Research Article

The Development of a Nano-based Approach to Alleviate Cisplatin-Induced Ototoxicity

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ABSTRACT

Cisplatin-induced hearing loss is experienced by a high percentage of patients with squamous cell carcinoma undergoing cisplatin chemotherapy. A novel nano-construct capable of sequestering extracellular cisplatin was developed to combat this problem. The nano-construct consisted of superparamagnetic iron oxide nanoparticles (SPIONs) entrapped within polymeric micelles, which were formed from a glutathione diethyl ester-conjugated amphiphilic diblock copolymer. The glutathione-micelles were analyzed at the cellular level and in an organotypic study for safety evaluation. All utilized methods indicated that the micelles do not cause cellular toxicity or organ damage. The micelles' ability to reduce cisplatin-induced cytotoxicity was then probed in an in vitro model. Cisplatin was pre-treated with the novel nano-construct before being added to growing cells. When compared to cells that were exposed to untreated cisplatin, cells in the pre-treated cisplatin group showed a significant increase in cell viability. This clearly demonstrates that the construct is able to protect the cells from cisplatin cytotoxicity and makes it highly likely that the novel nano-construct will be able to play a role in the protection of the inner ear from cisplatin-induced ototoxicity.

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INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum or CDDP) is a drug widely used in the treatment of squamous cell carcinoma (van den Berg et al. 2006) and testicular cancers (Einhorn 2002). Cisplatin's chemotherapeutic effect comes from its ability to bind to DNA's purine bases at the N7 position which can result in intrastrand binding and which in turn induces apoptosis (Jamieson and Lippard 1999). Cisplatin also damages cells by the generation of reactive oxygen species and other free radicals (Callejo et al. 2015). One of the major side effects associated with cisplatin chemotherapy is ototoxicity (Rybak et al. 2009). The use of a cisplatin chemotherapy regime results in permanent hearing loss for 10 to 25 % of adult patients and 26 to 90 % of pediatric patients (Lee et al. 2016). During cisplatin chemotherapy, the loss of hearing associated with cisplatin toxicity creates a dilemma for both patients and physicians that may significantly impact the outcome of the chemotherapy. The permanent loss of hearing is due to cisplatin generated DNA damage and oxidative stress in the hair cells of the inner ear, especially the outer hair cells (Callejo et al. 2015), which leads to caspase directed apoptosis (Karasawa and Steyger 2015). At present, there is no effective treatment for cisplatin-induced ototoxicity.

Surprisingly, although the majority of cisplatin's antitumor activity comes from its ability to form DNA

adducts (Rabik et al. 2006), only about 1 % of the cellular cisplatin is bound to DNA (Gonzalez et al. 2001). This is because the drug binds to other cellular nucleophiles (Gonzalez et al. 2001), with one of the most important physiological cisplatin interactions being with glutathione. Glutathione is an abundant thiol-containing tripeptide and is an extremely important cellular antioxidant (Gebicki 2016). Glutathione appears to play a major role in cellular cisplatin resistance (Jamali et al. 2015; Jamieson and Lippard 1999; Rabik and Dolan 2007) by forming highly stable complexes with cisplatin. It has been estimated that glutathione is responsible for the sequestration of approximately 60 % of intracellular cisplatin (Ishikawa and Ali-Osman 1993). Moreover, the export of the glutathione-cisplatin complex from the cell using MRP/GS-X pumps is one of the major mechanisms of cellular cisplatin detoxification (Ishikawa et al. 1996).

In its native form, cisplatin possesses limited therapeutic activity. However, it becomes highly active once one or both of its chloride groups are displaced by water (Rybak et al. 2009). This process is accelerated when cisplatin is transported by a range of membrane proteins (Ciarimboli 2014; Wensing and Ciarimboli 2013) from the high chloride environments of the perilymph and the endolymph to the lower chloride environment of the cytoplasm (Jamieson and Lippard 1999). Although cisplatin is less active in high-chloride environments, it is not inert, as it is still capable of binding to serum proteins (Ferraro et al. 2015).

