversely proportional to lipid NP size (39)
255 and in the cochlea the positively charged glycerol mono-oleate NP achieved a larger distribution
256 than neutral or negatively charged ones (34). The process responsible for this passage was firstly
257 described for lipid NPs as a paracellular pathway (33). Recent studies in rat RWM suggested that
258 the passage of liposome NP may occur either via the paracellular pathway or by endocytotic
259 mechanisms based on clathrin and caveolin (8).

260 In most studies NP were loaded or labelled with a fluorescent dye (Rhodamine B, Carboxycyanine,
261 Nile-red) or a contrast agent (gadolinium) for visualization of particles in cochlear cells or fluids by
262 imaging techniques: however, the reported data mostly detected the presence of NP in inner ear
263 tissues without a quantitative analysis. Liposome NP were detected in RWM until 11 days and in
264 the cochlea until 6 days post-injection (67); lipid nanocapsules were detected in the cochlea until 7
265 days post-injection (33). However, for treatment of sensorineural hearing loss, the cochlear target

cells are hair cells and spiral ganglion neurons: all populations are selectively reached by the functionalized NP tested (51). For example, the NP functionalized with nerve growth factor-derived peptides showed specificity for spiral ganglion neurons and nerve fibres (44).

The ability of NP to carry the drugs into the cochlea through the RWM was shown *in vivo* by several studies. Smaller supermagnetic iron oxide NP (SPION) coated with PGLA and conjugated with dexamethasone enabled the release of the drug in inner ear fluids, resulting in a higher concentration of dexamethasone in the perilymph compared to the pure drug diffusion (10% higher after 60 minutes, $p<0.01$) (65). The PLGA loaded with coumarin-6 enhanced up to 10.9 times the local bioavailability of the dye in the perilymph in comparison to pure drug solution (50). When the neutoxic agent disulfiram was loaded on liposomes and polymersomes, the number of spiral ganglion cells significantly decreased two days after administration (8). However, drug release by NP has not yet been examined by long-term studies.

Biocompatibility is one of the major concerns in NP clinical applications. Up to date no hearing impairment, loss of hair cells or histological damages were reported (31, 32, 45, 68), thus NP systems appear reasonably safe. However, SPION tend to aggregate when the magnetic field is removed and the long persistence of these nanoparticles on the inner ear may induce toxicity due to accumulation (8). The effects of LNC were evaluated 20 days post-injection and no toxicity was detected (67). Topic applications of liposomes in rats did not affect hearing, but a NP concentration-dependent toxicity was observed *in vitro* in primary cochlear cell cultures (32). A possible explanation was that a NP overload occurred in these cells, resulting in cytoplasm condensation and cell function impairment (69). In most of these studies a single intratympanic administration was employed: recently, in order to improve liposome efficiency, a continuous NP release was obtained through a high-performance polymide tubing (HPPT) equipped with an ALZET© micro-pump (DURECT Corp, CA; USA). Liposomes loaded with gadolinium-tetra-azacyclo-dodecane-tetra-acetic acid were visualized both *in vitro* by TEM and *in vivo* by MRI. *In vitro*, intact NP were free

291 to diffuse in the medium, and *in vivo* were detected in the cochlea without adverse effects within six
292 days (67). Again, no long-term effects of exposure to NP after multiple applications in the inner ear
293 have yet been evaluated.

294 The residence time of the drug within the middle ear cavity may be increased by NP, but this does
295 not guarantee direct contact between the loaded NP and the RWM, because the RWM is the access
296 point for the inner ear in the case of trans-tympanic administration. The NP also undergo middle ear
297 clearance through the Eustachian tube (70). A possible strategy to bypass these limits could be to
298 combine different DD systems together, for example using hydrogels. The incorporation of loaded
299 NP into hydrogels could increase their residence time in the middle ear, thus enhancing drug release
300 in the perilymph (26, 71).The hydrogel is applied near the RWM and releases the loaded NP in the
301 perilymph along with its degradation. This approach has been recently reported: a poloxamer 407
302 hydrogel combined with SPION was successfully applied on *ex vivo* models (human temporal
303 bones and explanted mouse inner ear cultures) (72). More recently, a nanohydrogel based on
304 chitosan polymer incorporating liposomes was tested *in vitro* and *in vivo* in the mouse model (73).
305 The *in vitro* results showed that NP persisted without significant degradation for at least two weeks
306 and were released in a controlled and continuous way by the nanohydrogel. The *in vivo* results
307 showed that the NP were successfully released by the nanohydrogel across the RWM and were able
308 to reach the perilymph and Organ of Corti cells (73).

309 Although NP research in local DD for inner ear therapy appears promising, there are still many
310 difficulties to overcome, mostly related to the inner ear anatomy, to the complexity of the cochlea
311 and its highly differentiated cell populations, as well as the possibility to cause hearing loss using
312 microsurgical approaches. The *in vivo* analyses of perilymph samples represent a technical
313 challenge (27), because of the small volume of inner ear fluids in animal models (the total volume
314 of perilymph in a Guinea pig amounts to about 10 µl) (74), and the possible contamination of
315 samples by cerebrospinal fluid (27). The pharmacokinetics of drugs in the inner ear is therefore still

316 unclear and there are no reliable quantitative data about local bioavailability, drug distribution and
317 RWM permeability (26). Only recently, a computer pharmacokinetic for inner ear fluids and drug
318 distribution has been developed (75). The software (Cochlear Fluids Simulator V3.083) outlines a
319 model based on inner ear anatomy in humans and rodents, pharmacokinetic and solute distribution
320 parameters. This model has been used to simulate the distribution of therapeutic drugs and other
321 compounds in the perilymph (24, 76). However, because of intraspecific variability of animal
322 models in the volume of inner ear fluids and RWM thickness and conditions, it is difficult to
323 compare the current studies using different DD systems and to draw quantitative conclusions about
324 drug pharmacokinetics in the inner ear.

325

326 **6. Conclusions and Future Perspectives**

327 The NP-based systems show a high potential for inner ear delivery of various therapeutic agents.
328 Their use could minimize the side effects of treatments, allow target specificity and provide a
329 sustained release of drugs in inner ear fluids. The type of NP may be adapted to the drug to be
330 carried and different formulations have been tested. The NP could also be combined with other
331 nanomaterials, such as hydrogels, to improve the local application of drugs. However, several
332 problems have yet to be solved and more *in vivo* studies are necessary to verify their bioavailability
333 and effectiveness before a successful clinical application.

334

335

Acknowledgements

We are grateful to the Foundation Onlus 'Staminali e Vita' (Padua, Italy) for providing support.

Funding

This work was supported by the Italian Ministry of Education, University and Research (MIUR), Italy [grant n. PRIN- 2010S58B38_004].

References

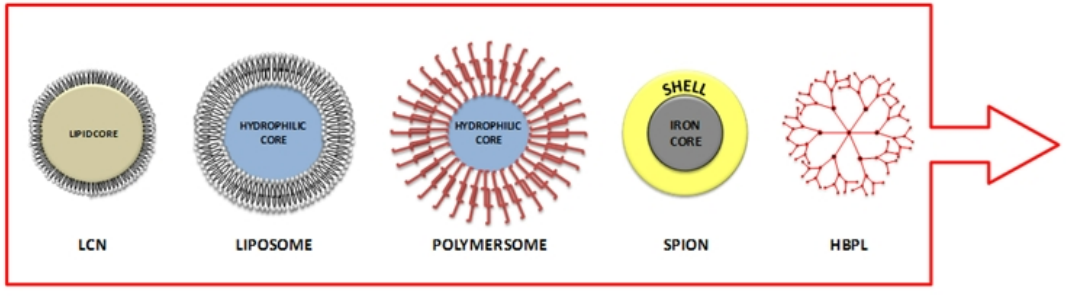
1. Chen G, Zhang X, Yang F and Mu L: Disposition of nanoparticle-based delivery system via inner ear administration. Curr Drug Metab 11: 886-897, 2010.
2. Juhn SK and Rybak LP: Labyrinthine barriers and cochlear homeostasis. Acta Otolaryngol 91: 529-534, 1981.
3. Inamura N and Salt AN: Permeability changes of the blood-labyrinth barrier measured in vivo during experimental treatments. Hear Res 61: 12-18, 1992.
4. Lefebvre PP and Staecker H: Steroid perfusion of the inner ear for sudden sensorineural hearing loss after failure of conventional therapy: a pilot study. Acta Otolaryngol 122: 698-702, 2002.
5. Minor LB, Schessel DA and Carey JP: Meniere's disease. Curr Opin Neurol 17: 9-16, 2004.
6. Malam Y, Loizidou M and Seifalian AM: Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. Trends Pharmacol Sci 30: 592-599, 2009.
7. Gelperina S, Kisich K, Iseman MD and Heifets L: The potential advantages of nanoparticle drug delivery systems in chemotherapy of tuberculosis. Am J Respir Crit Care Med 172: 1487-1490, 2005.
8. Buckiova D, Ranjan S, Newman TA*, et al*: Minimally invasive drug delivery to the cochlea through application of nanoparticles to the round window membrane. Nanomedicine (Lond) 7: 1339-1354, 2012.
9. Banerjee A and Parnes LS: The biology of intratympanic drug administration and pharmacodynamics of round window drug absorption. Otolaryngol Clin North Am 37: 1035-1051, 2004.
10. Goycoolea MV and Lundman L: Round window membrane. Structure function and permeability: a review. Microsc Res Tech 36: 201-211, 1997.
11. Shi X: Pathophysiology of the cochlear intrastrial fluid-blood barrier (review). Hear Res 338: 52-63, 2016.
12. Goycoolea MV: The round window membrane under normal and pathological conditions. Acta Otolaryngol Suppl 493: 43-55, 1992.
13. Salt AN: Pharmacokinetics of Drug Entry into Cochlear Fluids. Volta Rev 105: 277-298, 2005.
14. Jahnke K: [Permeability barriers of the inner ear. Fine structure and function]. Fortschr Med 98: 330-336, 1980.

