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# Pharmacokinetics and biodistribution of supraparticle-delivered neurotrophin 3 in the guinea pig cochlea

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## Abstract

Hearing loss is the most prevalent sensory disorder affecting nearly half a billion people worldwide. Aside from devices to assist hearing, such as hearing aids and cochlear implants, a drug treatment for hearing loss has yet to be developed. The neurotrophin family of growth factors has long been established as a potential therapy, however delivery of these factors into the inner ear at therapeutic levels over a sustained period of time has remained a challenge restricting clinical translation. We previously demonstrated that direct delivery of exogenous neurotrophin-3 (NT3) in the guinea pig cochleae via a bolus injection was rapidly cleared from the inner ear, with almost complete elimination 3 days post-treatment. Here, we explored the potential of suprapaticles (SPs) for NT3 delivery to the inner ear to achieve sustained delivery over time. SPs are porous spheroid structures comprised of smaller colloidal silica nanoparticles that provide a platform for long-term controlled release of therapeutics. This study aimed to assess the pharmacokinetics and biodistribution of SP-delivered NT3. We used a radioactive tracer (iodine 125: $^{125}$I) to label the NT3 to determine the loading, retention and distribution of NT3 delivered via SPs. Gamma measurements taken from $^{125}$INT3 loaded SPs revealed high drug loading (an average of 5.3 µg of NT3 loaded per SP weighing +/-50 µg) and elution capacities *in vitro* (67% cumulative release over one month). Whole cochlear gamma measurements from SP-implanted cochleae harvested at various time points revealed detection of $^{125}$INT3 in the guinea pig cochlea after one month, with 3.6 and 10% of the loaded drug remaining in the intracochlear and round window-implanted cochleae respectively. Autoradiography analysis of cochlear micro-sections revealed widespread $^{125}$INT3 distribution after intracochlear SP delivery, but more restricted distribution with the round window delivery approach. Collectively, drug delivery into the inner ear using SPs support sustained, long-term availability and release of neurotrophins in the inner ear.

**Keywords-** Hearing loss; SPs; Drug delivery; Neurotrophin-3; Pharmacokinetics

## 1. Introduction

Sensorineural hearing loss occurs due to damage or loss of cochlear hair cells, neurons and the synaptic connections between them in the inner ear. Currently there is no therapeutic treatment to repair or prevent the progression of sensorineural hearing loss. Whilst devices such as hearing aids and cochlear implants can aid hearing, they do not address the underlying cause of hearing loss or halt its progression. With the growing number of people affected by hearing loss, there is a significant need for a drug therapy for hearing loss that can repair the damage sensory cells and return lost function. Pre-clinical studies have identified potential drug candidates capable of restoring hearing, but one of the complications impeding their translation is the lack of a safe, efficacious drug delivery method. This is particularly pertinent for macromolecules whereby the effective drug half-life is short (minutes-hours *in vivo* - [1]) meaning that the therapeutic window for sensory cell repair is relatively narrow. As such, obtaining sufficient drug levels in the cochlea via systemic drug delivery of macromolecules is not a feasible option thus requiring local drug application to the cochlea. However, accessing the inner ear is challenging due to its location and complex architecture. The bony cochlea (hearing organ) is separated into three chambers filled with ionic fluids; perilymph and endolymph, that bathe the sensory and supporting cells. The semi-permeable membranes, the round and oval windows, are the main entry points into the cochlea from the middle ear. Effective therapy requires entry to these fluid-filled cavities to deliver bioactive drug to achieve therapeutically relevant concentrations.

Over a decade of research has established that administration of recombinant neurotrophins via mini pumps prevents the degeneration of auditory neurons (ANs) and elicits peripheral nerve fibre regrowth after aminoglycoside or noise-induced destruction of the organ of Corti in animal models [2-11]. Reduced excitation thresholds with the cochlear implant due to improved nerve-electrode interface are also observed upon neurotrophin administration [7, 12]. Chronic delivery of 30-50 μg/ml of NT3 using mini pumps have led to improved neural regeneration in guinea pigs [7, 9, 12]. Although mini pumps are a convenient experimental tool, concerns over drug formulation stability and safety have prevented their clinical uptake [3, 7, 9-11]. Emerging evidence has also revealed that acute local delivery (30 or 300 μg/ml) of neurotrophins promotes hair cell ribbon synapse protection and/or regeneration after acoustic trauma[13]. However, the extent of regeneration and functional improvement in animal deafness models was variable upon round window delivery of recombinant or viral-mediated overexpression of neurotrophins, with only half of the animals showing improvements [13, 14]. Insufficient protein transfer through the round window membrane and the short half-life of neurotrophins in cochlear fluids were attributed as potential factors contributing to these outcomes. There is a significant need to improve the neurotrophin delivery strategy to obtain improvements in hearing function outcomes.

The main approaches for localised drug delivery to the inner ear include diffusion across semi-permeable membranes (round or oval window), intracochlear infusion via an incision through the round window membrane or cochleostomy. Drug delivery across semi-permeable membranes has low risk to residual hearing but the efficacy is limited due to reduced drug concentrations entering the cochlea. Direct drug delivery approaches require invasive surgery and carries the risk of affecting residual hearing, therefore this approach could be considered if the cochlea is already being accessed such as during cochlear implant surgery. We previously reported that an acute bolus delivery of radiolabelled neurotrophin 3 ($^{125}$INT3) to guinea pig cochleae via direct intracochlear infusion resulted in an exponential decrease in NT3 in the cochlea over time, with almost complete elimination of NT3 in 3 days, as

evidenced from whole-cochlea gamma measurements of both cochlear fluids and tissue [15]. Autoradiography analysis of cochlear tissue corroborated the rapid clearance of NT3 from the cochlea and uncovered a basal to apical gradient of $^{125}$INT3 distribution across the cochlea, with greatest density in cells lining the scala tympani and lower density in the neural tissue.

Several strategies have been trialled to promote sustained neurotrophin delivery in the cochlea using cell-based therapies [16, 17], gene therapy [14, 18, 19], bisphosphonate agonists [20], electrode coating strategies or nanoparticle-based methods [21]. We have developed a highly biocompatible inner ear drug delivery system using mesoporous silica SPs. SPs are spheroid structures formed of smaller colloidal nanoparticles that provide a platform for long-term controlled drug release. They are advantageous as their physical properties such as size, porosity and composition can readily be tailored to modify performance (including drug loading kinetics, loading capacity, loading efficiency, and drug release) based on therapeutic requirement [22, 23]. We previously reported that SPs had the potential to load up to 7.2 μg/SP weighing +/-50 μg (when loaded with 10 μg /SP) and release 4.7 μg of brain derived neurotrophic factor (BDNF) over a period of 40 days [1, 22, 23]. Furthermore, intracochlear delivery of BDNF into guinea pigs deafened via exposure to aminoglycosides significantly promoted AN survival one-month post-treatment thus revealing its biological activity and therapeutic potential. For this delivery approach to be clinically translated, a thorough characterisation of the SPs, bioactivity of the released neurotrophin and analysis of the pharmacokinetics of the eluted drug is essential. Therefore, this study aims to analyse the pharmacokinetics of SP-mediated NT3 delivery in the guinea pig cochlea, including retention, distribution, and clearance, for SP delivery using the intracochlear and round window approaches.