The high incidence of ototoxicity among patients undergoing chemotherapy demonstrates that the inner ear structures are vulnerable to cisplatin-induced apoptosis (Lee et al. 2016). The drug is capable of diffusing through the labyrinth blood barrier into the cochlea (Laurell et al. 2000). Once the cisplatin has entered the cochlea, there is no mechanism to remove the cisplatin from the inner ear (Rabik and Dolan 2007). Many attempts have been made to combat cisplatin ototoxicity by delivering therapeutic agents to the inner ear. The thiol agent Amifostine has been used to treat cisplatin-induced ototoxicity. Amifostine, like glutathione, can form stable adducts with cisplatin (Thompson et al. 1995). Unfortunately, the benefit of Amifostine treatment has not been validated so it is not used in the clinic to treat ototoxicity (Katzenstein et al. 2009). Other antioxidants that have been used to reduce cisplatin toxicity include L-N-acetylcysteine (Yoo et al. 2014) and vitamin E (van den Berg et al. 2006) with little to no success.

Herein, we report the synthesis and characterization of a glutathione-functionalized novel nanoconstruct to sequester extracellular cisplatin and to reduce cisplatin-induced cytotoxicity. The nanoconstruct was prepared with superparamagnetic iron

oxide nanoparticles (SPIONs) encapsulated within the hydrophobic core of micelles, which were formed using the biodegradable amphiphilic diblock copolymer poly (ethylene glycol)-b-poly (caprolactone). Prior to micelle assembly, the poly (ethylene glycol) block of the diblock copolymer was conjugated to a glutathione diethyl ester to enable the sequestration of cisplatin. The physical chemical properties and cytotoxicity of the micelles were then characterized. Finally, the ability of the micelles to reduce cisplatin-induced cytotoxicity by binding cisplatin *in vitro* was evaluated. The procedure included the use of an external magnetic field to attract the micelle-encapsulated SPIONs and permanently remove the cisplatin-bound micelles from the growth media.

MATERIALS AND METHODS

Materials

All polyethylene glycol-poly(ϵ -caprolactone) (PEG-PCL) diblock copolymers were purchased from Advanced Polymer Materials Inc. The reduced glutathione diethyl ester was obtained from Bachem, N-Hydroxysuccinimide (NHS) was purchased from Sigma-Aldrich while 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Thermo Scientific. The PD-10 columns were manufactured by GE Healthcare and the CellEvent™ Caspase-3/7 Green ReadyProbes® Reagent by Thermo Fisher. Pharmaceutical grade cisplatin was supplied by Teva Pharmaceuticals. The C57BL/6J mice used in the cochlear organotypic culture experiments were purchased from Charles River Laboratories. The American Type Culture Collection's (ATCC's) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit was used for the measurement of cell viability. The platinum measurements were performed on a Spectro Genesis inductively coupled plasma optical emission spectrometry (ICP-OES) apparatus. Finally, the mouse anti-myosin VIIa primary antibody was obtained from Santa Cruz Biotechnology and the goat anti-mouse IgG (H+L) Alexa Fluor® 488 conjugate secondary antibody from ThermoFisher.

Synthesis of SPIONs

The method of Park et al. (Park et al. 2004) adapted by McQuade et al. (McQuade et al. 2015) was utilized to create and purify oleic acid stabilized hydrophobic SPIONs of approximately 7 nm in diameter. SPION size was determined using a JEOL 1010 transmission electron microscope, and the average size was deter-

mined by measuring the diameters of 50 individual particles.

Synthesis of the Glutathione Diethyl Ester-PEG PCL Conjugate

Carboxyl-PEG (5.2 k)-b-PCL (5.2 k), EDC, NHS and reduced glutathione diethyl ester were dissolved in dimethylformamide (DMF). A trace amount of trimethylamine was added to the mixture before it was allowed to incubate, with stirring, overnight. The reaction mixture was then dialyzed against a large excess of water with several water changes for 24 h. The conjugate was then lyophilized.

Synthesis of SPION Micelles

The micelles were created using a self-assembly method (Mahajan et al. 2013). Briefly, SPIONs (4 mg), glutathione diethyl ester-PEG PCL conjugate (2 mg), and PEG (2 k)-b-PCL (3 k) (2 mg) were dissolved in toluene (200 μ L). This mixture was added to water (4 mL) and the sample emulsified for at least 3 min in an ultrasonic water bath. The micelle sample was then allowed to incubate, with stirring, at 25 °C for 24 h. The unincorporated SPIONs and/or aggregates were removed from the sample firstly by centrifugation and then by passage through a 0.22- μ m filter.