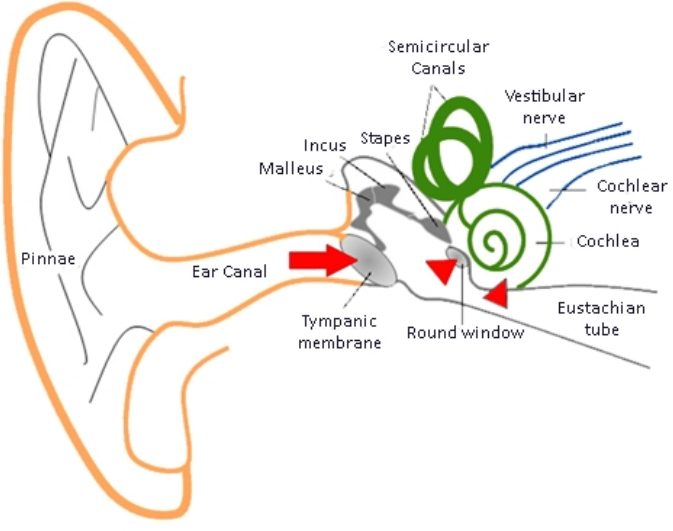
15. Saito T, Zhang ZJ, Tokuriki M, *et al*: Expression of p-glycoprotein is associated with that of multidrug resistance protein 1 (MRP1) in the vestibular labyrinth and endolymphatic sac of the guinea pig. Neurosci Lett 303: 189-192, 2001.
16. Liu H, Hao J, Li KS : Current strategies for drug delivery to the inner ear. Acta Pharmaceutica Sinica B 3: 86-96, 2013.
17. Swan EE, Mescher MJ, Sewell WF, Tao SL and Borenstein JT: Inner ear drug delivery for auditory applications. Adv Drug Deliv Rev 60: 1583-1599, 2008.
18. McCall AA, Swan EE, Borenstein JT, Sewell WF, Kujawa SG and McKenna MJ: Drug delivery for treatment of inner ear disease: current state of knowledge. Ear Hear 31: 156-165, 2010.
19. Tamura T, Kita T, Nakagawa T, *et al*: Drug delivery to the cochlea using PLGA nanoparticles. Laryngoscope 115: 2000-2005, 2005.
20. Horie RT, Sakamoto T, Nakagawa T, Ishihara T, Higaki M and Ito J: Stealth-nanoparticle strategy for enhancing the efficacy of steroids in mice with noise-induced hearing loss. Nanomedicine (Lond) 5: 1331-1340, 2010.
21. Bowe SN and Jacob A: Round window perfusion dynamics: implications for intracochlear therapy. Curr Opin Otolaryngol Head Neck Surg 18: 377-385, 2010.
22. De Ceulaer G, Johnson S, Yperman M, *et al*: Long-term evaluation of the effect of intracochlear steroid deposition on electrode impedance in cochlear implant patients. Otol Neurotol 24: 769-774, 2003.
23. Paulson DP, Abuzeid W, Jiang H, Oe T, O'Malley BW and Li D: A novel controlled local drug delivery system for inner ear disease. Laryngoscope 118: 706-711, 2008.
24. Hahn H, Kammerer B, DiMauro A, Salt AN and Plontke SK: Cochlear microdialysis for quantification of dexamethasone and fluorescein entry into scala tympani during round window administration. Hear Res 212: 236-244, 2006.
25. Ciorba A, Astolfi L, Jolly C, Martini A: Cochlear Implants and Inner Ear Based Therapy. European Journal of Nanomedicine 2: 4, 2009.
26. El Kechai N, Agnely F, Mamelle E, Nguyen Y, Ferrary E and Bochot A: Recent advances in local drug delivery to the inner ear. Int J Pharm 494: 83-101, 2015.
27. Salt AN and Plontke SK: Local inner-ear drug delivery and pharmacokinetics. Drug Discov Today 10: 1299-1306, 2005.
28. Jager E, Venturini CG, Poletto FS, *et al*: Sustained release from lipid-core nanocapsules by varying the core viscosity and the particle surface area. J Biomed Nanotechnol 5: 130-140, 2009.
29. Hureaux J, Lagarce F, Gagnadoux F, *et al*: Lipid nanocapsules: ready-to-use nanovectors for the aerosol delivery of paclitaxel. Eur J Pharm Biopharm 73: 239-246, 2009.
30. Hureaux J, Lagarce F, Gagnadoux F, *et al*: Toxicological study and efficacy of blank and paclitaxel-loaded lipid nanocapsules after i.v. administration in mice. Pharm Res 27: 421-430, 2010.
31. Scheper V, Wolf M, Scholl M, *et al*: Potential novel drug carriers for inner ear treatment: hyperbranched polylysine and lipid nanocapsules. Nanomedicine (Lond) 4: 623-635, 2009.
32. Zhang Y, Zhang W, Lobler M, *et al*: Inner ear biocompatibility of lipid nanocapsules after round window membrane application. Int J Pharm 404: 211-219, 2011.
33. Zou J, Saulnier P, Perrier T, *et al*: Distribution of lipid nanocapsules in different cochlear cell populations after round window membrane permeation. J Biomed Mater Res B Appl Biomater 87: 10-18, 2008.
34. Liu H, Chen S, Zhou Y, *et al*: The effect of surface charge of glycerol monooleate-based nanoparticles on the round window membrane permeability and cochlear distribution. J Drug Target 21: 846-854, 2013.
35. Chen G, Hou SX, Hu P, Hu QH, Guo DD and Xiao Y: [In vitro dexamethasone release from nanoparticles and its pharmacokinetics in the inner ear after administration of the drug-loaded nanoparticles via the round window]. Nan Fang Yi Ke Da Xue Xue Bao 28: 1022-1024, 2008.
36. Bozzuto G and Molinari A: Liposomes as nanomedical devices. Int J Nanomedicine 10: 975-999, 2015.

37. Wang AZ, Langer R and Farokhzad OC: Nanoparticle delivery of cancer drugs. Annu Rev Med 63: 185-198, 2012.
38. Ranjan S, Sood R, Dudas J, *et al*: Peptide-mediated targeting of liposomes to TrkB receptor-expressing cells. Int J Nanomedicine 7: 3475-3485, 2012.
39. Zou J, Sood R, Ranjan S, *et al*: Size-dependent passage of liposome nanocarriers with preserved posttransport integrity across the middle-inner ear barriers in rats. Otol Neurotol 33: 666-673, 2012.
40. Zou J, Sood R, Ranjan S, *et al*: Manufacturing and in vivo inner ear visualization of MRI traceable liposome nanoparticles encapsulating gadolinium. J Nanobiotechnology 8: 32, 2010.
41. Letchford K and Burt H: A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes. Eur J Pharm Biopharm 65: 259-269, 2007.
42. Farokhzad OC and Langer R: Nanomedicine: developing smarter therapeutic and diagnostic modalities. Adv Drug Deliv Rev 58: 1456-1459, 2006.
43. Lin JJ, Ghoroghchian PP, Zhang Y and Hammer DA: Adhesion of antibody-functionalized polymersomes. Langmuir 22: 3975-3979, 2006.
44. Roy S, Johnston AH, Newman TA, *et al*: Cell-specific targeting in the mouse inner ear using nanoparticles conjugated with a neurotrophin-derived peptide ligand: potential tool for drug delivery. Int J Pharm 390: 214-224, 2010.
45. Kim DK, Park SN, Park KH, *et al*: Development of a drug delivery system for the inner ear using poly(amino acid)-based nanoparticles. Drug Deliv 22: 367-374, 2015.
46. Yoon JY, Yang KJ, Kim da E, *et al*: Intratympanic delivery of oligoarginine-conjugated nanoparticles as a gene (or drug) carrier to the inner ear. Biomaterials 73: 243-253, 2015.
47. Kumari A, Yadav SK and Yadav SC: Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Surf B Biointerfaces 75: 1-18, 2010.
48. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A and Preat V: PLGA-based nanoparticles: an overview of biomedical applications. J Control Release 161: 505-522, 2012.
49. Grottkau BE, Cai X, Wang J, Yang X and Lin Y: Polymeric nanoparticles for a drug delivery system. Curr Drug Metab 14: 840-846, 2013.
50. Cai H, Wen X, Wen L, *et al*: Enhanced local bioavailability of single or compound drugs delivery to the inner ear through application of PLGA nanoparticles via round window administration. Int J Nanomedicine 9: 5591-5601, 2014.
51. Pritz CO, Dudas J, Rask-Andersen H, Schrott-Fischer A and Glueckert R: Nanomedicine strategies for drug delivery to the ear. Nanomedicine (Lond) 8: 1155-1172, 2013.
52. Le VH, Thuc CN and Thuc HH: Synthesis of silica nanoparticles from Vietnamese rice husk by sol-gel method. Nanoscale Res Lett 8: 58, 2013.
53. Sameti M, Bohr G, Ravi Kumar MN, *et al*: Stabilisation by freeze-drying of cationically modified silica nanoparticles for gene delivery. Int J Pharm 266: 51-60, 2003.
54. Ahola M, Rich J, Kortesuo P, Kiesvaara J, Seppala J and Yli-Urpo A: In vitro evaluation of biodegradable epsilon-caprolactone-co-D, L-lactide/silica xerogel composites containing toremifene citrate. Int J Pharm 181: 181-191, 1999.
55. Praetorius M, Brunner C, Lehnert B, *et al*: Transsynaptic delivery of nanoparticles to the central auditory nervous system. Acta Otolaryngol 127: 486-490, 2007.
56. Sun C, Lee JS and Zhang M: Magnetic nanoparticles in MR imaging and drug delivery. Adv Drug Deliv Rev 60: 1252-1265, 2008.
57. Cao ZG, Zhou SW, Sun K, Lu XB, Luo G and Liu JH: [Preparation and feasibility of superparamagnetic dextran iron oxide nanoparticles as gene carrier]. Ai Zheng 23: 1105-1109, 2004.
58. Ye F, Laurent S, Fornara A, *et al*: Uniform mesoporous silica coated iron oxide nanoparticles as a highly efficient, nontoxic MRI T(2) contrast agent with tunable proton relaxivities. Contrast Media Mol Imaging 7: 460-468, 2012.

