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Otoprotective properties of 6α-methylprednisolone-loaded nanoparticles against cisplatin: In vitro and in vivo correlation

Sergio Martín-Saldaña a, Raquel Palao-Suayb,c, Almudena Trinidad a, María Rosa Aguilar b, Rafael Ramírez-Camacho a,d, Julio San Románb, c
aDepartment of Otorhinolaryngology, Puerta de Hierro University Hospital, C/ Manuel de Falla, Majadahonda
bGroup of Biomaterials, Department of Polymeric Nanomaterials and Biomaterials, Institute of Polymer Science and Technology, CSIC, C/ Juan de la Cierva, Madrid, Spain
cNetworking Biomedical Research Centre in Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Spain
dUniversidad Autónoma de Madrid, Cantoblanco Campus University, Madrid

Abstract
6α-Methylprednisolone-loaded surfactant-free nanoparticles have been developed to palliate cisplatin ototoxicity. Nanoparticles were based on two different amphiphilic pseudo-block copolymers obtained by free radical polymerization and based on N-vinyl pyrrolidone and a methacrylic derivative of α-tocopheryl succinate or α-tocopherol. Copolymers formed spherical nanoparticles by nanoprecipitation in aqueous media that were able to encapsulate 6α-methylprednisolone in their inner core. The obtained nanovehicles were tested in vitro using HEI-OC1 cells and in vivo in a murine model. Unloaded nanoparticles were not able to significantly reduce the cisplatin ototoxicity. Loaded nanoparticles reduced cisplatin-ototoxicity in vitro being more active those based on the methacrylic derivative of vitamin E, due to their higher encapsulation efficiency. This formulation was able to protect hair cells in the base of the cochlea, having a positive effect in the highest frequencies tested in a murine model. A good correlation between the in vitro and the in vivo experiments was found.

Background
Cisplatin (CDDP) is a highly effective chemotherapeutic agent against a variety of solid tumors including head and neck, lungs, ovary, bladder and testicles; however, it presents severe side-effects. Marullo et al1 described a double way of CDDP-induced cytotoxicity: CDDP binding to guanine bases on nuclear DNA and the formation of inter- and intra-strand chain crosslinking trigger cell apoptosis because of replication and transcription blockage; CDDP also has a direct effect on mitochondrial DNA resulting in the impairment of electron 32 transport chain protein synthesis leading to ROS generation. Increasing doses incorporated into protocols, with the aim of increasing cure rates, are related with serious adverse effects that affect kidney function, nervous system and hearing. CDDP induces apoptosis of inner ear cell by binding to DNA, reactive oxygen species (ROS) generation, increased lipid peroxidation and Ca 2+ influx, and inflammation events.2 Hearing impairment begins in the high frequencies and progresses to midrange when patient receives doses higher than 100 mg/m 2. Patients who receive ultrahigh doses of CDDP (150-225 mg/m 42 2), show hearing loss in the high and extended high frequencies in 100% of cases. 44 3 There is substantial variability in susceptibility to the ototoxic effects of CDDP. Rapid intravenous bolus injections, high cumulative doses, pre-existing hearing loss, renal insufficiency, anemia, hypoalbuminemia, and prior cranial irradiation are some of the factors that can play a role in CCDP toxicity. The incidence and severity of hearing loss

after CDDP treatment vary considerably, and 40%-80% of patients develop Nanomedicine: Nanotechnology, Biology, and Medicine an elevated hearing threshold following CDDP treatment being a limiting factor in antineoplasic treatments.4 The molecular mechanism of CDDP ototoxicity has not been fully elucidated; however ROS accumulation plays a key role in it. González-García et al found a significant increase in total superoxide dismutase (SOD) activity and caspase-3/7 and caspase-9 expression in whole cochlear extracts that relates to an antioxidant response against platinum accumulation on the seventh day after a single dose of 5 mg/kg CDDP.5 A depletation in endogenous antioxidants enzymes like SOD, catalase or glutathione peroxidase and glutathione reductase was observed in animals that have received ototoxic doses of CDDP (16 mg/kg), leading to ROS accumulation and resulting in apoptosis.6 CDDP inhibition of antioxidant enzymes after early antioxidant cell response, promotes ROS accumulation in the cochlea that brings on the entry of Ca2+ in the inner ear cells triggering apoptosis. Thus, activation and redistribution of bcl-2-like protein 4 (Bax) in the cytosol promote the release of cytochrome c from damaged mitochondria. Caspases 3 and 9 are activated by cytochrome c causing DNA damage mediated by activation of DNAase with loss of outer hair cells (OHC) and spiral ganglia.7 Furthermore, some studies showed that CDDP induced apoptosis in inner hair cells (IHC) in a caspase independent way too.8,9 Utricle hair cells, with a common embryological origin with cochlea hair cells, minimizes oxidative stress by endogenous mechanisms and protective molecules such as glutathione, heat proteins (HSPs), heme oxigenase and adenosine A1 receptors that are proved to reduce CDDP-induced apoptosis in vitro.10 HSP-70 is over expressed in CDDP treated animals over 14 days,11 showing a correlation between its concentration and a decrease in caspase activity at the cochlea.12 Nevertheless, up-regulation of these protective molecules is not enough to settle CDDP-induced oxidative stress being necessary the systemic or local administration of protective drugs6 such as caspase inhibitors (caspase-3 inhibitor z-DEVD-fmk and caspase-9 inhibitor z-LEHD-fmk13, cannabinoid receptor 2 JWH-1514), antioxidants (Bucillamine15, caffeic acid,16 metformin17 Ginkgo biloba extract18) or corticoids (dexamethasone19, 6α-methylprednisolone20).

The systemic administration of drugs is related to variable penetration into the inner ear due to the presence of a blood–cochlea barrier and undesired side effects. Hydrocortisone, 6α-methylpred-nisolone (MP), and dexamethasone showed poor delivery to inner ear, and the systemic administration of higher doses of corticoids would be necessary to achieve otoprotective therapeutic concentrations.20 However, high concentrations of corticoids are accompanied by severe side-effects that need to be avoided, such as hyperglycemia, hypertension, hypokalemia, peptic ulcer disease, osteoporosis and immunosuppression.19,21 Intratympanic treatment presents several advantages if compared with systemic administration. It is possible to reach higher concentrations of drug in the inner ear, the target is locally treated minimizing the drug side effects, and CDDP antitumor activity is not affected. However, it is not patient friendly. The main challenge in intratympanic treatments is still to achieve sufficient concentration of drug in contact with the sensory auditory cells as any drug delivered through the middle ear has to cross the three-layered round window membrane (RWM), diffuse through the labyrinth fluids and finally enter the inner ear cells. RWM behaves like a semipermeable membrane and its permeability depends on

the size, concentration, structure, solubility and charge of the crossing molecule, enable agents and RWM thickness. 22

In the last years, nanoparticles (NPs) have emerged as promising vehicles to transport drugs to specific tissues or even a particular cell or organelle. NPs with diameters between 100 nm and 1 μm have used in drug delivery to the inner ear with good results. 23

Different NPs based on silica (unloaded NPs), polyethylene glycol (PEG) loaded with resveratrol or copolymers like poly-ε-caprolactone-PEG (PCL-PEG) loaded with furosemide were tested in vitro showing good internalization by auditory cells. 24–26

Different types of nanocarriers have been used in vivo to ameliorate sensorineural hearing loss. Lipid NPs showed a high capacity to incorporate hydrophobic or hydrophilic drugs, and improve stability of encapsulated drugs. Liposomes encapsulating gadolinium-tetra-azacyclo-dodecane-tetra-acetic acid (LPS + Gd-DOTA) showed an efficient diffusion through RWM, 27 and Solid Lipid NPs (SLNs) edaravone-loaded had certain protective effects against noise-induced hearing loss. 28 NPs based on copolymers like polyethylene glycol (PEG) provided immunologic benefits and remained longer in plasma. 29 Poly(D,L-lactide-co-glycolide acid) (PLGA) NPs were used for carrying single or multiple drugs through the RWM showing a significant improvement in drug distribution within the inner ear. 30

The aimof thisworkwas the development of a newtherapy against CDDP-induced ototoxicity based on MP-loaded self-assembled polymericNPs.TheNPswere designed not only to beMP nanocarriers, but also to be active as these were based on methacrylic derivatives of α-tocopherol (vitamin E), a verywell-known free radical scavenger that protects the cochlea from CDDP damage and prevents hearing loss.