Micelle Characterization

The diameters of the newly synthesized micelles were determined by dynamic light scattering using a Zetasizer Nano-ZS apparatus (Malvern Instruments). The micelles' iron concentration was determined by ICP-OES.

Glutathione Binding to Cisplatin in PBS

The Ellman's free thiol assays (Ellman 1959) containing 100 μ M 5,5'-dithio-bis-[-2-nitrobenzoic acid] (Ellman's Reagent) in 2.5 mM sodium acetate 100 mM Tris pH 8.0 were utilized. The Ellman's assays were used to indirectly measure the binding of glutathione to cisplatin. Reaction mixtures containing 1 mM glutathione and 1 mM cisplatin in phosphate-buffered saline (PBS) were prepared in triplicate. Control samples containing 1 mM glutathione or 1 mM cisplatin in PBS and PBS alone were also prepared in triplicate. The samples were then stored in the dark at 37 °C until required. At set time points, aliquots of each sample were added to Ellman's assays and 5 min later the Ellman's assays' absorption was monitored at 412 nm.

Micelle/Cisplatin Binding in PBS

Cisplatin does not absorb light in the 240- to 750-nm region of the spectrum and consequently, it also does not fluoresce, making it very difficult to determine cisplatin concentrations directly. Consequently, ICP-OES was utilized to measure the concentration of the platinum that is at the heart of the cisplatin molecule. This enabled the cisplatin concentration to be calculated. This method takes advantage of the observation that the concentration of platinum in both tissue and the environment is extremely low (Rudolph et al. 2005). As a result, the overwhelming majority of the platinum in the experimental samples detected by ICP-OES originated from cisplatin. Test solutions containing glutathione diethyl ester micelles with an iron concentration of 18 μ g/ml and 3 μ g/ml cisplatin concentration in 2 ml PBS were prepared. Control solutions were produced which contained: (a) micelles in PBS, (b) 3 μ g/ml cisplatin in PBS, and (c) PBS. The samples were allowed to incubate in the dark at room temperature for 48 h and were stirred occasionally by hand. The unbound cisplatin was then removed from the samples by passing them through PD-10 columns before the platinum content of the eluent was measured by ICP-OES ($n=3$).

HEI-OC1 Cells

The House Ear Institute-Organ of Corti 1 (HEI-OC1) cells were obtained from Dr. Kalinec at UCLA and chosen for the cytotoxicity studies because the cell line expresses many of the inner ear biomarkers (Devarajan et al. 2002). The HEI-OC1 cells were grown at 33 °C and 10 % CO₂ in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % penicillin (Kalinec et al. 2016). The half maximal inhibitory concentration (IC₅₀) for cisplatin interactions with HEI-OC1 cells was found to be between 1 and 2.5 μ M.

Organ of Corti Explant Organotypic Culture Cytotoxicity Assays

Animal care and use was in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of The University of Pennsylvania and of the National Institute of Health. Four-day-old C57BL/6J mouse pups were sacrificed and their cochleae removed. Cochlear explants were then cultured in DMEM/F12 medium at 37 °C in an atmosphere containing 5 % carbon dioxide. The samples were incubated for 48 h before the addition of glutathione-conjugated SPION micelles to the culture medium to produce a final iron concentration

of 278 μM . The explants were incubated with micelles for 72 h before being fixed in 10 % neutral buffered formalin for 20 min and then washed with PBS. The samples were blocked with 1 % bovine serum albumin in PBS and permeabilized with 0.25 % Triton X-100 before the addition of monoclonal mouse anti-Prestin primary antibodies (Santa Cruz). Finally, the hair cells were visualized using goat anti-mouse IgG secondary antibody, conjugated to Alexa Fluor® 488 (ThermoFisher). The hair cells' cilia were stained with Phalloidin 350 conjugate (CrusFluor). The cochlear cells' fluorescence was captured using a Zeiss LSM 880 confocal fluorescence microscope.

MTT Assays

The in vitro cytotoxicity of the thiol-conjugated SPION micelles was investigated using the ATCC's MTT cell proliferation assay kit and HEI-OC1 cells. The assay was performed according to the manufacturer's instructions with the cells being incubated at 33 °C. Three independent experiments were performed. The percentage of relative cell viability was calculated and the data presented as the mean $\pm$ standard error ($n=3$).