## 2. Materials and Methods

### *2.1 Neurotrophin labelling*

Human NT3 (PeproTech; 3–4.5μg) was radiolabelled with 0.25 mCi Na 125I (ICN Biomedicals Australasia) using the Chloramine-T method (ProSearch International) as previously described in [15]. Two different batches were used in the study, supplied in phosphate buffer containing 0.25% (w/v) BSA and 0.1% (w/v) sodium azide. Specific activity was 46 and 46.7 μCi/μg, with 82 and 84% incorporation. The supplied $^{125}$INT3 was purified and buffer exchanged to artificial perilymph using Amicon Ultra-2 3K filtration unit [15]. The final $^{125}$INT3 sample concentration was calculated using gamma counts and specific radiolabelled activity to be 2.7 and 3.1 μg/ml. Gamma counts were obtained using the Perkin Elmer WIZARD automatic gamma counter and measured over 1 min.

### *2.2 SP production*

The SPs were produced as described previously [22]. Briefly, primary silica particles with large pores (diameter, +/-1 μm) were distributed in an alginate aqueous solution (30 mg/ml in Milli-Q water). The suspension was ultra-sonicated (Qsonica, CT, USA) at an output amplitude of 30% for 40 s and then sonicated for 1 hour in an ultrasonic cleaner (Branson, USA) to ensure even distribution of the primary silica particles within the alginate solution. The suspension was then transferred into a syringe pump programmed to deliver a constant flow rate of 8 ml/h. An electrospray setup was used to drip the suspension at a 13-kV applied voltage into a gelling bath containing 1% $CaCl_2$ dissolved in Milli-Q water. The calcium chloride crosslinked with the alginate to form alginate beads. After 5 min, the alginate/silica SPs were collected from the bath using a strainer and washed in Milli-Q water 4-5 times. The alginate was then removed by calcination with the alginate beads placed into a furnace at 923 K for 30 h to produce the silica SPs. The SPs used in this study had a diameter of +/-500 μm.

### *2.3 SP sterilization*

SPs were sterilised during the furnacing stage (@ 923K for 30 hours) and transferred into sterile eppendorf tubes. Immediately prior to loading, the SPs were immersed in ethanol for 4 h, with subsequent rinsing with Milli-Q water six times. This wetting process is also necessary to improve particle loading.

To maintain sterility, the SPs were loaded in a laminar flow hood under PC2 conditions. All consumables were sterilized or autoclaved prior to use and surgical implantation was carried out using aseptic surgical techniques.

### *2.4 Neurotrophin loading*

Each SP was loaded in a solution containing 7.5 µg of NT3 per particle and loaded over 3 days at room temperature. On average, the SPs weighed +/- 50 µg [22]. Neurotrophin stocks were prepared by diluting 100 µg of NT3 with 50 µl of Milli Q water to obtain a stock concentration of 2mg/ml. Radiolabelled NT3 was added to unlabelled NT3 at a 1:1 volume ratio.

For the round window surgeries, SPs were loaded in batches of 5 SPs in a solution containing 18.75 µl of NT3 (stock 2mg/ml) and $^{125}$INT3 (stock 2.7 or 3.1 µg/ml) in a total volume of 37.5 µl. For the intracochlear surgeries, SPs were loaded in batches of 2 SPs in a solution containing 7.5 µl of NT3 (stock 2mg/ml) and 2.7 or 3.1 µg/ml of $^{125}$INT3 in a total volume of 15 µl.

Immediately prior to implantation, the SPs were washed three times with Milli-Q water and gamma measurements taken with the SPs in Milli-Q water to determine neurotrophin loading per particle.

### *2.5 Analysis of SP-released neurotrophin elution profile in vitro*

To assess the *in vitro* elution profile of the particles, the loaded particles were individually separated and transferred to 100 µl of phosphate buffered solution (PBS). The elution profile of the particles was analysed for 1 month. At specific timepoints (day 3, 7, 14, 21 and 28 days), the PBS solution and SPs were gamma counted to calculate the cumulative percentage and amount of NT3 eluted per SP. The PBS solution was replenished after each measurement.

### *2.6 In vitro tests to assess protein integrity and bioactivity of SP-released neurotrophin*

#### 2.6.1 Western blot

The samples were separated by SDS-PAGE (Bio-Rad Laboratories) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) for 1 hour and then incubated with NT3 primary antibody (Thermofisher, #PA5-14861) solution (1% BSA in TBST and 1:3000 primary antibody) overnight. After incubation with the primary antibody, membranes were washed with TBST and exposed to horseradish peroxidase-conjugated secondary antibody (BioRad) for 1 hour and subsequently washed with TBST. Immunoblot signals were detected with Pierce ECL Western blotting substrate or ECL plus (Thermo Fisher Scientific, Waltham, MA). Amersham Imager 600 (GE Healthcare Life Sciences) was used to image the Western Blot.

#### 2.6.2 TrkC cell line assay

The CHO K1 cell line with a CellSensor construct (TrkC-NFAT-bla) was obtained from Thermofisher and cultured in growth medium (DMEM with GlutaMAX) supplemented with 10% dialyzed FBS (Invitrogen), 1× MEM NEAA (minimum essential medium nonessential amino acid) solution (Sigma), 25 mM Hepes (Sigma), blasticidin (5 μg/ml) (Thermofisher), and Zeocin (200 μg/ml) (Thermofisher). The assay was performed according to the protocol provided by Thermofisher. Briefly, cells were seeded in 96-well plate at $1.0 \times 10^4$ cells per well in a medium without blasticidin and Zeocin overnight. The next day, the cells were treated with Neurotrophin-3 (freshly prepared control NT3 or SP-eluted NT3 adjusted to concentrations of 100 ng/mL) for 5 hours. Subsequently, the wells were loaded with β-lactamase LiveBLAzer-FRET B/G substrate (CCF4-AM, K1095, Invitrogen) for 2 hours. Fluorescence at 450 and 510 nm was recorded on a plate reader (Perkin Elmer; excitation wavelength, 410 nm). Control wells containing no cells or unstimulated cells (No NT3) were included in all experiments.