Our group has recently described the preparation, characterization and biological activity of surfactant-free NPs based on amphiphilic copolymeric drugs that self-assembled in aqueous media during nanoprecipitation giving rise to multimicellar nanoaggregates. 31 The hydrophilic segment of the copolymers was mainly based on N-vinyl pyrrolidone (VP), which was selected because of its hydrophilicity, biocompatibility and capability to avoid the reticuloendothelial system. 32 The hydrophobic segment was mainly formed by amethacrylic derivative of vitamin E (MVE) or a methacrylic derivative of α-tocopheryl succinate (MTOS).

Surfactant-free polymeric micelles nanoaggregates with hydrodynamic diameters between 96 and 220 nm were formed by self-assembling in aqueous media due to the appropriate hydrophilic/hydrophobic balance of these amphiphilic polymers. The hydrophobic core allowed the encapsulation of poorlywater-solublemolecules, such as coumarin-6 (C6) or additional α-TOS.

Our aim in this work was the encapsulation of hydrophobic MP in the inner core of theseNPs to be administered intratympanically in order to reach higher concentrations ofMP in the inner ear, improve MP stability, avoid the MP side effects, and prevent the systemic with CDDP.

Material and methods CDDP (1 mg/ml) and MP were purchased from Accord Healthcare and Sigma-Aldrich, respectively. Amphiphilic copolymers

A methacrylic derivative of α-TOS (MTOS) and a methacrylic derivative of Vitamin E (MVE), and the copolymers poly(VP-co-MTOS) (89:11) (from now on CO-MTOS) and

poly(VP-co-MVE) (60:40) (from now on CO-MVE) were synthesized as recently described by our group.31

Preparation of loaded nanoparticles MP-loaded NPs were prepared by nanoprecipitation as previously published.33 Briefly, CO-MVE and CO-MTOS were dissolved in dioxane (50 mg/ml) containing an appropriate amount of MP (NP-MVE-10 and NP-MTOS-10, with 10% w/w of drug respect to the polymer, and NP-MVE-15 and NP-MTOS-15 with 15% w/w of drug respect to the polymer).

The resulting solution was added drop-wise to PBS (10 ml) with 5 constant mechanical stirring (650 rpm) (Figure 1). C6-loaded NPs were also obtained by nanoprecipitation. C6 (1% w/w respect to the polymer) and the corresponding polymer (10 mg/mL) were dissolved in dioxane and added dropwise to PBS undermagnetic stirring. The final NPs concentration was 2.0 mg/mL.

The obtained NPs were dialyzed during 72 h, sterilized by filtration through 0.22 µm polyethersulfone membranes (Millipore Express®, Millex GP), and stored at 4 °C until used.

Characterization of NPs

The particle size distribution of the NPs suspensions was determined by dynamic light scattering (DLS) using a Malvern Nanosizer NanoZS, at 25 °C. Zeta potential of the NPs was determined using laser Doppler electrophoresis (LDE). The measurements were obtained for 0.2 mg/ml NPs suspension containing 10 mMNaCl. The zeta potentials were automatically calculated from the electrophoretic mobility using the Smoluchowski´s approximation. The statistical average and standard deviation of data were calculated from 8 measurements of 20 runs each one.

SEM and TEM analyses were performed with a Hitachi SU8000 TED, cold-emission FE-SEM microscope working with an accelerating voltage between 15 and 50 kV. Samples were prepared by deposition of one drop of the corresponding NPs suspension (0.02 mg/ml) over a small glass disk (12 mm diameter) or poly(vinyl formal)-coated copper TEM grid, and evaporation at room temperature. SEMsamples were coated with gold palladium alloy (80:20). An additional drop of Brilliant Black dye (Sigma-Aldrich, 1 mg/ml) was deposited on the grid and the excess was removed with filter paper and the grid was allowed to dry before TEM observation.

Encapsulation efficiency MP-loaded NP-MVE and NP-MTOS were freeze dried and an amorphous powder was obtained with a yield higher than 90 %in all cases. The powder was dissolved in chloroformand the solvent was evaporated at room temperature for 24 h. Ethanol (2 ml) was added to dissolve MP or C6, and stirred during 24 h. Samples were centrifuged at 10,000 rpm and supernatant was analyzed by UV or fluorescence spectroscopy (λabs = 244 nm for MP, and λexc = 485 nm, λemis = 528 for C6). The encapsulation efficiency (EE%) was calculated as follows:

Encapsulation efficiency (EE%) = ([loaded molecule]i/[loaded molecules]0) × 100,

With [loaded molecule]i being the concentration of MP or C6 encapsulated and detected experimentally, and [loaded molecule]0 the concentration of MP or C6 added in the nanoprecipitation process.

Esterase-mediated MP release 5 mL of MP-loaded NPs (NP-MVE-15 or NP-MTOS-15) 6 with 15 u/mL of esterase from porcine liver (Sigma-Aldrich) was dialyzed against 10 mL of PBS at 37 °C using a 3.5-5 kDa MWCO membrane (Spectrum Laboratories). After certain periods, 1 mL of the dialyzing medium was withdrawn and the same volume (1

mL) of PBS was replenished. MP concentration was measured by HPLC (Shimadzu). The separation was performed on a C18-column (4.6 mm × 250 mm, Agela Tech-nologies) at 30 °C. The mobile phase was a mixture of acetonitrile, and distillated water (80:20, v/v) pumped at a rate of 1 mL/min. The UV detector was set at λabs =244 nm. The experiment was carried out in triplicate.

Cell culture experiments

The HEI-OC1 cell line was a kind gift from Dr. Federico Kalinec (House Ear Institute, Los Angeles, CA). HEI-OC1 cells were maintained under permissive conditions: high-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Gibco), 5% L-Glutamine (Sigma-Aldrich) and Penicillin-G (Sigma-Aldrich)
at 33 °C and 10% CO2.34

Toxicity of CDDP

To assess the impact of CDDP on cell viability, the cells (3 × 104 cells/ml) were exposed to 10, 20, 30, 40, 50 and 100 µM of CDDP in DMEM/PBS for 24 h. DMEM without FBS was used to avoid uncontrolled cell growth.