59. Kopke RD, Wassel RA, Mondalek F, *et al*: Magnetic nanoparticles: inner ear targeted molecule delivery and middle ear implant. Audiol Neurootol 11: 123-133, 2006.
60. Ge X, Jackson RL, Liu J, *et al*: Distribution of PLGA nanoparticles in chinchilla cochleae. Otolaryngol Head Neck Surg 137: 619-623, 2007.
61. Mondalek FG, Zhang YY, Kropp B, *et al*: The permeability of SPION over an artificial three-layer membrane is enhanced by external magnetic field. J Nanobiotechnology 4: 4, 2006.
62. Barnes AL, Wassel RA, Mondalek F, Chen K, Dormer KJ and Kopke RD: Magnetic characterization of superparamagnetic nanoparticles pulled through model membranes. Biomagn Res Technol 5: 1, 2007.
63. Zou J, Zhang W, Poe D, *et al*: MRI manifestation of novel superparamagnetic iron oxide nanoparticles in the rat inner ear. Nanomedicine (Lond) 5: 739-754, 2010.
64. Dormer Kea: Magnetically-targeted, technetium 99m-labeled nanoparticles to the inner ear. J Biomed Nanotechnol 4: 10, 2008.
65. Du X, Chen K, Kuriyavar S, *et al*: Magnetic targeted delivery of dexamethasone acetate across the round window membrane in guinea pigs. Otol Neurotol 34: 41-47, 2013.
66. Roy S, Glueckert R, Johnston AH, *et al*: Strategies for drug delivery to the human inner ear by multifunctional nanoparticles. Nanomedicine (Lond) 7: 55-63, 2012.
67. Zou J, Sood R, Zhang Y, Kinnunen PK and Pyykko I: Pathway and morphological transformation of liposome nanocarriers after release from a novel sustained inner-ear delivery system. Nanomedicine (Lond) 9: 2143-2155, 2014.
68. Wu TH, Liu CP, Chien CT and Lin SY: Fluorescent hydroxylamine derived from the fragmentation of PAMAM dendrimers for intracellular hypochlorite recognition. Chemistry 19: 11672-11675, 2013.
69. Moss OR: Insights into the healthy effects of nanoparticles: why numbers matter. Int J Nanotechnol 5: 1-8, 2008.
70. Sheppard WM, Wanamaker HH, Pack A, Yamamoto S and Slepecky N: Direct round window application of gentamicin with varying delivery vehicles: a comparison of ototoxicity. Otolaryngol Head Neck Surg 131: 890-896, 2004.
71. El Kechai N, Mamelle E, Nguyen Y, *et al*: Hyaluronic acid liposomal gel sustains delivery of a corticoid to the inner ear. J Control Release 226: 248-257, 2016.
72. Thaler M, Roy S, Fornara A, *et al*: Visualization and analysis of superparamagnetic iron oxide nanoparticles in the inner ear by light microscopy and energy filtered TEM. Nanomedicine 7: 360-369, 2011.
73. Lajud SA, Nagda DA, Qiao P, *et al*: A novel chitosan-hydrogel-based nanoparticle delivery system for local inner ear application. Otol Neurotol 36: 341-347, 2015.
74. Wang X, Dellamary L, Fernandez R, *et al*: Dose-dependent sustained release of dexamethasone in inner ear cochlear fluids using a novel local delivery approach. Audiol Neurootol 14: 393-401, 2009.
75. Salt AN and Plontke SK: Principles of local drug delivery to the inner ear. Audiol Neurootol 14: 350-360, 2009.
76. Astolfi L, Guaran V, Marchetti N, *et al*: Cochlear implants and drug delivery: In vitro evaluation of dexamethasone release. J Biomed Mater Res B Appl Biomater 102: 267-273, 2014.

INNER EAR NANOPARTICLES DELIVERY





Nanoparticle drug delivery systems for inner ear therapy: an overview

Filippo Valente[1*], Laura Astolfi[1,2*], Edi Simoni[1], Serena Danti[3], Valeria Franceschini[2,4], Milvia Chicca[5], Alessandro Martini[1,2]

[1]Bioacoustics Research Laboratory, Department of Neurosciences, University of Padua, 35129 Padua, Italy; filippo.valente@studenti.unipd.it; laura.astolfi@unipd.it; edi.simoni@unipd.it; alessandromartini@unipd.it.

[2]Foundation Onlus 'Staminali e Vita', Padua, Italy; valeria.franceschini@unibo.it.

[3]Department. of Civil and Industrial Engineering, University of Pisa, Pisa, Italy; s.danti@med.unipi.it.

[4]Department of Biological, Geological and Environmental Sciences, University of Bologna, Bologna, Italy.

[5]Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy. milvia.chicca@unife.it.

*Both authors contributed equally to this work

Corresponding author

Dr. Laura Astolfi,

Bioacoustics Research Laboratory,

Department of Neurosciences,

University of Padua, 35129 Padua, Italy.

E-mail: laura.astolfi@unipd.it

Abstract

Local drug delivery based on nanoparticles (NP) represents a novel strategy to improve inner ear treatments. The intratympanic delivery of NP may be suitable to treat or prevent hearing loss originating from damage to hair cells and spiral ganglion neurons in the cochlea. Numerous experimental studies support *in vitro* and *in vivo* the biocompatibility of NP, their physical stability, target specificity, cell/tissue uptake and ability to internalize therapeutic agents. The topical use of NP helps to reduce the amount of drug required and avoid systemic side effects. This review focuses on recent findings and applications of different NP systems locally delivered to the inner ear. The perspectives for clinical application of NP in inner ear drug delivery are also discussed.

Keywords

Nanoparticles, inner ear, drug delivery, intratympanic administration, local administration

1. Introduction

The treatment of inner ear diseases through drug delivery (DD) faces numerous challenges (1), among which the limited blood flow to the inner ear (2), the presence of physical barriers acting as a selective filter for drug transportation to the inner ear from the circulatory system (3), the small size of the cochlea and its isolated location in the petrous bone. As a result, research in local drug applications and medications has recently attracted interest because it is a more effective and preferable treatment than the systemic one. Case studies involving steroids (4) and gentamicin treatment for Meniere's disease (5) have been documented, but these approaches could be improved for clinical protocols by the development of controlled and targeted delivery systems.

Nanoparticles (NP) are a possible option to improve existing therapeutic strategies (6). The NP with size between 10 and 200 nm are useful for application in biology and medicine for innovative DD systems. NP-based strategy could be more efficient and reduce drug-associated side effects because

of the ability to deliver the therapeutic agent to the target site. Moreover, the controlled release of compounds conjugated to NP results in a lower dose of drug required to achieve the therapeutic effects (1, 7).

The cochlea is a good model for studying the NP-based DD due to its isolated structure and the perilymph rheology. The intratympanic delivery of NP could be suitable to treat the hearing loss and prevent its progression when hair cells and spiral ganglion neurons are damaged (8).

Several works and reviews have been published in the past decade, focusing on NP type, pathology involved, delivery approach or a combination of these topics (1-4, 6-8). The goal of the present review is to provide an updated general overview of NP-based strategies and their advantages and disadvantages for local DD into the inner ear.

2. Ear barriers

The human inner ear consists of two main parts, the auditory system (the cochlea) and the vestibular system. The cochlea is a bony spiral canal, about 30 mm long and divided in three fluid-filled compartments: the scala tympani, the scala media and the scala vestibuli. The round window membrane (RWM), the blood inner ear barrier (BB) and the oval window are physical barriers that isolate the cochlea from the middle ear and from the circulatory system (Figure 1). The RWM is a three-layer semi-permeable membrane, composed of an outer epithelial cell layer, a middle connection layer and an inner connection layer facing the perilymph of the scala tympani (9). In humans, the variable thickness of RWM affects the response of patients to DD treatments. In animal models, its thickness is different among species but its composition is similar (10).