#### 2.6.3 Auditory nerve cultures

Time-mated pregnant rat mums were obtained (Albino Wistar). Rat pups were used at postnatal day 5. The use and care of the experimental animals in this study were approved by the St Vincent's Hospital (Melbourne) Animal Ethics Committee (#18-393AB). Cochleae were dissected in Hank's balanced salt solution and the modiolus containing ANs dissected. The cells were treated with TrypLE for 20 min at 37 °C. Single cells were obtained by mechanical trituration and filtration (40 μm filter) and resuspended in Neurobasal media (N2, B27, L-Glut, Pen/Strep- all from Thermofisher) containing fresh or SP-eluted NT-3 adjusted to concentrations of 50 ng/mL. The ANs were plated on polyornithine/laminin coated 8-well culture slides and cultured for 3 days *in vitro*. Two wells were fixed 4 h post-plating as a control. The cultures were fixed in 4% paraformaldehyde for 10 min at room temperature and then washed 3 times in PBS. To immunostain the cultures, the cells were blocked in 0.1% Triton X-100 and 10% heat inactivated donkey serum in PBS for 1 hour. Primary antibodies Tuj1(1:500, Biolegend, #801201) and S100 (1:100, Abcam, #ab52642) were diluted in blocking solution and applied overnight at 4 °C. The relevant secondary antibodies were diluted 1:500 in PBS. Nuclei were visualised using DAPI. All staining was visualised and imaged using the Zeiss Axiocam Microscope. The cultures were analysed by counting the total number of neurons in each well. Neurite lengths were analysed using the ImageJ neurite tracer software.

### *2.7 Experimental Animals*

We used young adult pigmented guinea pigs of either sex (mean 550 g) in this study (Table 1). All procedures were approved by the St. Vincent's Hospital Animal Research & Ethics Committee (18-393AB) in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and conformed to the Code of Practice of the National Health and Medical Research Council of Australia.

**Table 1:** Guinea pig numbers for SP implantation via the round window or intracochlear approaches

| **Delivery route** | **Timepoint** | **Number of guinea pigs (n=)** |
|---|---|---|
| **Intracochlear** | 3D | 4 |
| | 7D | 6 |
| | 14D | 4 |

| | | |
|---|---|---|
| | 28D | 4 |
| **Round window** | 7D | 3 |
| | 14D | 4 |
| | 28D | 4 |

### 2.7.1 Hearing function tests

The hearing status of animals was tested prior to surgery and at the specific time points post-implantation (Figure 1). The guinea pigs received a subcutaneous injection of Robinul (0.02 mg/mg) 15 minutes prior to being anaesthetized with isoflurane (3% per 1L for induction and 0.5-2% per 1L for maintenance. Click and tone -evoked auditory brainstem responses (ABRs) were recorded to evaluate the hearing status of the guinea pigs prior to, and at the completion of the treatment period. The anaesthetized guinea pigs were placed on a heat pad with the temperature maintained at 37°C in a sound attenuated room. Needle electrodes were positioned at the skull vertex, at the nape of the neck and on the abdomen. ABRs were recorded by delivering acoustic clicks and tones (1-24 KHz) delivered using a calibrated speaker at specific intensities up to 100 dB peak equivalent sound pressure level (SPL). The ABRs were amplified and averaged over 200 trials at the stimulus intensities ranging from 0 dB to 100 dB SPL. Hearing threshold was analysed and only guinea pigs with normal hearing thresholds in both ears were used in the study.

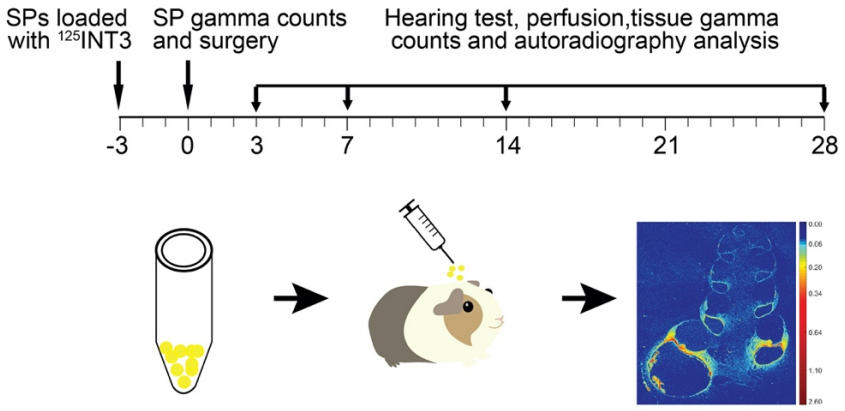



**Figure 1: Timeline used for the experiments.** The SPs were loaded with a 1:1 ratio of $^{125}$INT3 and unlabelled NT3. The SP gamma counts were obtained after NT3 loading. The animals underwent round window or intracochlear surgeries. After 3, 7, 14- and 28-days post-implantation, hearing function and whole-cochlear gamma counts were obtained. Cochlear sections were analysed using film and emulsion-based autoradiography techniques.

### 2.7.2 Implantation of SPs to guinea pig cochleae

The guinea pigs were bilaterally implanted with SPs via an intracochlear or round window approach. The surgeries were performed using aseptic surgical techniques. Guinea pigs were anaesthetized with isoflurane. A post-auricular approach was used to expose the bulla. For the round window surgeries, a hole was made in the bulla to expose the round window. SPs (n=4-5 SPs) were placed on the round window using a 21-gauge polyurethane catheter

(Optiva, Medex Medical, UK) and then a small silica plug was positioned above the round window to secure the SPs in place. For the intracochlear surgeries, a small cochleostomy was drilled to expose the basal turn of the cochlea. The cochleostomy was made using a 0.8 mm diamond drill and gentle suction was applied to remove bone debris from the cochlea. One SP was placed into the scala tympani of the basal turn of the cochlea using a 21-gauge polyurethane catheter. The cochleostomy was sealed using a small silica plug and a water-proof sealant (ACC Silicones). Dental cement was used to seal the bulla. The surrounding muscle layers were sutured, and the skin incision closed with staples. Hartmann's solution (10ml/kg; s.c.), the antibiotic Baytril (Bayer, Germany) (0.10mg/kg; s.c.), and the analgesic Temgesic (Reckitt-Benckiser, UK) (50 mg/kg; s.c.) were given after surgery and on the next day to aid recovery.

2.7.3 Tissue extraction and histology

At the completion of the treatment period, the animals were anaesthetised with isoflurane and ABRs were measured as described above. Following ABR recording the animals received an overdose of pentobarbital (150mg/kg; intraperitoneal) and intracardially perfused with 0.9% NaCl (37°C) followed by 10% Neutral Buffered Formalin (10% NBF; 4°C). The bulla was removed from the temporal bones and the cochlea dissected. The cochlea was placed in a radioimmunoassay tube containing formalin for gamma counting. Other tissues including brain, kidney, heart, intestine, liver, ear drum and semi-circular canal were also dissected and transferred into radioimmunoassay tubes for gamma counting.

The cochleae were then post-fixed in 10% NBF for one hour on a shaker and then transferred to 10% ethylenediamine tetraacetic acid (EDTA) in PBS at room temperature for decalcification. After 1-2 weeks of decalcification, the cochleae were cryo-protected in 15% and 30% sucrose solution, before being oriented and frozen in Tissue-Tek O.C.T. cryosectioning compound (Sakura, Japan). Cochleae were then sectioned at 12 µm onto Superfrost slides using a CM 1900 UV cryostat (Leica, Germany) at -22°C in the modiolar plane and mounted onto Superfrost-Plus slides (Menzel-Gläser, Braunschweig, Germany). Slides were stored at -20ºC until ready for use.

2.7.4 Autoradiography and imaging

To assess the drug distribution in the cochlea, we used two autoradiography analysis methods- film and emulsion techniques.