Apoptosis

Apoptotic cell death was qualitatively evaluated by Hoechst 33258 nuclear staining. Cells were incubated with 2 µg/ml of the Hoechst 33258 (Sigma-Aldrich) for 20 min. After washing twice with PBS, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were washed twice with distilled water and evaluated under a Nikon Eclipse TE 2000-S fluorescence microscope with a DS-U2 camera controller (Nikon). NPs toxicity and protection assay HEI-OC1 (3 × 104 cells/ml) cells were exposed to different concentrations of NPs solution (2.00, 1.00, 0.50, 0.25, 0.13, 0.06, 0.03, 0.02 and 0.01 mg/ml) in order to assess their impact on cell viability.
NPs were added to HEI-OC1 and 4 h later 20 or 30 µM CDDP was added to the cultures in DMEM without FBS to avoid uncontrolled cell growth for 24 h, in order to study the protection effect of the NPs against CDDP.
AlamarBlue® (Invitrogen) was carried out to determine cell viability using a Multi-Detection Microplate Reader Synergy HT (BioTek Instruments; λabs = 570 nm).
In vivo experiments Thirty six healthy female Wistar rats weighting 180-280 g were used. All animals were housed in plastic cages with water and food available ad libitum, and maintained on a 12 h light/ dark cycle. Rats with signs of present or past middle ear infection were discarded. Animals were randomly assigned to different groups (Table 1).
The animals were handled according to the guidelines of the Spanish law for Laboratory animals care registered in the "Real Decreto 53/2013" and the European Directive 2010/63/EU. The study was approved by the Clinical Research and Ethics Committee of the University Hospital Puerta de Hierro (dossier No. 013/2012).
Experimental procedure Animals were anesthetized with intraperitoneal ketamine (100 mg/kg) and diazepam (0.1 mg/kg). An initial auditory steady-state responses (ASSR) test was performed on all animals. An insert earphone (Etymotic Research ER-2) was placed directly into the external auditory canal. Subcutaneous electrodes were placed over the vertex (active) and in the pinna of each ear (reference). Ground

electrodes were placed in the right leg muscles. ASSR were recorded using an evoked potential averaging system (Intelligent Hearing System Smart-EP) in an electrically shielded, double-walled, sound-treated booth in response to 100 ms clicks or tone burst at 0.5, 1, 2, 4, 8, 12 and 16 kHz with 10 ms plateau and 1 ms rise/fall time. Intensity was expressed as decibels sound pressure level (dB SPL) peak equivalent. Intensity series were recorded, and an ASSR threshold was defined by the lowest intensity able to induce a replicable visual detectable response.
Following the ASSR measurements the right ear bulla was surgically approached and opened 301 35 and 50 µl of NPs solution was injected in the middle ear by bullostomy using a spinal needle (BD Whitecare 27G). Left ear was injected with PBS through the bullostomy as a control. After injection the anesthetized animals remained in lateral decubitus for 30 min to maximize the solution's contact time with the RWM and to prevent its leakage into the pharynx through the Eustachian tube.
After surgery, enrofloxacin and morphine (Braun 20 mg/ml) were administered subcutaneously for prevention of infection and postoperative analgesia, respectively. 310
CDDP-treated groups: after NPs administration in the right ear, an intraperitoneal slow infusion of CDDP (10 mg/kg) was carried out for 30 min. After CDDP infusion, animals were housed in individual cages with ad libitum access to water and food. ASSR were tested after 3 days, and rats were euthanized by CO2 suffocation.
In vivo distribution of coumarin-6-loaded NP NP-MVE-C6 and NP-MTOS-C6 were administered in vivo as described in the previous section. However, these animals were euthanized after 2 h. Cochlea was extracted and fixed in paraformaldehyde over 24 h and decalcified in 1% ethylenedi-aminetetraacetic acid (EDTA) at room temperature (with daily changes) for 10-12 days. Once the bone was completely decalcified, the cochlea was dissected in PBS, and cochlear surface extracts were visualized using an inverted microscope (Nikon Eclipse TE 2000-S) and a confocal laser fluorescence microscope (CLFM; Leica TCS-SP5 RS AOBS). Statistical analysis One-way ANOVA was used to analyze for statistical significance of all in vitro and in vivo results. Tukey test was used to identify significant differences between the paired treatments. P b 0.05 was considered statistically significant.

Results
NPs characterization Unloaded and MP-loaded NPs were obtained by nanopreci-pitation in PBS of the corresponding polymer solutions in dioxane and their principal characteristics are described in Table 2. EE% was higher for NP-MVE (48% for NP-MVE-10 and 53% for NP-MVE-15) than NP-MTOS (19% for NP-MTOS-10 and 26% for NP-MTOS-15).
All the synthesized NPs presented unimodal size distributions with apparent hydrodynamic diameters (by intensity, Dh) between 120 and 128 nm, with low PDI values. Dh slightly increased with the MP content in both families of NPs. All NPs presented slightly negative zeta potential.
SEM and TEM micrographs showed that both NP-MVE (Figure 2, A and C) and NP-MTOS (Figure 2, B and D) presented well-defined spherical morphology. Esterase-mediated in vitro MP release In vitro release of MP was studied by an esterase-mediated dialysis diffusion method. Figure 2, E shows the in vitro MP release profile at

37 °C during one week. About 20% of the loaded MP is released from the NP-MVE-15 and more than 40% from NP-MVE-15 within seven days.

In vitro experiments

HEI-OC1 auditory cell line is very sensitive to ototoxic drugs and expresses specific markers of Organ of Corti. 34 HEI-OC1 viability decreased with the concentration of CDDP in a dose dependent manner (see supplementary data Figure S2, A).

Cytotoxicity of unloaded and loaded-NPs was also tested using HEI-OC1 and the results demonstrated that only the highest concentration (2.0 mg/ml) of both NP-MVE and NP-MTOS was cytotoxic (viability b70%; ISO 10993-5:2009). Viability of cells treated with MVE formulations was reduced near to 40%, while MTOS formulations reduced viability near to 70%. MVE formulations (Figure 3, A) resulted to be more cytotoxic than MTOS formulations (Figure 3, B) because MVE at 1.0 mg/ml also reduced cell viability more than 20% and in a corticoid loading dependent manner (HEI-OC1 viability NP-MVE-0 N NP-MVE-10 N NP-MVE-15 at 1.0 mg/ml). However, no statistically significant differences were found between the formulations.

NP-MVE and NP-MTOS ameliorates CDDP-induced cytotoxicity in HEI-OC1 373

NPs were added to HEI-OC1, and 4 h later 20 or 30 μM CDDP was added for 24 h. Cell viability approximately decreased till 60%, and till 50% when 20 μM CDDP (Figures 4, A and 5, A) and 30 μM CDDP were added to the cells (Figures 4, B and 5, B), respectively. MP-loaded NP-MVE (NP-MVE-10 and NP-MVE-15) at concentrations between 0.5 and 0.13 mg/ml significantly reduced CDDP-induced cytotoxicity of 20 and 30 μM CDDP. NP-MVE concentrations of 2 and 1 mg/ml resulted to be cytotoxic per se, as shown in Figure 3, A and B and were not able to ameliorate CDDP cytotoxic effect even if cells were treated with MP-loaded NPs. NP-MVE-15 was apparently the most effective formulation although there were no statistically significant differences with NP-MVE-10. 2 mg/ml concentration of NP-MTOS resulted to be cytotoxic, but 1 mg/ml significantly protected HEI-OC1 from CDDP effects. No significant differences between NP-MTOS-0, NP-TOS-10 and NP-MTOS-15 were observed that could be explained due to the poor MP EE% with this copolymer.

NP-MVE-10, NP-MVE-15 and NP-MTOS-10 and NP-MTOS-15 significantly reduced CDDP cytotoxicity in a certain range of concentrations, being the most active MVE formulations. NP-MTOS-0 slightly increased cell viability of CDDP-treated cells.

In vivo experiments

Young adult animals were selected to test the possible protective effect of the synthesized NPs as CDDP ototoxicity is also related to the age of the patient and both elderly and pediatric patients are reportedly more sensitive to CDDP ototoxicity.

In order to maximize NP delivery to inner ear, the highest dose of NPs (2 mg/ml) was used even though it was toxic in the in vitro tests. Empty NPs were inoculated through a bullostomy in the right ear of 2 animals per formulation (NP-MVE-0 and NP-MTOS-0). These animals were not treated with CDDP after surgery although they did get the same palliative care as the animals that received the chemotherapeutic treatment. After 72 h of NPs exposure, no significant differences were observed between the auditory thresholds of the right ear and the left ear at all frequencies (see supplementary data Figure S3).