Both the RWM and the oval window membranes have been investigated for DD, as connections between the middle ear cavity and the cochlear perilymph. The DD strategies for the inner ear currently rely mostly on RWM (11). The passage of molecules across this membrane is not only

influenced by thickness, but also by its morphological integrity, inflammation and weight, concentration, liposolubility and external charge of the therapeutic compound (12). The drugs deposited topically in the middle ear cavity are internalized by pinocytosis and transported to the perilymph through blood vessels or by diffusion. Thus the direct application of drugs in the proximity of RWM is a suitable approach for treatment of inner ear pathologies (13).

The BB is a major barrier in the stria vascularis separating the cochlear tissues from the circulatory system (14). Its role is to maintain the homeostasis of cochlear fluids and protect the inner ear integrity. Its main components are principally the endothelial capillaries whose cells are connected by tight junctions, which lay over a basement membrane. However numerous accessory cells have recently been observed in the complex structure of the barrier, such as perycites and perivascular resident macrophage-like (11). The BB has been described to act as a physical and biochemical barrier through an efflux pump, the P-glycoprotein 1 (P-gp) (15). The BB is therefore considered a rate-limiting barrier in the passage of therapeutic agents from the circulatory system to the inner ear. However, the current knowledge about drug transportation processes through BB is still limited (16).

3. Administration routes

The clinical protocols for inner ear therapies mostly rely on systemic and local DD routes. The systemic administration represents a classical route for DD, but in the inner ear only few drugs may reach the target site at therapeutic concentrations. If high doses of systemic drugs are employed, often side effects are developed (17, 18). Systemic applications of NP in inner ear have been recently investigated: poly(lactic-co-glycolic acid) NP conjugated with rhodamine B and applied systemically were detected in the liver, but not in the cochlea (19). The limited bioavailability of NP after systemic administration could be due to the rapid clearance from the circulation in liver and spleen (20).

Local administration appears more suitable for inner ear DD (19). This approach allows a quick distribution of the drug inside the cochlea, improving their delivery to the target site; it also requires lower drug doses, avoiding side effects (21) (Figure 2). Two main routes are presently used for this purpose, the intratympanic (IT) or the intracochlear administration, but the second one is rarely performed because it is highly invasive and limited to surgery cases (22). On the contrary, the IT injection is minimally invasive and relies on passive diffusion of the active molecules through RWM to access the inner ear. This review focuses on development of these methods for DD with minimal trauma for the cochlea. However, local delivery trials show a high variability in results (23) because of some key factors: 1) the drug clearance within the middle ear through the Eustachian tube; 2) the permeability of RWM; and 3) the residence time of the drug in contact with RWM (24). A method to reduce variability of results and increase the drug concentration in the perilymph could be to better control the residence time of the drug at close range with RWM, using specific delivery systems based on NP (25).

4. Nanoparticle-based systems

The NP (also called nanocarriers or nanovectors) are artificial compounds with size at the nanoscale, which aim to compensate for adverse drug properties such as low solubility, degradation and short half-life (26). The NP may also be adapted to target a specific tissue of the inner ear. However, when injected in the middle ear as a liquid suspension, NP will undergo clearance through the Eustachian tube (27), thus significantly reducing their residence time near RWM. The NP suitable for DD systems should therefore increase the residence time, together with the ability to cross RWM and their biocompatibility (Figure 3). A detailed description of physico-chemical characteristics of NP and their applications is reported.

4.1. Lipid Core NP

Lipid Core NP (LCN) possess a lipid core matrix (usually triglycerides) with a surrounding shell of lecithin, polyethylene glycol or poloxamers as stabilizing agents. The LCN structure can be changed to include different drugs and control the kinetics of drug release (28). It has been shown to be stable up to six months in aerosol dispersion (29). These NPs did not induce toxicological effects *in vivo* in mice after systemic applications (12 mg/kg intravenously for five days) (30) and their cell uptake and cell viability was *in vitro* verified on fibroblasts by confocal scanner laser microscopy (31). In rat animal models LCN were able to cross RWM and reach inner ear targets after middle ear application *in vivo*, while not affecting hearing capacity (32). Their preferred pathway to diffuse inside the cells was also investigated: they followed a "nerve pathway", diffusing from the perilymph in the scala tympani to the spiral ganglion, nerve fibres and later approaching the inner and the outer hair cells (33). Their variability in diffusion and ability to cross RWM depends on their lipid composition, size and external charge. The ability to cross the RWM has been shown to be size-dependent, because the percentage of particle diffusion was inversely proportional to their size (31). Surface charge may also affect the uptake and biodistribution of LNC. Some NP candidates based on glycerol mono-oleate were studied under different external charges: after an *in vivo* application to RWM, LCN expressing stronger positive charges were detected in the deeper turns of the cochlea (34). The LCN were also tested as a drug carrier, delivering dexamethasone in the inner ear through IT injection and comparing the results with a systemic application of the same LCN. The amount of dexamethasone detected in cochlear fluid after local LCN application was significantly higher compared to the systemic application, also increasing the half-life and the average residence time of the drug in the perilymph by 1.9 folds (35). All these results indicate a great potential for LCN for sustained drug release and targeting of inner ear tissues after local administration.

4.2. Liposomes

144 Liposomes are artificial phospholipid bilayers, similar to those found in the cell membrane, but
145 surrounding an aqueous core. They exhibit a wide size range (between 50 nm and 5 μm) and
146 morphology, depending on the phospholipid used and the preparation method (36). Liposomes can
147 encapsulate either hydrophobic molecules in the phospholipid bilayer or hydrophilic molecules in
148 their aqueous core (37). The uptake of these NP *in vitro* or *in vivo* usually relies on the passive
149 diffusion inside the cells, but their surface can be modified with polyethylene glycol, antibodies,
150 peptides, carbohydrates, hyaluronic acid and folic acid (35). Such modified liposomes successfully
151 targeted cells expressing tropomyosin receptor-B (TrkB) by using 18-mer peptides to promote
152 cellular uptake (38). Liposomes labelled with fluorescent markers applied *in vivo* to a mouse model
153 with a single IT injection were identified in all cochlear turns, with a concentration gradient
154 decreasing from the base to the apex and, to a lesser extent, in the lateral wall and in the organ of
155 Corti. No morphological or functional damages to the inner ear were detected 24 hours after the
156 application (8). Disulfiram, a neurotoxic agent, was used as model payload for DD analysis: NP
157 loaded with Disulfiram damaged the spiral ganglion 48 hours after application, with an associated
158 threshold shift reaching 35 dB. No significant effects were observed with a similar application of a
159 pure Disulfiram solution (8). To test the drug delivery efficiency of liposome nanocarriers, NP of
160 different size (95, 130, 240 nm) encapsulating the contrast agent gadolinium-tetra-azacyclo-
161 dodecane-tetra-acetic acid (Gd-DOTA) were applied in the middle ear and analyzed with MRI: the
162 results showed that the liposome carrier efficiency was inversely proportional to NP size (39, 40).

163 **4.3. Polymersomes and copolymers**

164 The polymersomes (also called multifunctional NP) are a wide class of amphiphilic copolymers,
165 consisting of a self-assembled membrane of hydrophobic units, surrounding an aqueous core, and of
166 a hydrophilic corona (41). Structurally they are similar to liposomes, with the advantages that the
167 membrane thickness can be controlled by the molecular weight of the hydrophobic block of
168 copolymer to achieve stronger, thicker and more stable membranes. The hydrophilic corona can be

169 modified to regulate the biodistribution of polymersomes and induce specific cellular uptake (42).
170 Hydrophilic drugs can be loaded in the core, while hydrophobic ones in the membrane (43).

171 Different multifunctional polymersomes were studied for inner ear DD targeting specific tissue or
172 conjugated with ferromagnetic materials.

173 In a mouse model, poly(ethylene glycol)-*b*-poly (ε-caprolactone) NP (PEG-*b*-PCL) labeled with
174 fluorescent markers were detected in the spiral ganglion, in the organ of Corti and in the lateral wall
175 after 24 hours from RWM application *in vivo* (8). Tissue specificity was also investigated: PEG-*b*-
176 PCL were conjugated with a nerve growth factor derived peptide and tested *ex-vivo* on explanted
177 mouse cochleae and *in vitro* on PC12 cells. No significant toxic effect was observed and a specific
178 targeting to spiral ganglion neurons, Schwann cells and nerve fibres was achieved by conjugating
179 the NP with tyrosin kinase and p75 neurotrophin receptors (44).

180 Poly(2-hydroxyethyl aspartamide) NP (PHEA) were observed to enter *in vitro* the immortalized
181 mouse organ of Corti cell line (HEI-OC1) and the human middle ear cell line (HMEEC). When
182 applied *in vivo* near the RWM in a mouse model, PHEA were also detected in the inner ear tissue
183 (45). In order to improve NP uptake, PHEA were modified with oligoarginine peptide, a positively
184 charged copolymer, and conjugated with fluorescent Nile red as a hydrophobic model drug (46). In
185 these conditions the NP uptake *in vitro* on HEI-OC1 and HMEEC cells was significantly improved
186 after 15 and 24 hours, compared to pure Nile red solution. Modified PHEA were detected after 24
187 hours from application in the inner hair cells and supporting cells (47).