*Film*

The application of a film-based analysis provides a quantitative assessment of the distribution and relative concentration of $^{125}$INT3 in the cochlea. Standards were prepared to obtain a NT3 concentration gradient. They were prepared using Whatman filter paper disks (5mm diameter), numbered from 1-10. A 1/2 serial dilution of $^{125}$INT3 was prepared and each disk inoculated with 5 ul of the sample- with disk 1 being the most concentrated. The disks were aligned on sticky tape and then positioned on a cardboard containing the slides for exposure to film.

The slides were defrosted and dried prior to being exposed to Biomax MR film (Kodak). The films were developed after 6 weeks in a medical film processor (SRX-101A; Konica Minolta Medical and Graphic, Inc). The autoradiographs were subsequently scanned using an Epson v800 photo scanner (grayscale, 16-bit, 2400 dpi with no compression).

The autoradiographs were analysed by calibration of the standards in ImageJ using the Rodbard function [15]. Using the freehand tool, insets were drawn within regions of interest

(organ of Corti, Rosenthal's canal, nerve fibres) and measurements of the area, mean, grey value, standard deviation and integrated density measurements were taken. Background measurements were subtracted from measurements obtained at the regions of interest.

*Emulsion*

Defrosted and dried slides were placed in water to remove the OCT. They were subsequently dehydrated in an increasing gradient of ethanol and then air dried. Some slides were pre-stained with H&E prior to coating with emulsion using standard protocols but with incubation times in both dyes doubled as there are additional washing steps in the developing process [24]. The fronts of the slides were then coated with Kodak NTB emulsion at 42 °C (back-to-back coating technique). The slides were dried and then placed in a light-tight box for 1 hour at room temperature. Anhydrous silica was then added to the boxes before being placed at 4ºC for 6weeks.

After 6 weeks, the slides were brought to room temperature and then developed in dark room conditions. The slides were immersed in Dektol developer (Kodak; diluted 1:1 with water) for 5 min, a water stop bath for 30 s and fixer (Kodak) for 10 min. The slides were then rinsed in running water for 15 minutes and subsequently dehydrated using graded ethanol (90% and 100% v/v), xylene and cover slipped in DPX. Images of the entire cochlear mid-modiolar cross sections and higher magnification images of the organ of Corti and Rosenthal's canal were taken using a Zeiss Axio Imager M2 upright microscope. Identical settings were used when acquiring the images including exposure and light settings. The images were analysed using ImageJ using previously described protocols [15].

*2.8 Data analysis*

The amount of NT3 loaded into the SPs is expressed as a percentage of the concentration of the loading solution. The amount eluted by the SPs is expressed as a percentage of the loaded NT3 into the SPs. Retention of $^{125}$INT3 in the cochlea is expressed as a percentage of the dose injected (e.g. the amount of NT loaded into the SPs). Data is presented as individual points or as mean ± standard deviation or error of mean.

There were 33 sets of SP batches loaded for animal implantation. Of the 33 batches, 4 batches had lower loading percentages <20% and had to be excluded from the data analysis. We postulate that these variabilities may be from the SP manufacturing process (e.g., inconsistency in porosity or particle calcination). Clinical implementation will require a quality-controlled manufacturing process. Current development work is underway to develop this manufacturing system.

Animals that had very low or no whole-cochlear gamma counts (retention percentages of 0-1.28%) post-transplantation were excluded (n=3 IC implanted cochleae; 2x 7D and 1x 28D and n=3 RW implanted cochleae; 2x 7D and 1x 14D). We believe this may be attributed to surgical complications during SP delivery via the cochleostomy or failure to deliver the SPs on the RWM given the narrow space available and thus difficulty in visualising all the SPs post-implantation. Future experiments will focus on improving surgical delivery into the cochlea and onto the round window membrane.

All gamma counts were corrected for radioactive decay (half-life of $^{125}$I is 59.5 days). Statistical comparison of groups was carried out with one-way ANOVA and Tukeys multi comparison test. Curve fitting analysis was performed to compare clearance over time.

Autoradiographic film analysis was performed in ImageJ with MATLAB used for image visualisation. Analysis of emulsion coated slides was performed in ImageJ with background

subtracted and data expressed as normalised relative integrated density [15]. Slides were selected for analysis avoiding sections that contained the SPs.

Statistical analysis of the hearing function tests was done using two-way repeated measures ANOVA (Frequency and Pre/Post as factors) with Holm-Sidak post hoc test.

## 3. Results

### *3.1 SPs possess high drug loading and sustained drug release capacities*

SPs are three-dimensional structures (+/-500 µm in diameter, averaging +/-50 µg in weight) formed by self-assembly of nanoparticles (Figure 2A and B). The loading and elution capacity of the SPs with radiolabelled neurotrophin was initially examined *in vitro*. The SPs were loaded with a 1:1 volume ratio of unlabelled and radiolabelled NT3 at 7.5 µg/SP for three days. The particles were loaded in batches of two (intracochlear; 15 µg in loading solution) or five (round window, 37.5 µg in loading solution). Gamma counts were obtained from the particles and post-loading solution after three days to obtain the amount and percent loaded into the particles. On average 70% of the loading solution was taken up by the particles (Figure 2C), corresponding to an average NT3 loading amount of 5.28 µg/SP (Figure 2D). The loaded particles were subsequently transferred to PBS for the *in vitro* elution analysis and maintained at 37ºC for 28 days. At the specific time points (Figure 2E and F), the SP eluates were collected, gamma counted, and the same volume of PBS replenished. The gamma counts revealed an *in vitro* accumulative release of 67% of the loaded NT3 solution at 28 days, corresponding to a release of 4 µg NT3 per SP. The eluted samples were stored at -20ºC and used to test drug bioactivity.