NP-MVE-15 decreased CDDP-induced hearing loss in an in vivo model NP-MVE-0 and NP-MTOS-0 were not able to decrease CDDP-induced ototoxicity. Auditory thresholds 72 h after CDDP treatment increased in the same way in both ears
(Figure 6).
NP-MVE-10 and NP-MTOS-10 showed no otoprotection (data not shown). However, NP-MTOS-15 and NP-MVE-15 were active in vivo and showed protection against CDDP-induced hearing loss. NP-MTOS-15 protected in a significant way only in one medium frequency (Figure 7, A), while NP-MVE-15 was able to decrease CDDP-induced ototoxicity in frequencies between 10 and 16 kHz, being statistically signif-icant from 14 to 16 kHz. In view of the results, the size of the group was increased from 4 animals to 12 to corroborate the preliminary results. Animals that received this treatment showed that auditory thresholds in high frequencies (14 and 16 kHz) were significantly lower in the protected right ear (Right ear: CDDP + NP-MVE-15) when compared with the auditory thresholds of the left ear (left ear: PBS + CDDP) (Figure 8, A).

NP-MVE-C6 and NP-MTOS-C6 accumulation in IHC and OHC NP-MTOS-C6 (Figure 7, B, C and D) and NP-MVE-C6 (Figure 8, B, C and D) were preferentially accumulated in the IHC than in OHC of the basal turn of the cochlea after 2 h post-administration in the middle ear. Green fluorescence due to the accumulation of C6-loaded NP progressively decreased from the basal turn to the apical turn (see supplementary data Figure S4).

Discussion

CO-MVE and CO-MTOS are amphiphilic copolymers that present an appropriate hydrophilic/hydrophobic balance to self-organize in aqueous media forming spherical NPs with a hydrophilic shell (mainly VP sequences) and a hydrophobic core (mainly MVE or MTOS monomer, respectively). 31

MP was incorporated in the core of the NPs in order to locally deliver the corticoid in the inner ear at appropriate concentrations and to increase its activity and decrease its undesired side effects.

Encapsulation efficiency was higher for NP-MVE than NP-MTOS.

This was probably due to a different hydrophobic/hydrophilic balance (MVE system is poly(VP-co-MVE)(60:40) and MTOS system is poly(VP-co-MTOS)(89:11)). CO-MVE has a higher content in the hydrophobicmonomerMVEthat could favor the encapsulation ofMP.

Bowe et al 22 studied the RWM perfusion dynamics and stated that NPs with sizes lower than 200 nm were able to pass through this membrane by rapid diffusion making them ideal candidates for drug delivery across the RWM. Cai et al 459 30 demonstrated the potential of PLGA-based NPs with sizes between 135 and 154 nm for carrying drugs and crossing the RWMin guinea pigs.

Sizes measured by DLS were between 120 and 128 nm and therefore were adequate for this purpose.

All NPs presented slightly negative zeta potential, indicating an almost neutral charge surface. These values have been previously described for other authors and were also corrobo-rated by our group with α-TOS-loaded NPs based on CO-MVE and CO-MTOS. This fact indicates that the shell of the NPs is constituted by VP-rich hydrophilic domains.

Esterase-mediated MP release profiles of both systems followed a zero order kinetic during the first 80 h, followed by a non-linear period (Figure 2, E). The experimental conditions are far from the in vivo environment; however, this experiment probes that MP release takes place in the presence of esterase in a sustained manner during, at least, one week. The biological half-life of MP in the inner ear is around 24 h20 and therefore, its protection and sustained release are of great interest to its application as otoprotector of CDDP-induced toxicity.

Non-loaded NPs (CO-MTOS and CO-MVE) were tested in order to check if they could effectively reduce ROS in CDDP treated HEI-OC1 cells as they incorporate α-tocopherol or α-tocopheryl succinate derivatives covalently attached to their macromolecular chain.36

Kruspig et al37 studied the effects of the combinatorial administration of α-TOS and other antiproliferative molecules, and demonstrated the antagonic effects of low concentrations of α-TOS and CDDP. Therefore, NP-MTOS were tested in order to check if the α-TOS of the copolymer could effectively reduce the pro-apoptotic effect of CDDP in HEI-OC1 cells. NPs based on both MVE and MTOS with concentrations between 1 and 0.01 mg/ml resulted to be non-cytotoxic to HEI-OC1. NP-MVE-15 was the most effective formulation in vitro, although there was no statistically significant difference with NP-MVE-10 (Figure 4, A and B). NP-MTOS had less protector effect against CDDP-induced cytotoxicity than NP-MVE treated group (Figure 5, A and B). NP-MTOS-0 slightly increased cell viability of CDDP-treated cells. This could indicate certain activity of the polymer although this effect was not statistically significant. Therefore, NP-MVE-0 and NP-MTOS-0 cannot be considered active against CDDP cytotoxicity in vitro, but are good nanocarriers to encapsulate and transport MP. As it has been commented before, the differences between both systems could be ascribed to the different composition of the copolymers better than the chemical structure of both MVE and MTOS components. Despite the development of new regimens and dosage limits, the ototoxic effect of CDDP treatment is still unavoidable. A single injection of high doses of CDDP rapidly causes ototoxicity with a high incidence. CDDP-induced hearing loss firstly affects the high frequencies and continues to medium frequencies so an increase in auditory threshold is shown for frequencies from 8 to 16 kHz. NP-MVE-0 and NP-MTOS-0 were not able to decrease CDDP-induced ototoxicity (Figure 6). This fact could be related to the high doses that were used in this experiment to simulate aggressive CDDP-based therapies used in clinical patients.

Corticoids have been systemically administered to protect against sensorineural hearing loss with good results.38 However, some authors have called into question the efficacy of systemic steroids due to the controversial results in clinical studies because

of the side effects. Other problem associated with corticoid systemic administration has been the wide range of pharmacokinetic factors that influence the concentration of the drug inside the inner ear including: difference in distribution, variability to cross the blood–labyrinth barrier, different drug metabolic pathways and varied routes of excretion.39 Due to these related problems intratympanic administration is an accepted alternative to systemic administration in patients with contraindications for systemic corticoids.20,40 A good correlation between the in vitro and in vivo experiments was found when unloaded and MP-loaded nanoparticles were tested to palliate the ototoxicity of CDDP.

Unloaded-NP reduced the ototoxicity of the CDDP but this effect was not statistically significant, although both copolymers incorporated vitamin E or α-TOS in their structure. Loaded-NPs were active and their activity mainly depended on the encapsulation efficiency. NP-MVE-15 was the most active formulation both in vitro and in vivo. In the in vitro experiments, concentrations between 0.13 and 0.50 mg/mL significantly reduced cytotoxicity of CDDP on HEI-OC1 cells. In the in vivo experiments, high frequencies (14-16 kHz) were protected by the addition of the NP suspension intratympanically by bullostomy (Figure 8, A). In addition, NP-MVE-C6 (Figure 8, B,C andD) andNP-MTOS-C6 (Figure 8,B,CandD)were accumulated in the area of sensory auditory cells by crossing the three-layered RWMafter 2 h of the administration. Accumulation preferentially occurred in IHC in the basal turn of the cochlea associated with higher frequency hearing.

In conclusion, these results indicate that NP-MVE-15 could be a good candidate to deliver MP in the inner ear to palliate, at least in part, the CDDP ototoxicity. Moreover, both kinds of polymers could also be used to encapsulate and deliver other hydrophobic drugs (antioxidants, anti-inflammatory agent, or anticaspase drugs) as both NP crossed the round window membrane and enter the inner ear.

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Figura 1.