188 Poly(lactic-co-glycolic acid) (PLGA) NP are copolymers among the novel carrier developed for
189 DD. The Food and Drug Administration (FDA) and the European Medicines Agency (EMA)
190 approved PLGA NP for parenteral administration (47). PLGA NP are interesting because of their
191 hydrophilicity, biocompatibility and easy derivatization by functional groups on the surface or
192 inside the polymer. Their surface may be modified for target specificity by PEGylation, chitosan

193 absorption and binding of antibodies and oligopeptides (48), and different molecules (proteins,
194 steroids, antibiotics and nucleic acids) have been successfully encapsulated and delivered by PLGA
195 NP (49). Programmed degradation of the polymer may therefore yield quantitative delivery of
196 drugs, plasmids or other bioactive molecules. The PLGA NP tested in the inner ear were first
197 conjugated with rhodamine B, a red fluorescent dye, and applied via IT injection: they were
198 identified in the scala tympani, showing that PLGA NP are able to cross RWM by diffusion and
199 their clearance depends on the perilymph flow rate (19). A quantitative pharmacokinetic study
200 recently showed that PLGA NP applied locally *in vivo* in Guinea pigs significantly improved the
201 drug distribution within the inner ear (52). When PLGA NP were loaded with the fluorescent dye
202 coumarin-6 and applied through IT injection, the concentration of the compound after 96 hours
203 from treatment was 10.9-fold higher in the perilymph than when administered in pure solution.
204 Similar results were obtained for other therapeutic payloads such as antioxidants and antiapoptotic
205 drugs (50). Thus PLGA NP are an useful DD system for inner ear because of their high versatility
206 in adaptation to drug properties and tissue targets (51).

207

208 **4.5. Silica NP**

209 Silica NP are modified colloidal silica particles (52) used to transfect *in vitro* plasmid DNA (53) but
210 also as a DD system (54). A pilot study in mice tested the efficacy of diffusion of Cy3-labeled silica
211 NP administered near the RWM: these NP were found inside the inner hair cells, the vestibular hair
212 cells, the spiral ganglion neurons and the supporting cells, without any hearing impairment. Since
213 the NP also reached the dorsal cochlear nucleus and the superior olivary complex, the authors
214 suggested a retrograde axonal transport and concluded that silica NP could be applied for safe drug
215 deliver in the auditory system (55).

216 **4.6. Supermagnetic iron oxide NPs (SPIONs)**

Magnetic NP are synthetic Fe_3O_4 (magnetite) particles, with a core diameter around 15 nm, that can be widely applied for magnetic targeting of cells (56). Unlike large ferromagnetic materials, the smaller supermagnetic iron oxide NP (SPION) are characterized by the absence of residual magnetic interactions when the magnetic field is not active, thus they are more suitable for biomedical applications (57). The SPION derivatized to increase biocompatibility and cell interactions could be guided by an external magnetic field to a specific biological target, but they cannot encapsulate any drug (58). For *in vivo* applications, to prevent particle aggregation and favour dispersion SPION were coated by organic compounds (59). In inner ear drug delivery, SPION have been encapsulated in PLGA (60), silica (58) and dextran (61) and their biocompatibility was tested and verified *in vitro* and *in vivo* (59). The mobility of SPION induced by a magnetic field was also quantified and the results of flux density, gradients and NP properties were compared between *in vitro* and *in vivo* models (62). The magnetic force required for SPION to cross RWM *in vivo* in Guinea pigs was significantly lower than that of the *in vitro* RWM model (63). Another study *in vivo* in Guinea pigs revealed that the concentration of coated SPION inside the cochlea significantly increased (330% above control) when a magnetic field was active (64). Recently, SPION coated with PGLA NP were tested as drug carriers with dexamethasone-acetate (Dex-Ac) as a payload: the levels of Dex-Ac detected in the inner ear fluids after 1 hour from treatment were significantly higher compared with those in absence of a magnetic field (65). All these results support the application of SPION for inner ear drug delivery protocols.

4.7. Hyperbranched poly-L-lysine NP

Hyperbranched poly-L-lysine (HBPL) are high cationic charged dendrimers widely used for non-viral gene transfer (67, 68). The HBPL were applied *in vivo* in Guinea pig inner ears without any sign of cell toxicity or permanent hearing loss (31): they were detected in the stria vascularis and hair cells (31). Nanoparticles based on HBPL and conjugated with fluorescein isothiocyanate were

242 tested *ex-vivo* on freshly frozen human temporal bones, placing them near the intact RWM: HBPL
243 were detected in hair cells, nerve fibres and other cochlear tissues (66).

244

245 **5. Key aspects for nanoparticle-based drug delivery in the inner ear**

246 There are several key parameters to consider for NP-based local DD in the inner ear: the RWM
247 permeability; the NP cochlear targeting; their payload ability and the controlled drug release; their
248 biocompatibility and their stability in cochlear fluids and tissues. All these aspects were evaluated
249 with different NP systems *in vitro, ex-vivo* or *in vivo* in animal models. However, studies on their
250 therapeutic efficacy are still in progress (26).

251 The RWM is considered the main access to the inner ear after the administration in the middle ear
252 (67). NP with different composition and size between 10 and 640 nm were able to cross RWM. The
253 size and the surface charge are determinant factors that affect NP diffusion through RWM. The
254 number of NP crossing from middle ear to inner ear was inversely proportional to lipid NP size (39)
255 and in the cochlea the positively charged glycerol mono-oleate NP achieved a larger distribution
256 than neutral or negatively charged ones (34). The process responsible for this passage was firstly
257 described for lipid NPs as a paracellular pathway (33). Recent studies in rat RWM suggested that
258 the passage of liposome NP may occur either via the paracellular pathway or by endocytotic
259 mechanisms based on clathrin and caveolin (8).

260 In most studies NP were loaded or labelled with a fluorescent dye (Rhodamine B, Carboxycyanine,
261 Nile-red) or a contrast agent (gadolinium) for visualization of particles in cochlear cells or fluids by
262 imaging techniques: however, the reported data mostly detected the presence of NP in inner ear
263 tissues without a quantitative analysis. Liposome NP were detected in RWM until 11 days and in
264 the cochlea until 6 days post-injection (67); lipid nanocapsules were detected in the cochlea until 7
265 days post-injection (33). However, for treatment of sensorineural hearing loss, the cochlear target

cells are hair cells and spiral ganglion neurons: all populations are selectively reached by the functionalized NP tested (51). For example, the NP functionalized with nerve growth factor-derived peptides showed specificity for spiral ganglion neurons and nerve fibres (44).

The ability of NP to carry the drugs into the cochlea through the RWM was shown *in vivo* by several studies. Smaller supermagnetic iron oxide NP (SPION) coated with PGLA and conjugated with dexamethasone enabled the release of the drug in inner ear fluids, resulting in a higher concentration of dexamethasone in the perilymph compared to the pure drug diffusion (10% higher after 60 minutes, $p<0.01$) (65). The PLGA loaded with coumarin-6 enhanced up to 10.9 times the local bioavailability of the dye in the perilymph in comparison to pure drug solution (50). When the neutoxic agent disulfiram was loaded on liposomes and polymersomes, the number of spiral ganglion cells significantly decreased two days after administration (8). However, drug release by NP has not yet been examined by long-term studies.

Biocompatibility is one of the major concerns in NP clinical applications. Up to date no hearing impairment, loss of hair cells or histological damages were reported (31, 32, 45, 68), thus NP systems appear reasonably safe. However, SPION tend to aggregate when the magnetic field is removed and the long persistence of these nanoparticles on the inner ear may induce toxicity due to accumulation (8). The effects of LNC were evaluated 20 days post-injection and no toxicity was detected (67). Topic applications of liposomes in rats did not affect hearing, but a NP concentration-dependent toxicity was observed *in vitro* in primary cochlear cell cultures (32). A possible explanation was that a NP overload occurred in these cells, resulting in cytoplasm condensation and cell function impairment (69). In most of these studies a single intratympanic administration was employed: recently, in order to improve liposome efficiency, a continuous NP release was obtained through a high-performance polymide tubing (HPPT) equipped with an ALZET© micro-pump (DURECT Corp, CA; USA). Liposomes loaded with gadolinium-tetra-azacyclo-dodecane-tetra-acetic acid were visualized both *in vitro* by TEM and *in vivo* by MRI. *In vitro*, intact NP were free

to diffuse in the medium, and *in vivo* were detected in the cochlea without adverse effects within six days (67). Again, no long-term effects of exposure to NP after multiple applications in the inner ear have yet been evaluated.

The residence time of the drug within the middle ear cavity may be increased by NP, but this does not guarantee direct contact between the loaded NP and the RWM, because the RWM is the access point for the inner ear in the case of trans-tympanic administration. The NP also undergo middle ear clearance through the Eustachian tube (70). A possible strategy to bypass these limits could be to combine different DD systems together, for example using hydrogels. The incorporation of loaded NP into hydrogels could increase their residence time in the middle ear, thus enhancing drug release in the perilymph (26, 71).The hydrogel is applied near the RWM and releases the loaded NP in the perilymph along with its degradation. This approach has been recently reported: a poloxamer 407 hydrogel combined with SPION was successfully applied on *ex vivo* models (human temporal bones and explanted mouse inner ear cultures) (72). More recently, a nanohydrogel based on chitosan polymer incorporating liposomes was tested *in vitro* and *in vivo* in the mouse model (73). The *in vitro* results showed that NP persisted without significant degradation for at least two weeks and were released in a controlled and continuous way by the nanohydrogel. The *in vivo* results showed that the NP were successfully released by the nanohydrogel across the RWM and were able to reach the perilymph and Organ of Corti cells (73).