Apoptosis Study

The CellEvent™ Caspase reagent was used to study apoptosis. HEI-OC1 cells were added to 96-well fluorescence microplates. The following day, micelles containing up to 96 μM SPIONs were added to the cells in triplicate before the cells were incubated for a further 24 h. The Caspase-3/7 reagent was then added to the wells. Finally, the plate was incubated for another 4 h before the samples' fluorescence was measured on a Tecan Infinite M1000 plate reader ($n=3$).

Glutathione-Conjugated Micelle Protection from Cisplatin Ototoxicity

The protocol used is illustrated in Fig. 1. Cisplatin was added to growth medium to produce a final cisplatin concentration of either 2.5 or 5 μM . Then, where appropriate, unconjugated or thiol-conjugated SPION micelles were added to the cisplatin containing growth media, with each micelle/cisplatin sample containing 50 μM iron. The micelle/cisplatin samples were then incubated for 4 h before being placed adjacent to an electromagnet for 1 h to remove the majority of the micelles. The cisplatin growth media was then filtered using a 50-K centrifuge filter (Amicon) to remove the remaining micelles. The cisplatin-containing medium was then added to HEI-OC1 cells and the cell samples grown for up to 3 days.

Immediately prior to cell counting, the plates were washed thoroughly with sterile PBS and then trypsinized for 5 min in 33 °C and 10 % CO_2 using 0.05 % Trypsin-EDTA (Life Technologies). The cells were counted using a hemocytometer. The experiment was done in triplicate with a total of three observations per group.

Statistical Analysis

The histograms show the data's mean values $\pm$ standard error. A single-factor ANOVA test was used to determine whether the differences between data groups were statistically significant. The threshold of statistical significance was set at $p>0.05$. The statistical analysis was performed using the Microsoft Excel Analysis ToolPak.

RESULTS

SPION and Micelle Characterization

The newly created SPIONs were imaged using a JEOL 1010 transmission electron microscope. Analysis of the resulting image indicated that the SPIONs' size was consistent at 6.82 ± 0.08 nm. The SPIONs were then successfully encapsulated within glutathione-functionalized diblock copolymers. Dynamic light scattering (DLS) indicated that the average diameter of the resulting micelle was 97.82 ± 0.34 nm. The stability of the micelles was then investigated. The DLS profile of a micelle sample was measured before the sample was reserved for 14 days when a second DLS profile was obtained (Fig. 2). The experimental data indicated that no significant changes in the profile were observed during the course of the experiment. In particular, there was no evidence of aggregation that might have occurred due to the formation of inter-micelle disulfide bonds. A 20-day-old micelle sample was then viewed under an electron microscope (Fig. 2). The resulting images demonstrated that the micelles carried a large SPION payload and that there was no evidence of micelle instability or aggregation. Taken together, the dynamic light scattering and electron microscopy data indicate that the micelles are stable in vitro for at least 2 weeks.

In Vitro Safety Studies Using the Novel Nano-construct

MTT cell proliferation assays (Fig. 3) indicated that the presence of glutathione-micelles resulted in an insignificant decrease in cell viability. In addition, the presence of the micelles did not affect the cellular levels of caspase activity (Fig. 4). This demonstrates

Fig. 1. The protocol used to measure the effect of SPION sequestration of cisplatin on cell growth. Cisplatin was added to the DMEM media (A), then mixed with SPION micelles with or without conjugated thiols for up to 4 h (B). An electromagnet was applied to the samples for 1 h to remove the majority of the micelles (C). The remaining micelles were then

removed using a 50-k centrifuge filter (D). The cisplatin laden media were then added to HEI-OC1 cells and incubated at 33 °C and 10 % CO₂ (E). Finally, cell samples were trypsinized daily over a 3-day period before being counted using a hemocytometer (F).

that the micelles do not induce apoptosis. Finally, the cochlear organotypic culture study clearly showed that the thiol micelles did not alter the cochlear hair cell morphology nor cause any observable damage, even after 72 h incubation (Fig. 5). Taken together, the three techniques demonstrate that the micelles are safe to be used in in vivo inner ear studies.