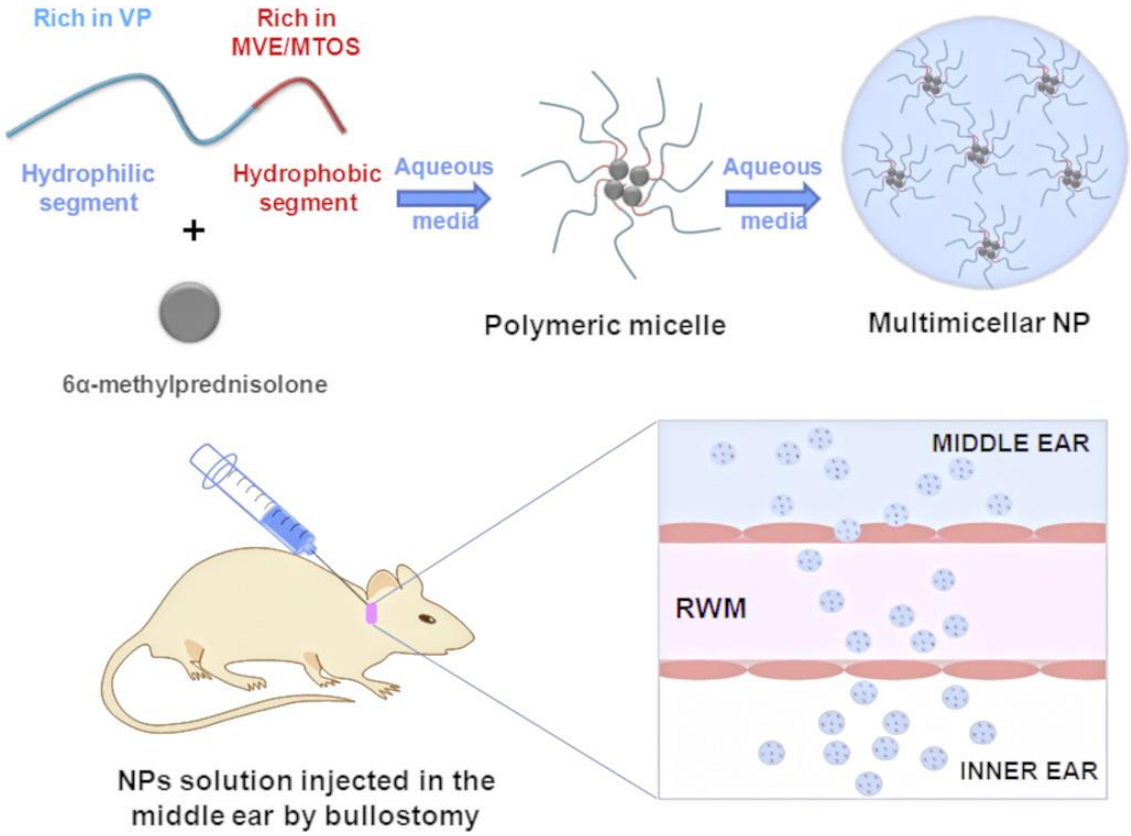

Rich in VP
Rich in MVE/MTOS
Hydrophilic segment
Hydrophobic segment
Aqueous media
Aqueous media
Polymeric micelle
Multimicellar NP
6α-methylprednisolone
MIDDLE EAR
RWM
INNER EAR
NPs solution injected in the middle ear by bullostomy

Figura 2

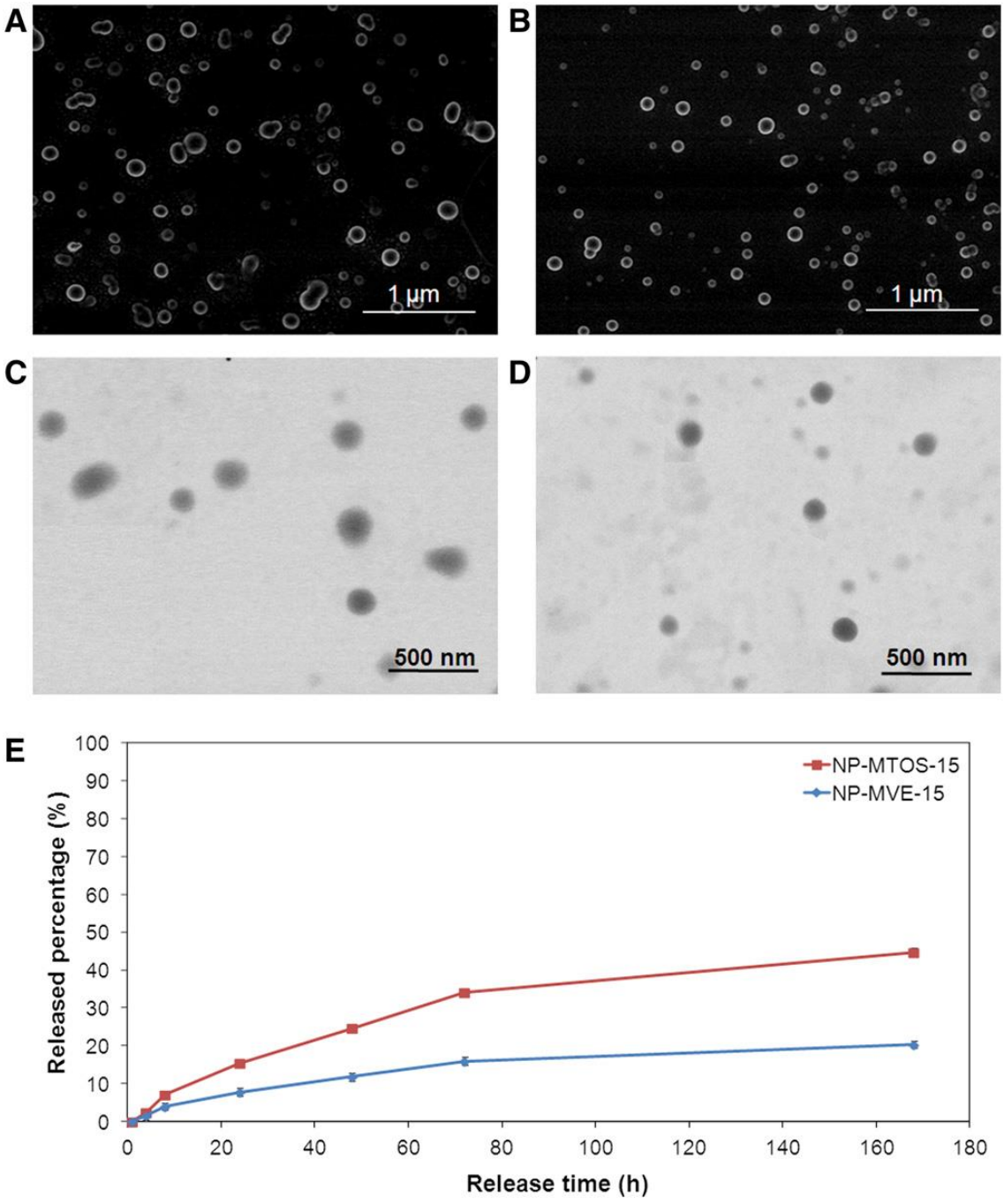

A
1 μm
B
1 μm
C
500 nm
D
500 nm
E
NP-MTOS-15
NP-MVE-15
Released percentage (%)
Release time (h)
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
160
180