Although NP research in local DD for inner ear therapy appears promising, there are still many difficulties to overcome, mostly related to the inner ear anatomy, to the complexity of the cochlea and its highly differentiated cell populations, as well as the possibility to cause hearing loss using microsurgical approaches. The *in vivo* analyses of perilymph samples represent a technical challenge (27), because of the small volume of inner ear fluids in animal models (the total volume of perilymph in a Guinea pig amounts to about 10 µl) (74), and the possible contamination of samples by cerebrospinal fluid (27). The pharmacokinetics of drugs in the inner ear is therefore still

316 unclear and there are no reliable quantitative data about local bioavailability, drug distribution and
317 RWM permeability (26). Only recently, a computer pharmacokinetic for inner ear fluids and drug
318 distribution has been developed (75). The software (Cochlear Fluids Simulator V3.083) outlines a
319 model based on inner ear anatomy in humans and rodents, pharmacokinetic and solute distribution
320 parameters. This model has been used to simulate the distribution of therapeutic drugs and other
321 compounds in the perilymph (24, 76). However, because of intraspecific variability of animal
322 models in the volume of inner ear fluids and RWM thickness and conditions, it is difficult to
323 compare the current studies using different DD systems and to draw quantitative conclusions about
324 drug pharmacokinetics in the inner ear.

325

326 **6. Conclusions and Future Perspectives**

327 The NP-based systems show a high potential for inner ear delivery of various therapeutic agents.
328 Their use could minimize the side effects of treatments, allow target specificity and provide a
329 sustained release of drugs in inner ear fluids. The type of NP may be adapted to the drug to be
330 carried and different formulations have been tested. The NP could also be combined with other
331 nanomaterials, such as hydrogels, to improve the local application of drugs. However, several
332 problems have yet to be solved and more *in vivo* studies are necessary to verify their bioavailability
333 and effectiveness before a successful clinical application.

334

335

Acknowledgements

We are grateful to the Foundation Onlus 'Staminali e Vita' (Padua, Italy) for providing support.

Funding

This work was supported by the Italian Ministry of Education, University and Research (MIUR), Italy [grant n. PRIN- 2010S58B38_004].

References

1. Chen G, Zhang X, Yang F and Mu L: Disposition of nanoparticle-based delivery system via inner ear administration. Curr Drug Metab 11: 886-897, 2010.
2. Juhn SK and Rybak LP: Labyrinthine barriers and cochlear homeostasis. Acta Otolaryngol 91: 529-534, 1981.
3. Inamura N and Salt AN: Permeability changes of the blood-labyrinth barrier measured in vivo during experimental treatments. Hear Res 61: 12-18, 1992.
4. Lefebvre PP and Staecker H: Steroid perfusion of the inner ear for sudden sensorineural hearing loss after failure of conventional therapy: a pilot study. Acta Otolaryngol 122: 698-702, 2002.
5. Minor LB, Schessel DA and Carey JP: Meniere's disease. Curr Opin Neurol 17: 9-16, 2004.
6. Malam Y, Loizidou M and Seifalian AM: Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. Trends Pharmacol Sci 30: 592-599, 2009.
7. Gelperina S, Kisich K, Iseman MD and Heifets L: The potential advantages of nanoparticle drug delivery systems in chemotherapy of tuberculosis. Am J Respir Crit Care Med 172: 1487-1490, 2005.
8. Buckiova D, Ranjan S, Newman TA*, et al*: Minimally invasive drug delivery to the cochlea through application of nanoparticles to the round window membrane. Nanomedicine (Lond) 7: 1339-1354, 2012.
9. Banerjee A and Parnes LS: The biology of intratympanic drug administration and pharmacodynamics of round window drug absorption. Otolaryngol Clin North Am 37: 1035-1051, 2004.
10. Goycoolea MV and Lundman L: Round window membrane. Structure function and permeability: a review. Microsc Res Tech 36: 201-211, 1997.
11. Shi X: Pathophysiology of the cochlear intrastrial fluid-blood barrier (review). Hear Res 338: 52-63, 2016.
12. Goycoolea MV: The round window membrane under normal and pathological conditions. Acta Otolaryngol Suppl 493: 43-55, 1992.
13. Salt AN: Pharmacokinetics of Drug Entry into Cochlear Fluids. Volta Rev 105: 277-298, 2005.
14. Jahnke K: [Permeability barriers of the inner ear. Fine structure and function]. Fortschr Med 98: 330-336, 1980.

15. Saito T, Zhang ZJ, Tokuriki M, *et al*: Expression of p-glycoprotein is associated with that of multidrug resistance protein 1 (MRP1) in the vestibular labyrinth and endolymphatic sac of the guinea pig. Neurosci Lett 303: 189-192, 2001.
16. Liu H, Hao J, Li KS : Current strategies for drug delivery to the inner ear. Acta Pharmaceutica Sinica B 3: 86-96, 2013.
17. Swan EE, Mescher MJ, Sewell WF, Tao SL and Borenstein JT: Inner ear drug delivery for auditory applications. Adv Drug Deliv Rev 60: 1583-1599, 2008.
18. McCall AA, Swan EE, Borenstein JT, Sewell WF, Kujawa SG and McKenna MJ: Drug delivery for treatment of inner ear disease: current state of knowledge. Ear Hear 31: 156-165, 2010.
19. Tamura T, Kita T, Nakagawa T, *et al*: Drug delivery to the cochlea using PLGA nanoparticles. Laryngoscope 115: 2000-2005, 2005.
20. Horie RT, Sakamoto T, Nakagawa T, Ishihara T, Higaki M and Ito J: Stealth-nanoparticle strategy for enhancing the efficacy of steroids in mice with noise-induced hearing loss. Nanomedicine (Lond) 5: 1331-1340, 2010.
21. Bowe SN and Jacob A: Round window perfusion dynamics: implications for intracochlear therapy. Curr Opin Otolaryngol Head Neck Surg 18: 377-385, 2010.
22. De Ceulaer G, Johnson S, Yperman M, *et al*: Long-term evaluation of the effect of intracochlear steroid deposition on electrode impedance in cochlear implant patients. Otol Neurotol 24: 769-774, 2003.
23. Paulson DP, Abuzeid W, Jiang H, Oe T, O'Malley BW and Li D: A novel controlled local drug delivery system for inner ear disease. Laryngoscope 118: 706-711, 2008.
24. Hahn H, Kammerer B, DiMauro A, Salt AN and Plontke SK: Cochlear microdialysis for quantification of dexamethasone and fluorescein entry into scala tympani during round window administration. Hear Res 212: 236-244, 2006.
25. Ciorba A, Astolfi L, Jolly C, Martini A: Cochlear Implants and Inner Ear Based Therapy. European Journal of Nanomedicine 2: 4, 2009.
26. El Kechai N, Agnely F, Mamelle E, Nguyen Y, Ferrary E and Bochot A: Recent advances in local drug delivery to the inner ear. Int J Pharm 494: 83-101, 2015.
27. Salt AN and Plontke SK: Local inner-ear drug delivery and pharmacokinetics. Drug Discov Today 10: 1299-1306, 2005.
28. Jager E, Venturini CG, Poletto FS, *et al*: Sustained release from lipid-core nanocapsules by varying the core viscosity and the particle surface area. J Biomed Nanotechnol 5: 130-140, 2009.
29. Hureaux J, Lagarce F, Gagnadoux F, *et al*: Lipid nanocapsules: ready-to-use nanovectors for the aerosol delivery of paclitaxel. Eur J Pharm Biopharm 73: 239-246, 2009.
30. Hureaux J, Lagarce F, Gagnadoux F, *et al*: Toxicological study and efficacy of blank and paclitaxel-loaded lipid nanocapsules after i.v. administration in mice. Pharm Res 27: 421-430, 2010.
31. Scheper V, Wolf M, Scholl M, *et al*: Potential novel drug carriers for inner ear treatment: hyperbranched polylysine and lipid nanocapsules. Nanomedicine (Lond) 4: 623-635, 2009.
32. Zhang Y, Zhang W, Lobler M, *et al*: Inner ear biocompatibility of lipid nanocapsules after round window membrane application. Int J Pharm 404: 211-219, 2011.
33. Zou J, Saulnier P, Perrier T, *et al*: Distribution of lipid nanocapsules in different cochlear cell populations after round window membrane permeation. J Biomed Mater Res B Appl Biomater 87: 10-18, 2008.
34. Liu H, Chen S, Zhou Y, *et al*: The effect of surface charge of glycerol monooleate-based nanoparticles on the round window membrane permeability and cochlear distribution. J Drug Target 21: 846-854, 2013.
35. Chen G, Hou SX, Hu P, Hu QH, Guo DD and Xiao Y: [In vitro dexamethasone release from nanoparticles and its pharmacokinetics in the inner ear after administration of the drug-loaded nanoparticles via the round window]. Nan Fang Yi Ke Da Xue Xue Bao 28: 1022-1024, 2008.
36. Bozzuto G and Molinari A: Liposomes as nanomedical devices. Int J Nanomedicine 10: 975-999, 2015.