Cisplatin Sequestration by Glutathione

The binding of cisplatin to glutathione was followed indirectly using Ellman's free thiol assays (Ellman 1959) (Fig. 6). The assays indicated that a large reduction in free thiol concentration occurred in samples that contained both glutathione and cis-

Fig. 2. Characterization and stability of the thiol micelles. **a** Dynamic light scattering (DLS) spectra obtained from thiol functionalized SPION micelles on day one (blue) and day 14 (red) shows an unchanged size distribution after 2 weeks. **b** Transmission electron microscopy (TEM) image of an SPION micelle after 20 days in vitro. The two techniques confirm that the SPION micelles are stable for at least 2 weeks.

Fig. 3. The effect of SPION/thiol micelles on cell viability. Micelles were added to HEI OC1 cells at various concentrations. The micelles were incubated with the cells for 4 (blue) or 24 (red) h before the addition of MTT. The cells catalyzed the reduction of MTT to formazan, whose concentration was determined by measuring the absorption at 570 nm. The data indicate that the micelles possess no significant toxicity even at relatively high micelle concentrations ($n = 3$).

platin. This suggests that cisplatin was binding to glutathione via its free thiol. It was, however, possible that the free thiol concentration could have decreased due to glutathione oxidation. It is therefore significant that Ellman's assays indicate that the concentration of free thiol in the samples containing only 1 mM glutathione barely changed over the course of the experiment. This demonstrates that minimal glutathione oxidation took place in the control sample and confirms that glutathione was binding to cisplatin in the test assays ($F(3, 8) = 21,725.94$, $P = 5.58E-16$).

Cisplatin Sequestration by the Novel Nano-construct

ICP-OES. The ICP-OES data (Fig. 7) indicate that the platinum concentration of the test sample, where the thiol micelles had been incubated with cisplatin, was approximately fivefold higher than the platinum levels found in the control samples.

Fig. 4. HEI-OC1 caspase activity in the presence of thiol-conjugated micelles. Differing amounts of the thiol-conjugated micelles were added to the caspase assay. The data indicate that the addition of micelles to HEI-OC1 cells did not result in increased caspase activity. This strongly suggests that the micelles do not induce apoptosis ($n = 3$).

The binding studies thus clearly show that the thiol micelles sequester cisplatin (Fig. 7). The platinum concentration in the control samples was routinely too low for the ICP-OES apparatus to accurately measure the platinum concentration ($F(3, 14) = 109.92$, $P = 5.73E-10$).

In Vitro Cisplatin Detoxification. In order to observe maximal detoxification effects, a concentration of cisplatin above the IC 50 was used. The experimental aim was to conjugate the cisplatin to the micelles. This would significantly lower the free cisplatin concentration, hopefully below the IC50 leading to a large increase in cell viability. HEI-OC1 cells were exposed to growth medium containing 5 μM cisplatin which, where appropriate, had been incubated with either unconjugated or thiol-conjugated micelles (Fig. 8). The 48- and 72-h data points indicated that only the thiol-conjugated micelles significantly reduced cisplatin cytotoxicity. The hemocytometer cell counts show that at both time points the number of cells in the wells exposed to thiol-conjugated micelles was almost double the number found in either the control group ($F(2, 6) = 10.38$, $P = 0.011$). It is noteworthy that the data illustrated in Fig. 9 demonstrate that the number of cells in the wells where the cisplatin laden growth medium was added directly to the cells and in the wells where the medium was exposed to the magnet and then filtered were essentially the same. This shows that the cisplatin concentration was not affected by the magnetic or the filtration steps ($F(3, 8) = 23.30$, $P = 0.0002$).

DISCUSSION

The physical characterization of the SPIONs and the micelles demonstrated that the desired products had been synthesized. It was then essential to prove that the micelles were not cytotoxic. The MTT assays found that the addition of the thiol micelles to HEI-OC1 cells resulted in an insignificant fall in cell viability. However, it is noteworthy that a dose dependency was not observed at either the 4- or the 24-h time points. Furthermore, the presence of the micelles did not induce an increase in caspase activity, strongly suggesting that the addition of thiol-conjugated micelles does not promote caspase-induced apoptosis. Finally, the addition of the micelles had no effect on the inner ear hair cells for up to 72 h in organotypical assays. Taken together, the three toxicity evaluation assays strongly suggest that the thiol/SPION micelles will possess no cytotoxicity in inner ear applications.