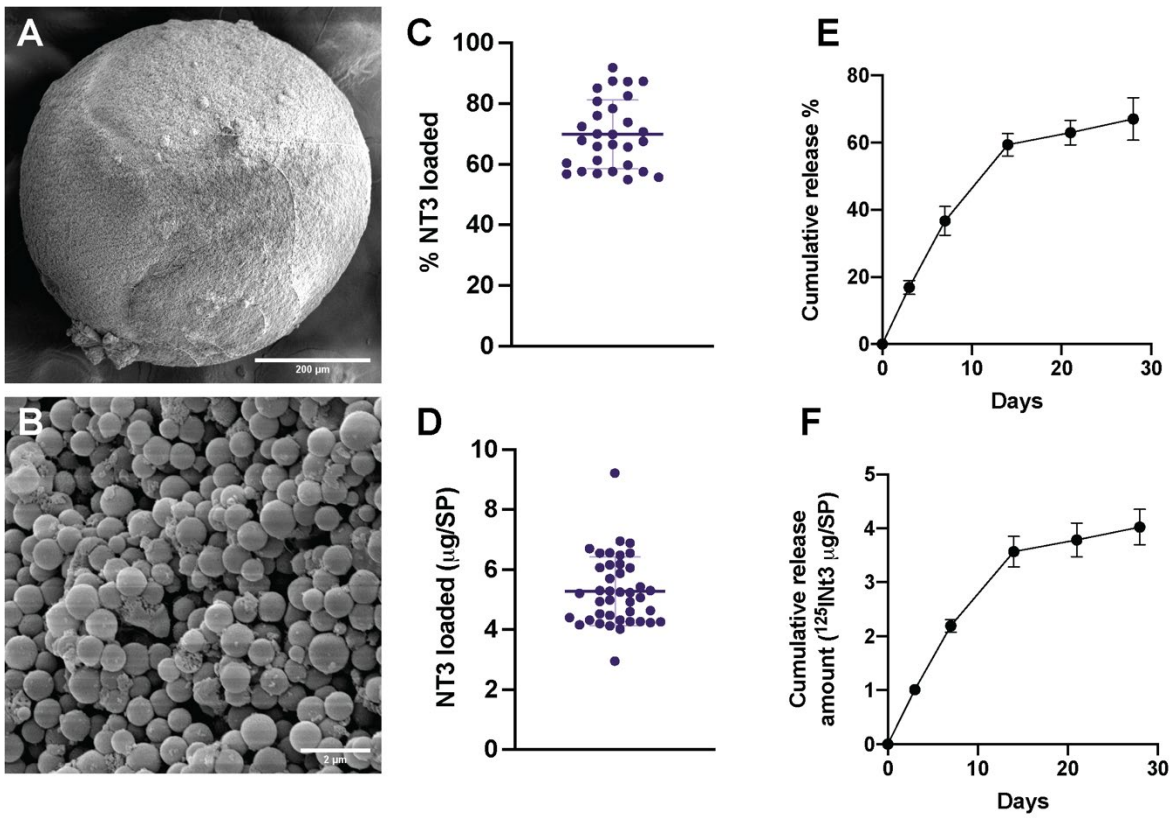


**Figure 2: Characterization of the loading and elution profiles of the SPs** (A) Scanning electron microscope low magnification image of SPs, Scale bar= 200 μm. (B) Higher magnification scanning electron microscopy image of the surface of the SPs, scale bar= 2 μm. (C & D) The SPs were loaded with standard NT3 and $^{125I}$NT3 for 3 days and gamma counted to obtain percentage of the loading solution taken up by the particles and the amount of NT3 loaded. (D) Each particle was placed in a loading solution containing NT3 (labelled and unlabelled). Uptake was calculated as a function of particle counts relative to loading solution and particle wash counts. An average of 5.5 μg/particle was loaded after 3 days. (E & F) The *in vitro* elution profile of the particles over a period of 1 month was analysed. The loaded particles were transferred to phosphate buffered solution and maintained at 37ºC. Over the time period analysed, samples of the solution were gamma counted to calculate cumulative percentage (E) and amount (F) of NT3 eluted per SP. Data presented as mean ± standard deviation.

*3.2 SPs maintain the integrity and bioactivity of NT3*

A series of *in vitro* tests were undertaken to test drug bioactivity. We have previously demonstrated that iodination of NT3 does not affect the integrity and activity of the protein [24]. To determine if the integrity of the $^{125}$I NT3 protein was affected upon loading the protein into SPs, a western blot was performed using a 3- day SP-eluted NT3 sample. In both the fresh NT3 (unpackaged) and SP-eluted NT3, bands were observed at the expected sizes of 15 and 17 kD (Figure 3A). No evidence of any degradation products was visualised on the gel.

To examine the bioactivity of the protein over extended periods of time, we employed a commercially available TrkC CellSensor assay. The cell line contains a beta-lactamase reporter gene expressed upon stimulation of the TrkC receptor with NT3. In the absence of beta lactamase, the cells fluoresce green. Upon activation of beta lactamase, the cells fluoresce blue. Expression of beta lactamase is measured using a fluorescence plate reader and response ratios calculated as a measure of NT3 activity. We initially conducted a dose response experiment to assess the effective concentration of freshly prepared NT3. Calculation of the response ratio revealed an $EC_{100}$ of ~100 ng/ml for the NT3 (Figure 3B). Based on this data, subsequent experiments were performed by diluting both the fresh and SP-eluted NT3 to a concentration of 100 ng/ml. We next tested the bioactivity of the SP-eluted NT3 at multiple time-points up to 28 days. When compared to the bioactivity of the fresh NT3, there was no statistically significant difference between the SP-eluted NT3 at any of the timepoints analysed, thus indicating maintained bioactivity of the NT3 as assessed using the TrkC activation assay (Figure 3C).

To further evaluate the bioactivity of the SP-eluted neurotrophins, we next examined the ability of eluted NT3 to maintain AN survival- the cell population to be targeted with this therapeutic approach. The ANs were isolated from early postnatal rat pups and cultured with freshly prepared or SP-eluted NT3 for 3 days. Control samples were prepared and contain cells fixed 4 hours post-culture (to obtain AN seeding density) and cells cultured without NT3 for 3 days. After 3 days, in the samples that did not receive NT3, only 3.4% of the neurons remained, when compared to the 4-hour control (Figure 3D). In the samples treated with freshly prepared NT3, there was a statistically significant increase in the number of neurons surviving, averaging 35% in culture, relative to the untreated ($p<0.001$***). For the SP-eluted NT3 treated samples, maintained neuronal survival was observed when treated with drug eluted over 3, 14 and 28 days (24%, 30% and 15%, respectively). In the samples treated with day 28 SP-eluted NT3, there was a small, but statistically significant decline in

the number of neurons surviving, when compared to the freshly prepared or day 14 eluted neurotrophin (Figure 3D; $p<0.05$*).