37. Wang AZ, Langer R and Farokhzad OC: Nanoparticle delivery of cancer drugs. Annu Rev Med 63: 185-198, 2012.
38. Ranjan S, Sood R, Dudas J, *et al*: Peptide-mediated targeting of liposomes to TrkB receptor-expressing cells. Int J Nanomedicine 7: 3475-3485, 2012.
39. Zou J, Sood R, Ranjan S, *et al*: Size-dependent passage of liposome nanocarriers with preserved posttransport integrity across the middle-inner ear barriers in rats. Otol Neurotol 33: 666-673, 2012.
40. Zou J, Sood R, Ranjan S, *et al*: Manufacturing and in vivo inner ear visualization of MRI traceable liposome nanoparticles encapsulating gadolinium. J Nanobiotechnology 8: 32, 2010.
41. Letchford K and Burt H: A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes. Eur J Pharm Biopharm 65: 259-269, 2007.
42. Farokhzad OC and Langer R: Nanomedicine: developing smarter therapeutic and diagnostic modalities. Adv Drug Deliv Rev 58: 1456-1459, 2006.
43. Lin JJ, Ghoroghchian PP, Zhang Y and Hammer DA: Adhesion of antibody-functionalized polymersomes. Langmuir 22: 3975-3979, 2006.
44. Roy S, Johnston AH, Newman TA, *et al*: Cell-specific targeting in the mouse inner ear using nanoparticles conjugated with a neurotrophin-derived peptide ligand: potential tool for drug delivery. Int J Pharm 390: 214-224, 2010.
45. Kim DK, Park SN, Park KH, *et al*: Development of a drug delivery system for the inner ear using poly(amino acid)-based nanoparticles. Drug Deliv 22: 367-374, 2015.
46. Yoon JY, Yang KJ, Kim da E, *et al*: Intratympanic delivery of oligoarginine-conjugated nanoparticles as a gene (or drug) carrier to the inner ear. Biomaterials 73: 243-253, 2015.
47. Kumari A, Yadav SK and Yadav SC: Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Surf B Biointerfaces 75: 1-18, 2010.
48. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A and Preat V: PLGA-based nanoparticles: an overview of biomedical applications. J Control Release 161: 505-522, 2012.
49. Grottkau BE, Cai X, Wang J, Yang X and Lin Y: Polymeric nanoparticles for a drug delivery system. Curr Drug Metab 14: 840-846, 2013.
50. Cai H, Wen X, Wen L, *et al*: Enhanced local bioavailability of single or compound drugs delivery to the inner ear through application of PLGA nanoparticles via round window administration. Int J Nanomedicine 9: 5591-5601, 2014.
51. Pritz CO, Dudas J, Rask-Andersen H, Schrott-Fischer A and Glueckert R: Nanomedicine strategies for drug delivery to the ear. Nanomedicine (Lond) 8: 1155-1172, 2013.
52. Le VH, Thuc CN and Thuc HH: Synthesis of silica nanoparticles from Vietnamese rice husk by sol-gel method. Nanoscale Res Lett 8: 58, 2013.
53. Sameti M, Bohr G, Ravi Kumar MN, *et al*: Stabilisation by freeze-drying of cationically modified silica nanoparticles for gene delivery. Int J Pharm 266: 51-60, 2003.
54. Ahola M, Rich J, Kortesuo P, Kiesvaara J, Seppala J and Yli-Urpo A: In vitro evaluation of biodegradable epsilon-caprolactone-co-D, L-lactide/silica xerogel composites containing toremifene citrate. Int J Pharm 181: 181-191, 1999.
55. Praetorius M, Brunner C, Lehnert B, *et al*: Transsynaptic delivery of nanoparticles to the central auditory nervous system. Acta Otolaryngol 127: 486-490, 2007.
56. Sun C, Lee JS and Zhang M: Magnetic nanoparticles in MR imaging and drug delivery. Adv Drug Deliv Rev 60: 1252-1265, 2008.
57. Cao ZG, Zhou SW, Sun K, Lu XB, Luo G and Liu JH: [Preparation and feasibility of superparamagnetic dextran iron oxide nanoparticles as gene carrier]. Ai Zheng 23: 1105-1109, 2004.
58. Ye F, Laurent S, Fornara A, *et al*: Uniform mesoporous silica coated iron oxide nanoparticles as a highly efficient, nontoxic MRI T(2) contrast agent with tunable proton relaxivities. Contrast Media Mol Imaging 7: 460-468, 2012.

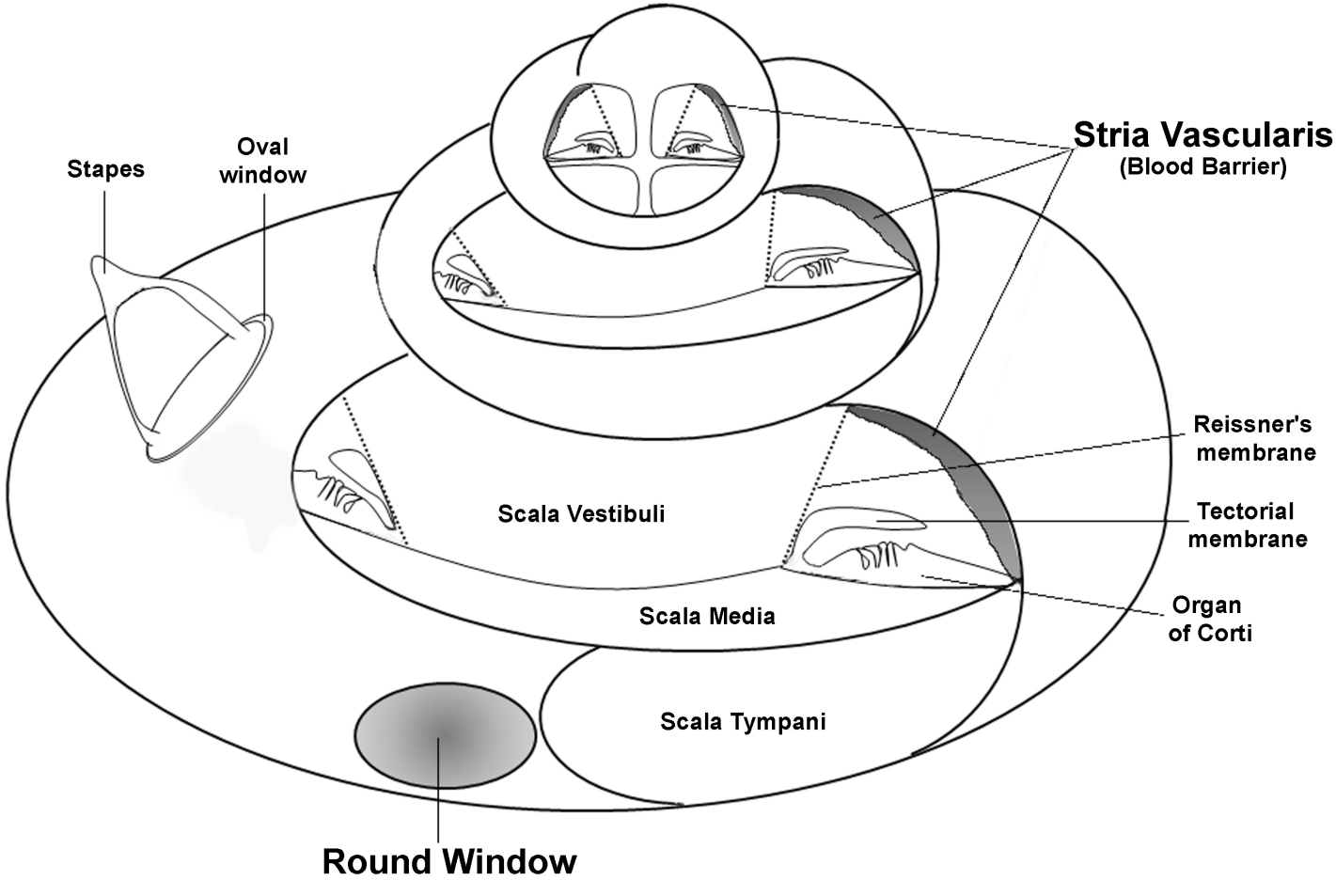
59. Kopke RD, Wassel RA, Mondalek F, *et al*: Magnetic nanoparticles: inner ear targeted molecule delivery and middle ear implant. Audiol Neurootol 11: 123-133, 2006.
60. Ge X, Jackson RL, Liu J, *et al*: Distribution of PLGA nanoparticles in chinchilla cochleae. Otolaryngol Head Neck Surg 137: 619-623, 2007.
61. Mondalek FG, Zhang YY, Kropp B, *et al*: The permeability of SPION over an artificial three-layer membrane is enhanced by external magnetic field. J Nanobiotechnology 4: 4, 2006.
62. Barnes AL, Wassel RA, Mondalek F, Chen K, Dormer KJ and Kopke RD: Magnetic characterization of superparamagnetic nanoparticles pulled through model membranes. Biomagn Res Technol 5: 1, 2007.
63. Zou J, Zhang W, Poe D, *et al*: MRI manifestation of novel superparamagnetic iron oxide nanoparticles in the rat inner ear. Nanomedicine (Lond) 5: 739-754, 2010.
64. Dormer Kea: Magnetically-targeted, technetium 99m-labeled nanoparticles to the inner ear. J Biomed Nanotechnol 4: 10, 2008.
65. Du X, Chen K, Kuriyavar S, *et al*: Magnetic targeted delivery of dexamethasone acetate across the round window membrane in guinea pigs. Otol Neurotol 34: 41-47, 2013.
66. Roy S, Glueckert R, Johnston AH, *et al*: Strategies for drug delivery to the human inner ear by multifunctional nanoparticles. Nanomedicine (Lond) 7: 55-63, 2012.
67. Zou J, Sood R, Zhang Y, Kinnunen PK and Pyykko I: Pathway and morphological transformation of liposome nanocarriers after release from a novel sustained inner-ear delivery system. Nanomedicine (Lond) 9: 2143-2155, 2014.
68. Wu TH, Liu CP, Chien CT and Lin SY: Fluorescent hydroxylamine derived from the fragmentation of PAMAM dendrimers for intracellular hypochlorite recognition. Chemistry 19: 11672-11675, 2013.
69. Moss OR: Insights into the healthy effects of nanoparticles: why numbers matter. Int J Nanotechnol 5: 1-8, 2008.
70. Sheppard WM, Wanamaker HH, Pack A, Yamamoto S and Slepecky N: Direct round window application of gentamicin with varying delivery vehicles: a comparison of ototoxicity. Otolaryngol Head Neck Surg 131: 890-896, 2004.
71. El Kechai N, Mamelle E, Nguyen Y, *et al*: Hyaluronic acid liposomal gel sustains delivery of a corticoid to the inner ear. J Control Release 226: 248-257, 2016.
72. Thaler M, Roy S, Fornara A, *et al*: Visualization and analysis of superparamagnetic iron oxide nanoparticles in the inner ear by light microscopy and energy filtered TEM. Nanomedicine 7: 360-369, 2011.
73. Lajud SA, Nagda DA, Qiao P, *et al*: A novel chitosan-hydrogel-based nanoparticle delivery system for local inner ear application. Otol Neurotol 36: 341-347, 2015.
74. Wang X, Dellamary L, Fernandez R, *et al*: Dose-dependent sustained release of dexamethasone in inner ear cochlear fluids using a novel local delivery approach. Audiol Neurootol 14: 393-401, 2009.
75. Salt AN and Plontke SK: Principles of local drug delivery to the inner ear. Audiol Neurootol 14: 350-360, 2009.
76. Astolfi L, Guaran V, Marchetti N, *et al*: Cochlear implants and drug delivery: In vitro evaluation of dexamethasone release. J Biomed Mater Res B Appl Biomater 102: 267-273, 2014.