Fig. 5. The effect of micelles on Cochlear Organotypic Cultures. The cochlear samples were cultured in defined medium for 48 h before the addition of thiol/SPION micelles to produce a final iron concentration in the medium of 278 μ M. The cochlear explants were incubated with the micelles for 72 h before the explants were fixed, blocked, and washed. The hair cells were then visualized using mouse anti-Prestin and a goat

anti-mouse secondary antibody conjugated to Alexa Fluor® 488. The hair cells' cilia were stained with Phalloidin 350 conjugate (CrusFluor). The resulting images of the apex (a), middle turn (b), and basal turn (c) of the test sample that had been exposed to the micelles clearly show no alter of the cochlear hair cell morphology nor cause any observable damage.

The Ellman's assay was successfully used to demonstrate that the addition of cisplatin to glutathione resulted in a

large decrease in the free thiol concentration. This was almost certainly due to the glutathione binding to cisplatin.

Fig. 6. The time course of glutathione binding to cisplatin. Reaction mixtures containing 1 mM glutathione and 1 mM cisplatin in PBS were prepared in triplicate (red dotted line) together with control solutions, also prepared in triplicate, containing 1 mM glutathione only (red solid line) or 1 mM cisplatin in PBS (blue) and PBS only (green). The samples were stored in the dark at 37 °C until required. The samples' free thiol concentration was measured by transferring aliquots, at intervals, into fresh cuvettes Ellman's assay

reagents. The assays indicate that a large decrease in free thiol concentration occurred in the samples containing both glutathione and cisplatin. The standard error bars are so small that they are covered by the data markers. There is a significant difference after 8 h in the free thiol content of the samples containing glutathione only and the glutathione/cisplatin mixture ($F(3, 8) = 21,725.94$, $P = 5.58E-16$).

Fig. 7. Demonstration that the thiol micelles sequester cisplatin in PBS. Thiol/SPION micelles containing 18 $\mu\text{g/ml}$ iron were incubated with 3 $\mu\text{g/ml}$ cisplatin in PBS for 48 h. Once the free cisplatin was removed using PD-10 columns, the platinum content of the column eluents from the test sample and three control samples was

determined using ICP-OES. The experimental data demonstrated that the test samples' platinum content were much higher than in the control samples, proving that the thiol-conjugated micelles were capable of sequestering cisplatin ($F(3, 14) = 109.92$, $P = 5.73\text{E-}10$).

The ICP-OES binding data then demonstrated that the cisplatin bound strongly to the thiol-conjugated micelles. The cisplatin/micelle interactions had to be strong otherwise the cisplatin would have dissociated from the micelles during size exclusion chromatography step and then be retained by the PD-10 columns. In many of the ICP-OES experiments, the levels of platinum in the control samples were so low that the ICP-OES was unable to produce accurate platinum

measurements. Consequently, a mean platinum concentration with standard error values could not be determined with the control samples.

It is noteworthy that only the micelles labeled with glutathione were capable of protecting cells from cisplatin challenge in the cell culture experiments. The unlabeled micelles exhibited no protection against cisplatin-induced cytotoxicity. This strongly

Fig. 8. Protection by thiol-conjugated micelles. Unconjugated or thiol-conjugated SPION micelles were incubated with growth medium containing 5 μM (final concentration) cisplatin for 4 h. The micelles were then removed and HEI-OC1 cells were grown in the cisplatin-containing media for up to 3 days. Cell samples: Blue = cisplatin only, Red = cisplatin plus unconjugated micelles, Green = cisplatin plus thiol-conjugated micelles ($n = 3$). The data indicate that the thiol-conjugated micelles partially protected the growing cells from cisplatin-induced cytotoxicity ($F(2, 6) = 10.38$, $P = 0.011$).