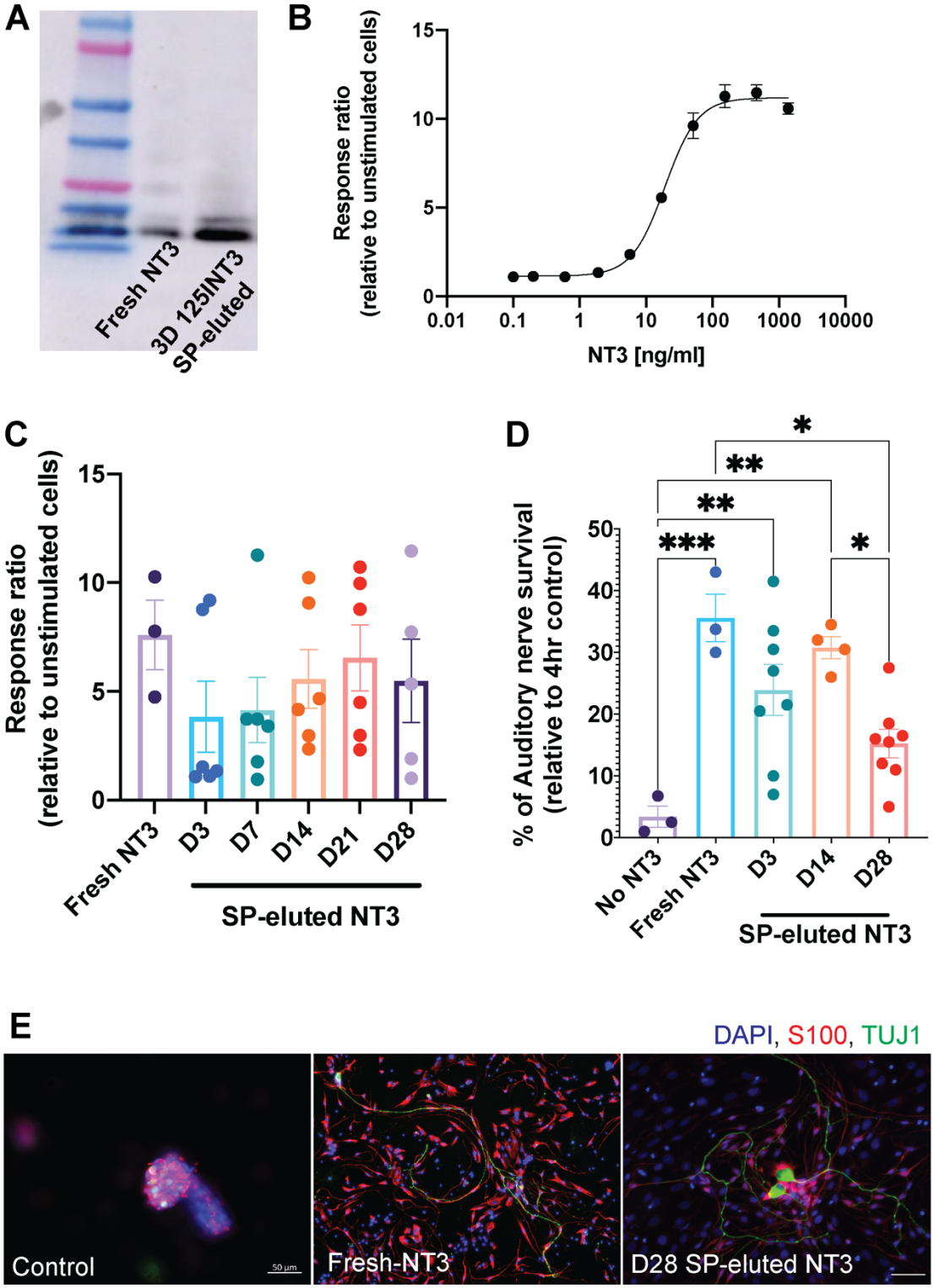



**Figure 3: Bioactivity of the SP eluted NT3** (A) Western blot for freshly prepared NT3 and SP-eluted NT3. (B) CellSensor TrkC assay to obtain dose response standard curve for fresh NT3. Data obtained from three experiments, 8 replicates per concentration per experiment. (C) CellSensor TrkC assay to determine bioactivity of freshly prepared-NT3 versus SP-eluted NT3 collected at day 3, 7, 14, 21 and 28 days. Gamma counts were used to estimate drug concentrations at the specific time points and all samples were diluted to 100 ng/mL.

Response ratios were calculated relative to the unstimulated cells (No NT3). Data obtained from 6 experiments, 8 replicates per concentration per experiment. (D) Auditory nerve culture assay to assess neuronal survival after treatment with freshly prepared NT3 and SP-eluted NT3 collected at day 3, 14 and 28 days. Data obtained from 3 experiments, 4 samples per timepoint run in duplicates. (E). Images of ANs labelled with Tuj1 (green), glia labelled with S100 (red) and DAPI (blue). Scale bars= 50 μm. All data presented as mean ± standard error of mean.

### *3.4 Intracochlear delivery of SP-loaded neurotrophin extends drug availability in the cochlea by up to 1 month*

One SP was implanted directly into each cochlea bilaterally via a cochleostomy. The animals were terminated at the specific time points post-implantation and whole-cochlear gamma counts obtained to calculate drug retention. Note that the gamma counts obtained here represent both the amount of NT3 present within the SP and the NT3 that was released from the particle and still present within the cochlear tissues and fluids at the time of tissue collection. A non-linear regression exponential growth curve was fitted to the retention data. The data showed an exponential decline in the radiolabelled NT3 levels with increased post-surgery time (Figure 4A; $p<0.001$). After 3 days, NT3retention of 55%, corresponding to 3.1 μg of NT3 was observed respectively (Figure 4A and B). Significant clearance of the radiolabelled neurotrophin was observed between 3-28 days, with approximately 3.6% of the injected dose from the implanted SP remaining after 1 month (0.18 μg; $p<0.001$, Tukey's). The gamma counts at 28 days were 332-fold above background measurements, defined as average cpm value of cochlea not exposed to $^{125}$I.

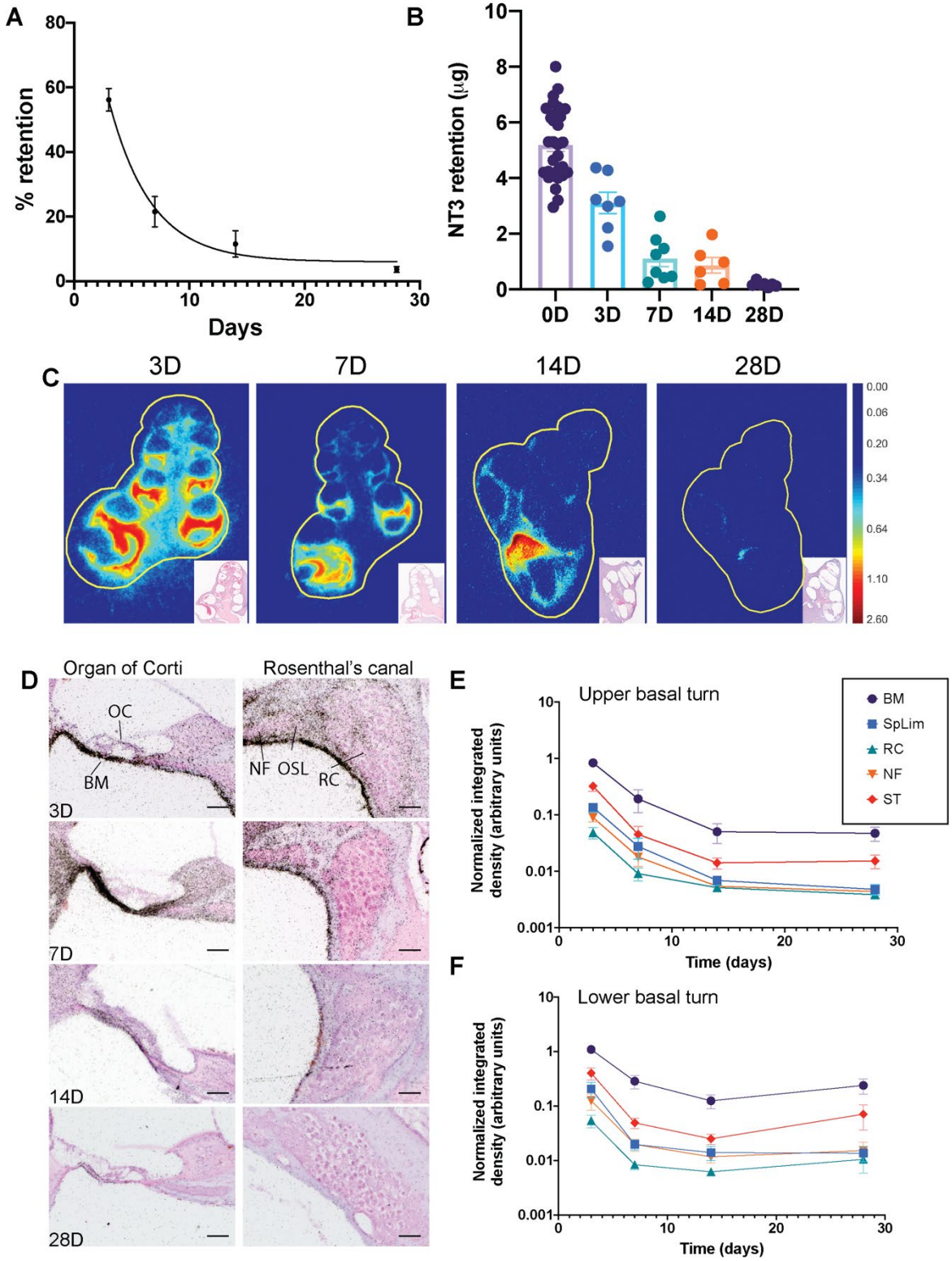
### *3.5 Widespread $^{125I}$NT3 distribution in the cochlea following intracochlear delivery of SPs*

