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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

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Cover letter 1.docx [Cover Letter]

Answer_to_Reviewers1.docx [Response to Reviewers]

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

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NP_Figure_Legends1.docx [Figure]

Figure 1 inner ear barrier.jpg [Figure]

Figure 2 administration routes.jpg [Figure]

Figura 3 nanoparticles.jpg [Figure]

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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.

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

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- 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.
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Nanoparticle drug delivery systems for inner ear therapy: an overview

Filippo Valente^{1*}, Laura Astolfi^{1,2*}, Edi Simoni¹, Serena Danti³, Valeria Franceschini^{2,4}, Milvia Chicca⁵, Alessandro Martini^{1,2}

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*Both authors contributed equally to this work

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22 **Abstract**

23 Local drug delivery based on nanoparticles (NP) represents a novel strategy to improve inner ear
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25 originating from damage to hair cells and spiral ganglion neurons in the cochlea. Numerous
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28 NP helps to reduce the amount of drug required and avoid systemic side effects. This review
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30 ear. The perspectives for clinical application of NP in inner ear drug delivery are also discussed.

31

32 **Keywords**

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

34 **1. Introduction**

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

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

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

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

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

56

57 2. Ear barriers

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

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

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

75 The BB is a major barrier in the stria vascularis separating the cochlear tissues from the circulatory
76 system (14). Its role is to maintain the homeostasis of cochlear fluids and protect the inner ear
77 integrity. Its main components are principally the endothelial capillaries whose cells are connected
78 by tight junctions, which lay over a basement membrane. However numerous accessory cells have
79 recently been observed in the complex structure of the barrier, such as pericytes and perivascular
80 resident macrophage-like (11). The BB has been described to act as a physical and biochemical
81 barrier through an efflux pump, the P-glycoprotein 1 (P-gp) (15). The BB is therefore considered a
82 rate-limiting barrier in the passage of therapeutic agents from the circulatory system to the inner ear.
83 However, the current knowledge about drug transportation processes through BB is still limited
84 (16).

85

86 **3. Administration routes**

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

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

108

109 **4. Nanoparticle-based systems**

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

118 **4.1. Lipid Core NP**

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

142

143 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 μ m) 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)

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

236

237 **4.7. Hyperbranched poly-L-lysine NP**

238 Hyperbranched poly-L-lysine (HBPL) are high cationic charged dendrimers widely used for non-
239 viral gene transfer (67, 68). The HBPL were applied *in vivo* in Guinea pig inner ears without any
240 sign of cell toxicity or permanent hearing loss (31): they were detected in the stria vascularis and
241 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

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

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

278 Biocompatibility is one of the major concerns in NP clinical applications. Up to date no hearing
279 impairment, loss of hair cells or histological damages were reported (31, 32, 45, 68), thus NP
280 systems appear reasonably safe. However, SPION tend to aggregate when the magnetic field is
281 removed and the long persistence of these nanoparticles on the inner ear may induce toxicity due to
282 accumulation (8). The effects of LNC were evaluated 20 days post-injection and no toxicity was
283 detected (67). Topic applications of liposomes in rats did not affect hearing, but a NP concentration-
284 dependent toxicity was observed *in vitro* in primary cochlear cell cultures (32). A possible
285 explanation was that a NP overload occurred in these cells, resulting in cytoplasm condensation and
286 cell function impairment (69). In most of these studies a single intratympanic administration was
287 employed: recently, in order to improve liposome efficiency, a continuous NP release was obtained
288 through a high-performance polyimide tubing (HPPT) equipped with an ALZET[®] micro-pump
289 (DURECT Corp, CA; USA). Liposomes loaded with gadolinium-tetra-azacyclo-dodecane-tetra-
290 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 μ l) (74), and the possible contamination of
315 samples by cerebrospinal fluid (27). The pharmacokinetics of drugs in the inner ear is therefore still

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

325

326 **6. Conclusions and Future Perspectives**

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

334

335

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342

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43

INNER EAR NANOPARTICLES DELIVERY

Nanoparticle drug delivery systems for inner ear therapy: an overview

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22 **Abstract**

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

31

32 **Keywords**

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

34 **1. Introduction**

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

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

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

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

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

56

57 **2. Ear barriers**

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

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

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

75 The BB is a major barrier in the stria vascularis separating the cochlear tissues from the circulatory
76 system (14). Its role is to maintain the homeostasis of cochlear fluids and protect the inner ear
77 integrity. Its main components are principally the endothelial capillaries whose cells are connected
78 by tight junctions, which lay over a basement membrane. However numerous accessory cells have
79 recently been observed in the complex structure of the barrier, such as pericytes and perivascular
80 resident macrophage-like (11). The BB has been described to act as a physical and biochemical
81 barrier through an efflux pump, the P-glycoprotein 1 (P-gp) (15). The BB is therefore considered a
82 rate-limiting barrier in the passage of therapeutic agents from the circulatory system to the inner ear.
83 However, the current knowledge about drug transportation processes through BB is still limited
84 (16).