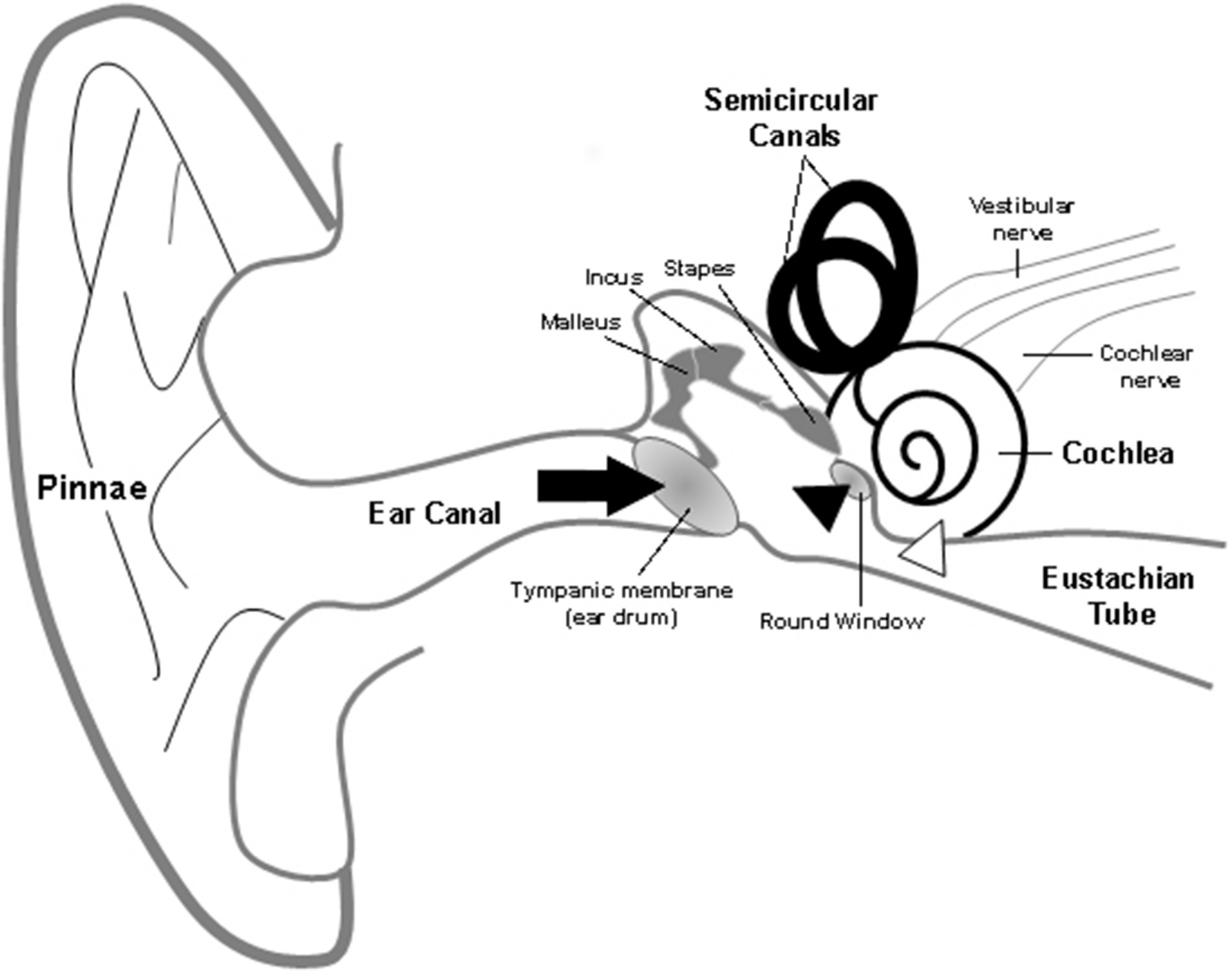
Figure Legends

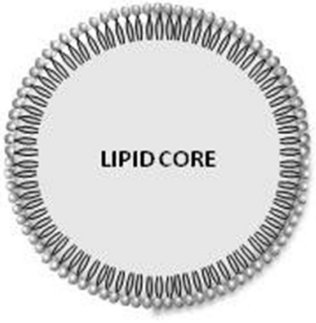
Fig. 1. Scheme of the cochlea structure, highlighting the cochlear barriers, the round window and the stria vascularis.

Fig. 2. Scheme of nanoparticle administration routes. Arrow: intratympanic route; black arrowhead: intracochlear route by round window; white arrowhead: intracochlear route by cochleostomy.

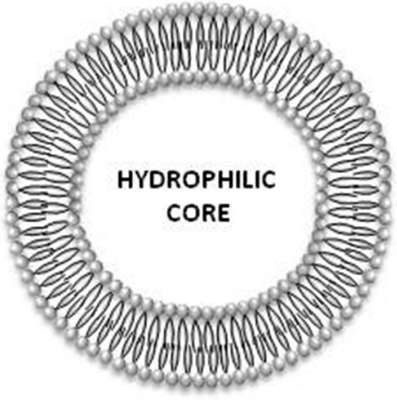
Fig. 3. Structures of nanoparticles (NP) useful for drug delivery. LCN: lipid core NP; SPION: supermagnetic iron oxide NP; HBPL: hyperbranched poly-L-lysine NP; P: hydrophilic region; NP: hydrophobic region.


Stapes
Oval window
Stria Vascularis
(Blood Barrier)
Reissner's membrane
Tectorial membrane
Organ of Corti
Scala Vestibuli
Scala Media
Scala Tympani
Round Window

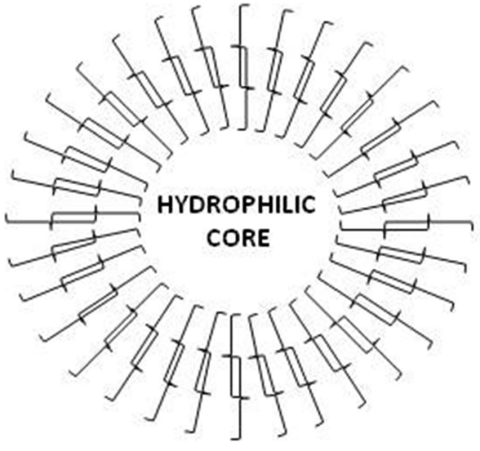

Semicircular Canals
Vestibular nerve
Incus
Stapes
Malleus
Cochlear nerve
Cochlea
Pinnae
Ear Canal
Tympanic membrane (ear drum)
Round Window
Eustachian Tube



LCN

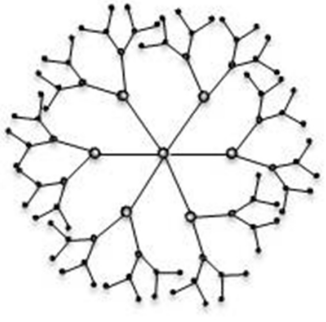


LIPOSOME



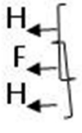
POLYMEROSOME

SPION



HBPL

Phospholipids



Polymers