Figura 3

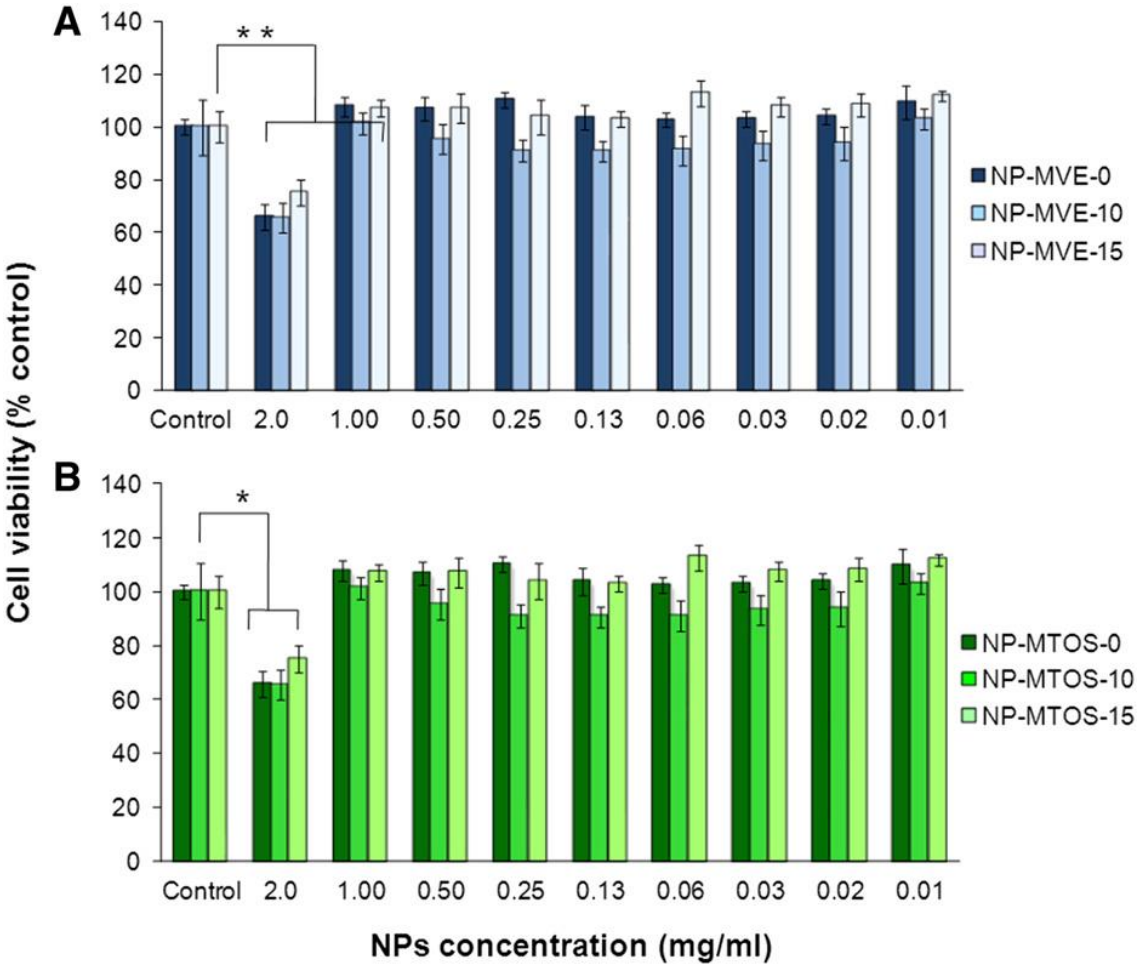

A
B
Cell viability (% control)
NPs concentration (mg/ml)
Control
2.0
1.00
0.50
0.25
0.13
0.06
0.03
0.02
0.01
NP-MVE-0
NP-MVE-10
NP-MVE-15
NP-MTOS-0
NP-MTOS-10
NP-MTOS-15

Figura 4

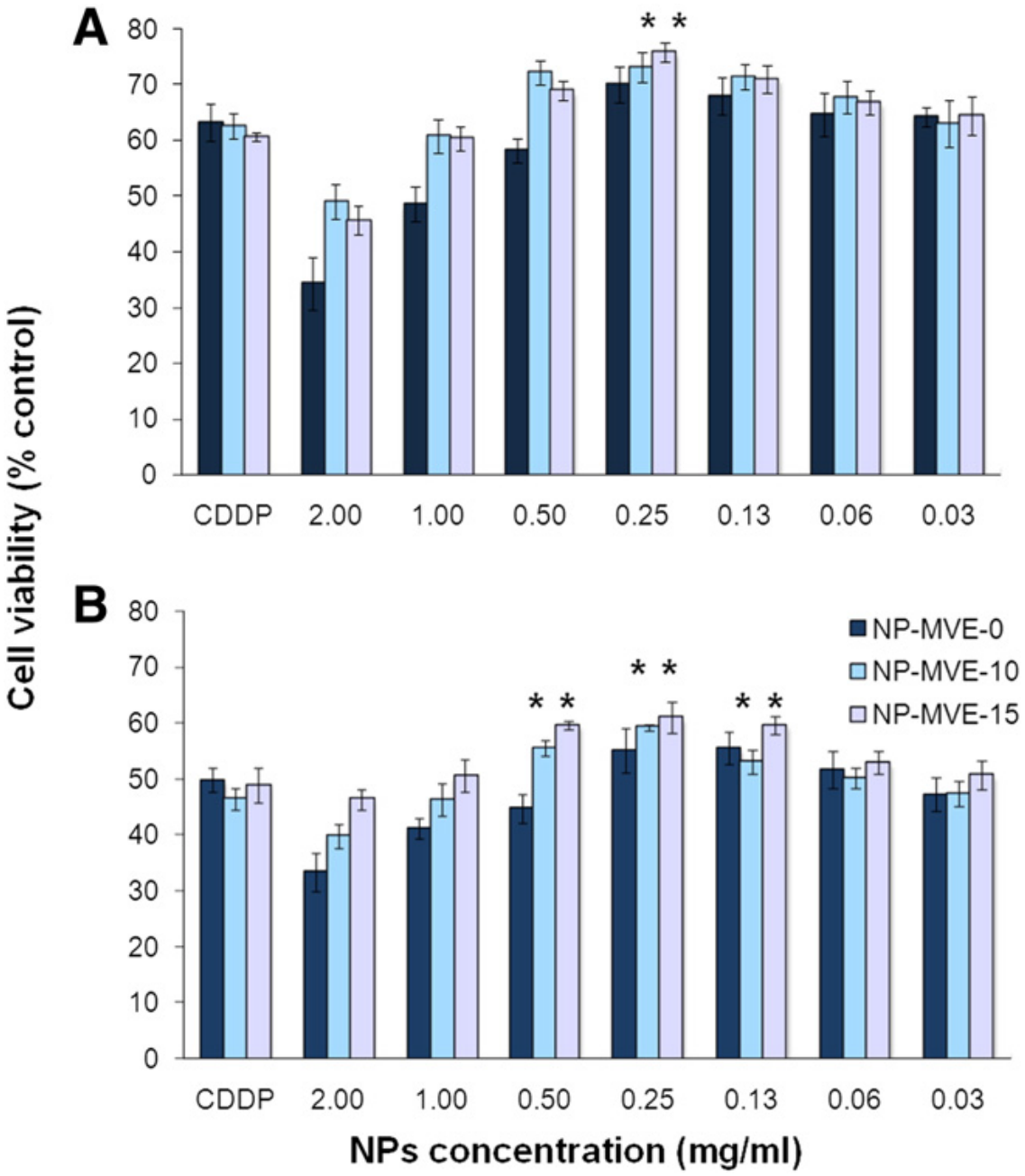

A
B
Cell viability (% control)
NP-MVE-0
NP-MVE-10
NP-MVE-15
CDDP
2.00
1.00
0.50
0.25
0.13
0.06
0.03
NPs concentration (mg/ml)