Autoradiography film and emulsion-based methods were used to assess the biodistribution of $^{125}$INT3 within the cochlea. Cochlear sections exposed to film revealed the distribution of the $^{125}$INT3 signal throughout all the cochlear turns after 3- and 7-days post-surgery, with the strongest signal observed in the lower and upper basal turns (Figure 4C). With increased post-surgery time, clearance of $^{125}$INT3 was evident in the apical and mid-cochlear turns, with a weak signal still detectable in the basal turns after 1 month.

The$^{125}$I signal was detected in several cochlear regions, including the medial and lateral cochlear bone, organ of Corti (region of the hair cells and nerve fibres) and underlying basilar membrane, spiral limbus and spiral ligament. Autoradiographic emulsion quantification of the $^{125}$INT3 signal within regions of interest in cochlear sections were expressed as integrated density (density x area- in arbitrary units a.u) across the time points analysed and normalized to background. Comparison of the intracochlear $^{125}$I integrated densities between the lower and upper basal cochlear turns revealed the highest $^{125}$INT3 accumulation in the basilar membrane region and the mesothelial cells lining the scala tympani in both turns. With increased post-surgery time, there was a decrease in the $^{125}$I signal strength within the cochlea, although a signal was still detectable (above background) particularly in the basilar membrane and scala tympani regions after 28 days (figure 4D).

There was a clear and obvious $^{125}$INT3 signal within the regions around the neural tissue; the AN, including Rosenthal's canal which houses the cell bodies of the neurons and the internal auditory canal (osseous spiral lamina) containing the nerve fibres projecting to the central auditory organs particularly after 3 days. The strongest $^{125}$INT3 was still observed in the basilar membrane region, having an average signal 20-21-fold higher than the integrated density of Rosenthal's canal at the peak intensity observed at 3 days in both basal turns

(Figure 4E; Rosenthal's canal -UB: 0.04 and LB:0.05 a.u, basilar membrane UB: 0.8 and LB: 1.09 a.u). However, this signal declined with post-surgery time, with almost non-detectable levels after 1 month.



14- and 28-days post-surgery and whole-cochlea gamma counts obtained. (A) The percentage of NT3 retained inside the cochlea. (B) The amount of NT3 injected at day 0 and retained at day 3, 7, 14 and 28 days. These were calculated from the gamma counts of the administered SPs and gamma counts of the whole-cochleae post-surgery. (C) Film-based analysis of whole-cochlear sections with MATLAB image processing to visualise the spread of drug. Insets show H&E stained sections of the processed images. (D) High magnification images of H&E stained autoradiographs of the cochlear upper basal turn showing representative images of the organ of Corti region (OC) and Rosenthal's canal (RC). The black areas represent the $^{125}$INT3 signal. Scale bars= 50 μm. (E & F) Autoradiography analyses of $^{125}$I signal density in the upper and lower basal turns of cochlear regions of interest over the time course analysed. Basilar membrane (BM), osseous spiral lamina (OSL), nerve fibres (NF), spiral Limbus (SpLim), scala tympani (ST). Data presented as mean ± SEM**,** n=18.

*3.6 Neurotrophin availability with round window implantation of SPs*

The round window is a semipermeable membrane covering the opening into the basal cochlea and separating the internal cochlear environment from the middle ear. To surgically implant SPs onto the round window membrane the left and right bulla were exposed and SPs (n= 4-5) positioned on the membrane. The animals were terminated at 7, 14- and 28-days post-implantation. Whole cochlear gamma counts were obtained to measure the total drug availability at each time point, as the gamma counts obtained here represent the amount of neurotrophin present both inside the cochlea and within the particles that are external to the cochlea. A non-linear regression exponential growth curve was fitted to the retention data. There was a significant interaction between time and percentage retained (Figure 5A, $p<0.001$). After 7 days of treatment there was an average availability of 43% of the loaded NT3, but with increased post-surgery time, there was a statistically significant decline in percentages of NT3 available with 10% (2.3 μg NT3; Figure 5B) remaining after 28 days ($p<0.001$, Tukey's). At 28 days, the average cochlea gamma count was 2675x the background measurement of 30 cpm (defined as average cpm value of cochleae not exposed to $^{125}$INT3).

*3.7 Limited biodistribution of $^{125}$INT3 upon SP implantation via the round window*

Autoradiography analysis of cochlear sections revealed a strong $^{125}$I signal from the SPs within the round window niche external to the cochlea, with limited diffusion of the NT3 into the cochlear fluids (Figure 5C). $^{125}$INT3 was detected in the lower and upper segments of the basal turn in the organ of Corti and Rosenthal's canal (Figure 5D). Comparison of the $^{125}$I integrated densities between the basal turn segments revealed that the upper basal turn had a weaker signal compared to the lower basal turn. Specifically, in the basilar membrane, the $^{125}$I signal in the upper basal turn after one week was 12-fold lower than the integrated density in the lower basal turn (UB- 0.006 and LB- 0.07 a.u; Figure 5E and F). With increased post-surgery time, a decrease in the $^{125}$I signal in the lower basal turn was observed. A weak $^{125}$I signal was detected in the AN including Rosenthal's canal and the nerve fibres but decreased over time. The integrated density of the $^{125}$I signal in the nerve fibres was 0.03 a.u at 2 weeks in the lower basal turn, but by 4 weeks, the signal was 10-fold lower (0.003 a.u).