Fig. 9. Demonstration that the micelles are responsible for the reduction in cisplatin cytotoxicity. Cisplatin was added to the growth medium and then, where appropriate, thiol-conjugated SPION micelles were incubated with the cisplatin containing media for 4 h. The micelles were then removed and HEI-OC1 cells exposed to the cisplatin containing media for up to 3 days. Cell samples: Green = 2.5 μM cisplatin, Purple = 5 μM cisplatin, Blue = 5 μM cisplatin that had been through the micelle extraction steps, Red = 5 μM cisplatin plus thiol-conjugated micelles ($n = 3$) ($F(3, 8) = 23.30$, $P = 0.0002$).

suggests that the micelles' thiol groups were sequestering the cisplatin rather than cisplatin binding non-specifically to the micelles. Finally, an essential control experiment demonstrated that the degree of cisplatin-induced cytotoxicity was unaffected by the passage of the cisplatin infused growth medium through a centrifuge filter. This confirmed that the cellular protection observed when thiol-conjugated micelles were used in the pre-incubation experiments was due to the micelles sequestering the cisplatin and not by cisplatin being absorbed by the filter membrane.

We have now demonstrated that thiol-conjugated micelles of the desired composition have been created. We have further shown that the conjugated micelles are capable of sequestering cisplatin and can protect cells from the cytotoxic effects of cisplatin. Consequently, we have reached a point where we can begin to measure the thiol micelles' ability to protect the inner ear from cisplatin ototoxicity. This will necessarily involve the delivery of the nanoparticle to the inner ear without damaging any inner ear structures and causing further hearing loss. It is also probable that the nanoparticles can be utilized to combat other forms of cisplatin-induced toxicity experienced by patients undergoing a course of cisplatin-based chemotherapy.

The development of an effective treatment of cisplatin or other ototoxic drug-induced hearing loss is an important task for both hearing scientists and clinicians. A significant amount of effort was expended investigating the ability of Amifostine to protect the inner ear from the ototoxic effects of cisplatin. Unfortunately, the compound was found to be unable to significantly protect the inner ear from the anticancer agent (Katzenstein et al. 2009). However, a phase three trial where sodium thiosulfate was intravenously administered to pediatric oncology patients 6 h after every cisplatin injection produced promising results (Freyer et al. 2017). The administration of sodium thiosulfate resulted in the lowering of the percentage of patients developing hearing loss from 56.4 % in the control group to 28.6 % in the test group. Regrettably, the study identified some potentially significant problems. The most important is that more deaths were reported in the test group patients with non-localized tumors than in the corresponding control group. This suggests that sodium thiosulfate may possess a limited ability to protect the tumor from cisplatin, even when the sodium thiosulfate was administered 6 h after cisplatin treatment (Freyer et al. 2017). Finally, sodium thiosulfate administration resulted in almost a doubling in the incidence of nephrotoxicity, with 25 % of the patients in the test group displaying symptoms (Freyer et al. 2017).

The micelles developed during the course of this study should possess many advantages over the

thiosulfate used in the above study. The most significant advantage being that they are designed to be administered topically to the round window niche. This will result in a localized high concentration of the micelles only in the cochlea, ensuring that the micelles will not interfere with cisplatin's antitumor activity or pose a potential threat to other organs. Sodium thiosulfate has to be administered 4–8 h after cisplatin administration in order to minimize its impairment of cisplatin's antitumor activity. It is anticipated that as tumor protection should not be an issue for the micelles, they could be given to the patient at any point in the cisplatin regimen. They could, if necessary, be administered prior to the injection of cisplatin. Finally, the micelles are designed to be extracted from the inner ear once they have sequestered the cisplatin that has defused into the inner ear. This should prevent the development of any potential long-term adverse interactions between the micelles and the cochlear tissue.

The current study has demonstrated, using in vitro and ex vivo techniques, that the nano-constructs do not induce cytotoxicity. However, the nano-constructs are able to bind strongly to cisplatin and protect cells from cisplatin-induced ototoxicity. This suggests that the constructs will be able to protect the inner ear cells from cisplatin-induced ototoxicity by sequestering the drug before it enters the cell. This will fulfill an important clinical need because there is no current treatment for cisplatin-induced ototoxicity. The use of the nano-constructs should increase the utilization of cisplatin-based chemotherapy which is an effective treatment for many cancers and will solve a dilemma encountered by physicians and patients.

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COMPLIANCE WITH ETHICAL STANDARDS

Animal care and use was in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of The University of Pennsylvania and of the National Institute of Health.

Conflict of Interest The authors declare that they have no conflicts of interest.

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