Figura 5

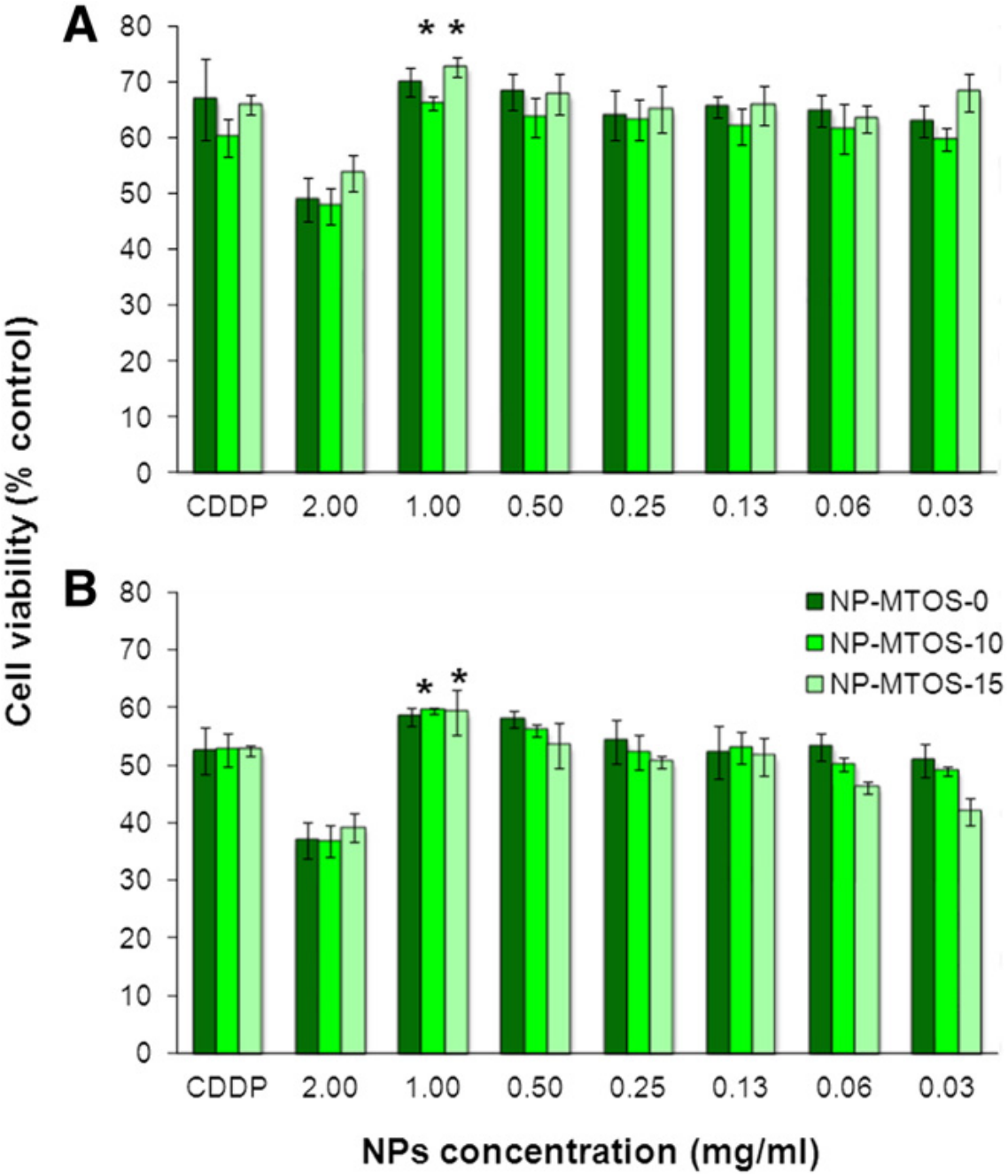

A
B
Cell viability (% control)
0
10
20
30
40
50
60
70
80
* *
CDDP
2.00
1.00
0.50
0.25
0.13
0.06
0.03
NP-MTOS-0
NP-MTOS-10
NP-MTOS-15
NPs concentration (mg/ml)

Figura 6

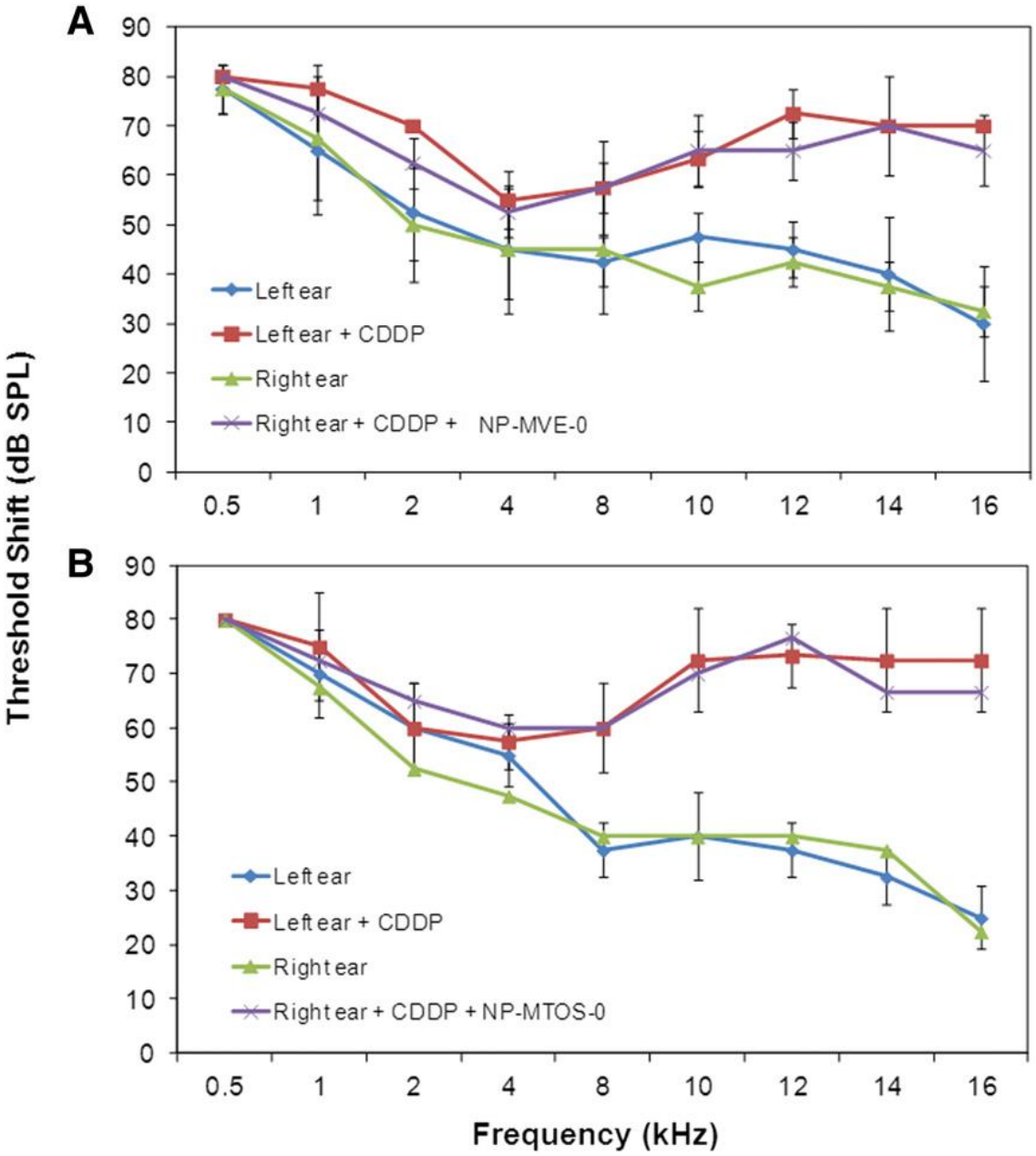

A
B
Threshold Shift (dB SPL)
Frequency (kHz)
0
10
20
30
40
50
60
70
80
90
0.5
1
2
4
8
10
12
14
16
Left ear
Left ear + CDDP
Right ear
Right ear + CDDP + NP-MVE-0
Right ear + CDDP + NP-MTOS-0