85

86 **3. Administration routes**

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

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

108

109 **4. Nanoparticle-based systems**

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

118 **4.1. Lipid Core NP**

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

142

143 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 μ m) 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)**

217 Magnetic NP are synthetic Fe₃O₄ (magnetite) particles, with a core diameter around 15 nm, that can
218 be widely applied for magnetic targeting of cells (56). Unlike large ferromagnetic materials, the
219 smaller supermagnetic iron oxide NP (SPION) are characterized by the absence of residual
220 magnetic interactions when the magnetic field is not active, thus they are more suitable for
221 biomedical applications (57). The SPION derivatized to increase biocompatibility and cell
222 interactions could be guided by an external magnetic field to a specific biological target, but they
223 cannot encapsulate any drug (58). For *in vivo* applications, to prevent particle aggregation and
224 favour dispersion SPION were coated by organic compounds (59). In inner ear drug delivery,
225 SPION have been encapsulated in PLGA (60), silica (58) and dextran (61) and their
226 biocompatibility was tested and verified *in vitro* and *in vivo* (59). The mobility of SPION induced
227 by a magnetic field was also quantified and the results of flux density, gradients and NP properties
228 were compared between *in vitro* and *in vivo* models (62). The magnetic force required for SPION to
229 cross RWM *in vivo* in Guinea pigs was significantly lower than that of the *in vitro* RWM model
230 (63). Another study *in vivo* in Guinea pigs revealed that the concentration of coated SPION inside
231 the cochlea significantly increased (330% above control) when a magnetic field was active (64).
232 Recently, SPION coated with PGLA NP were tested as drug carriers with dexamethasone-acetate
233 (Dex-Ac) as a payload: the levels of Dex-Ac detected in the inner ear fluids after 1 hour from
234 treatment were significantly higher compared with those in absence of a magnetic field (65). All
235 these results support the application of SPION for inner ear drug delivery protocols.

236

237 **4.7. Hyperbranched poly-L-lysine NP**

238 Hyperbranched poly-L-lysine (HBPL) are high cationic charged dendrimers widely used for non-
239 viral gene transfer (67, 68). The HBPL were applied *in vivo* in Guinea pig inner ears without any
240 sign of cell toxicity or permanent hearing loss (31): they were detected in the stria vascularis and
241 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

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

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

278 Biocompatibility is one of the major concerns in NP clinical applications. Up to date no hearing
279 impairment, loss of hair cells or histological damages were reported (31, 32, 45, 68), thus NP
280 systems appear reasonably safe. However, SPION tend to aggregate when the magnetic field is
281 removed and the long persistence of these nanoparticles on the inner ear may induce toxicity due to
282 accumulation (8). The effects of LNC were evaluated 20 days post-injection and no toxicity was
283 detected (67). Topic applications of liposomes in rats did not affect hearing, but a NP concentration-
284 dependent toxicity was observed *in vitro* in primary cochlear cell cultures (32). A possible
285 explanation was that a NP overload occurred in these cells, resulting in cytoplasm condensation and
286 cell function impairment (69). In most of these studies a single intratympanic administration was
287 employed: recently, in order to improve liposome efficiency, a continuous NP release was obtained
288 through a high-performance polyimide tubing (HPPT) equipped with an ALZET[®] micro-pump
289 (DURECT Corp, CA; USA). Liposomes loaded with gadolinium-tetra-azacyclo-dodecane-tetra-
290 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 μ l) (74), and the possible contamination of
315 samples by cerebrospinal fluid (27). The pharmacokinetics of drugs in the inner ear is therefore still

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

325

326 **6. Conclusions and Future Perspectives**

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

334

335

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342

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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.

LCN

LIPOSOME

POLYMEROSOME

SPION

HBPL