Figura 7

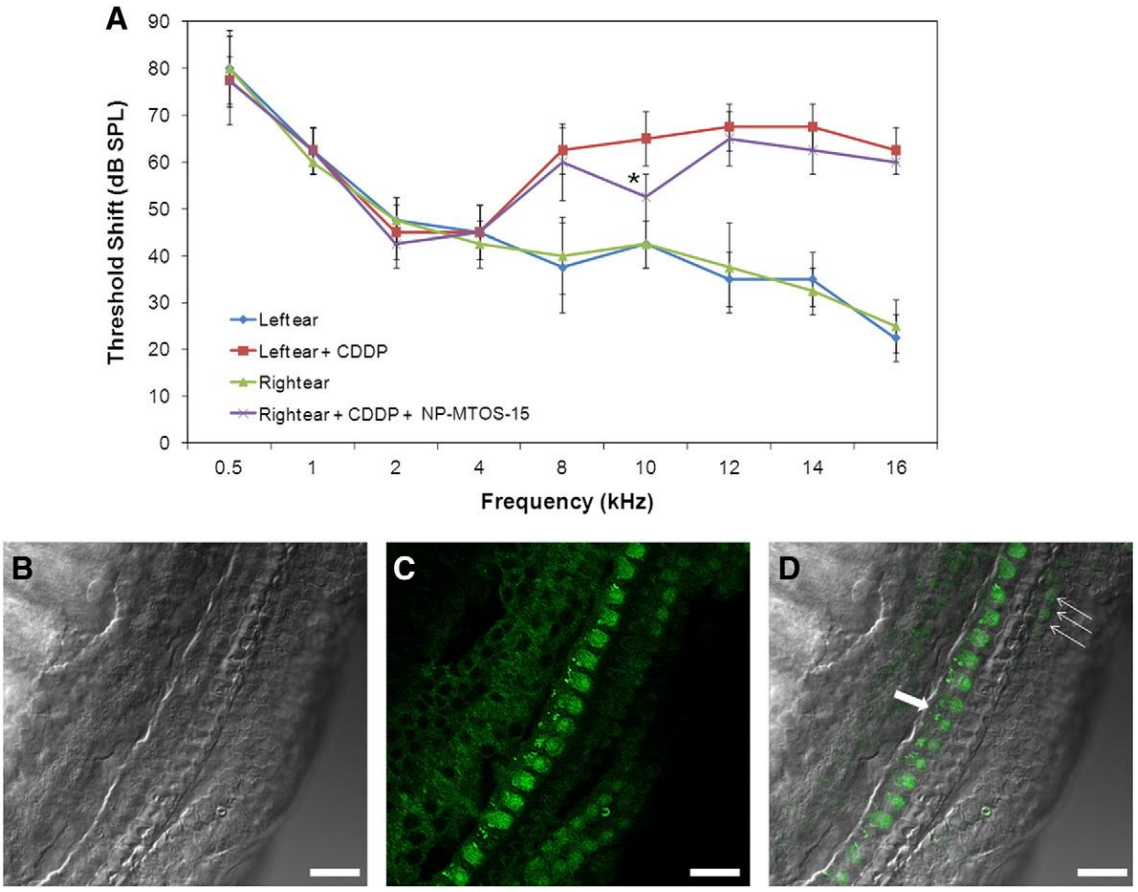
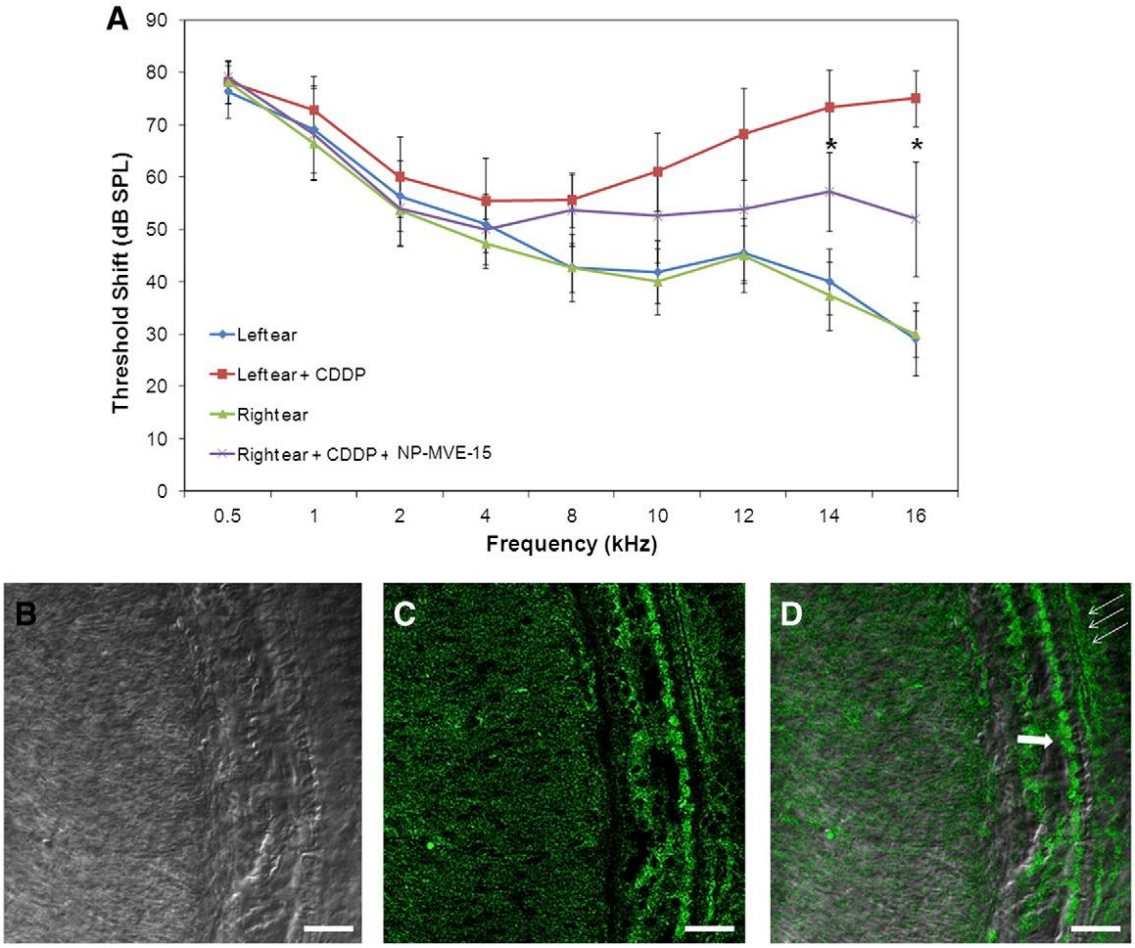

A
Threshold Shift (dB SPL)
90
80
70
60
50
40
30
20
10
0
0.5
1
2
4
8
10
12
14
16
Frequency (kHz)
Left ear
Left ear + CDDP
Right ear
Right ear + CDDP + NP-MTOS-15
*
B
C
D

Figura 8


A
Threshold Shift (dB SPL)
90
80
70
60
50
40
30
20
10
0
0.5
1
2
4
8
10
12
14
16
Frequency (kHz)
Left ear
Left ear + CDDP
Right ear
Right ear + CDDP + NP-MVE-15
*
*
B
C
D