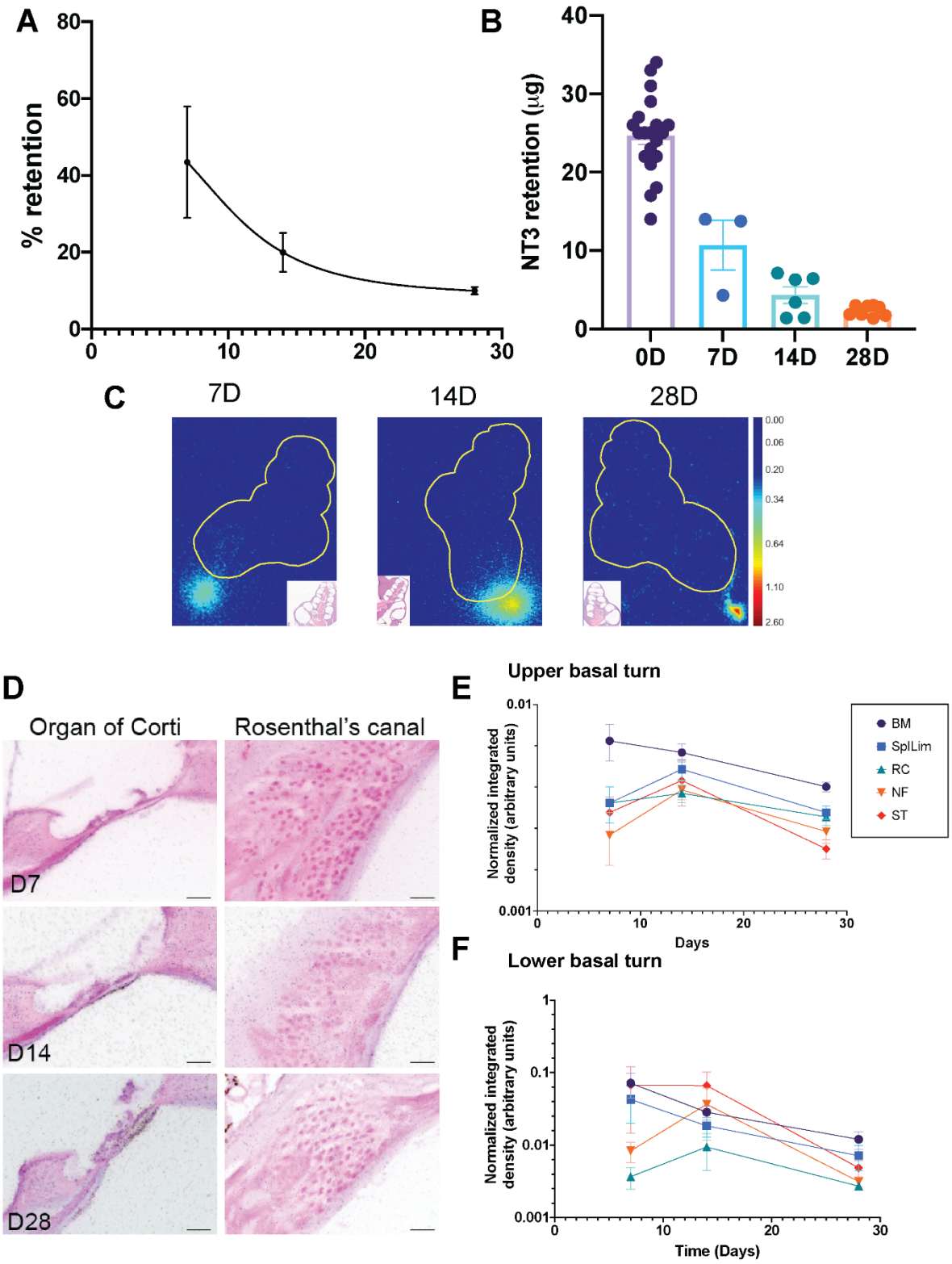



**Figure 5: Retention and biodistribution of NT3 after round window implantation of SPs.** The animals were implanted with four-five particles per cochlea. They were then terminated 7, 14- and 28-days post-surgery and whole-cochlea gamma counts obtained. (A) The percentage of neurotrophin retained inside the cochlea and within the round window

membrane niche post-surgery. (B) The amount of neurotrophin injected at day 0 and retained at day 7, 14 and 28 days. These were calculated from the gamma counts of the administered SPs and gamma counts of the whole-cochleae post-surgery. (C) Film-based analysis of whole-cochlea sections with MATLAB image processing showed retention of the SPs within the round window niche. Insets show H&E stained sections of the processed images. (D) High magnification images of H&E stained autoradiographs of the cochlear upper basal turn showing representative images of the organ of Corti region (OC) and Rosenthal's canal (RC). Scale bars= 50 μm. (E & F). Autoradiography analyses of $^{125}$I signal density in the upper and lower basal turns of cochlear regions of interest over the time course analysed. Basilar membrane (BM), osseous spiral lamina (OSL), nerve fibres (NF), spiral Limbus (SpLim), scala tympani (ST). Data presented as mean $\pm$ SEM, n=11.

### *3.8 Intracochlear implantation causes some loss of hearing at the high frequencies, while the round window approach has minimal impact on hearing*

In order to assess the surgical impact of both the intracochlear and round window delivery approaches on auditory function, auditory brainstem responses (ABRs) were recorded prior to surgical implantation of the SPs and remeasured at the conclusion of the treatment period (1 week, 2 weeks or 4 weeks). Auditory thresholds were measured for tone pip acoustic stimuli at a range of frequencies (1 kHz to 24 kHz).

All animals had normal hearing thresholds prior to surgery (Figure 6; thresholds within the grey region). For the intracochlear approach, there was a statistically significant elevation in ABR thresholds across all timepoints, with a significant interaction between frequency and pre/post ABR thresholds (Figure 6A; 1 week- $p<0.041$, 2 weeks- $p<0.002$ and 4 weeks- p ($p<0.026$). Post hoc analysis indicated a significant elevation in thresholds at frequencies above 8 kHz after 4 weeks of treatment ($p<0.05$, Holm Sidak). Average threshold shifts of 29 dB SPL at the mid-high frequencies (>8 kHz) was observed after 1 week, which improved slightly after 2 weeks, but did not recover to pre-implantation levels after 4 weeks. A maintained average loss of 13 dB SPL was observed across the mid-high frequencies upon delivery of SPs using the intracochlear method.

For the round window approach, a statistically significant increase in ABR thresholds were observed after 1 week, with a significant interaction between frequency and pre/post ABR thresholds ($p<0.037$; Figure 6B). Post hoc analysis indicated a significant elevation in thresholds at 24 kHz ($p<0.001$, Holm Sidak). Following 2 weeks of treatment, there was no statistical difference in pre verses post implantation thresholds (or any interaction). After 4 weeks of implantation, there was a small but significant elevation (average 3.5 dB shift across the frequency range) in ABR thresholds ($p<0.038$). The average difference in ABR threshold (post minus pre ABR threshold) for each frequency tested revealed minimal impact on functional hearing in normal hearing guinea pigs upon delivery of SPs using the round window method.

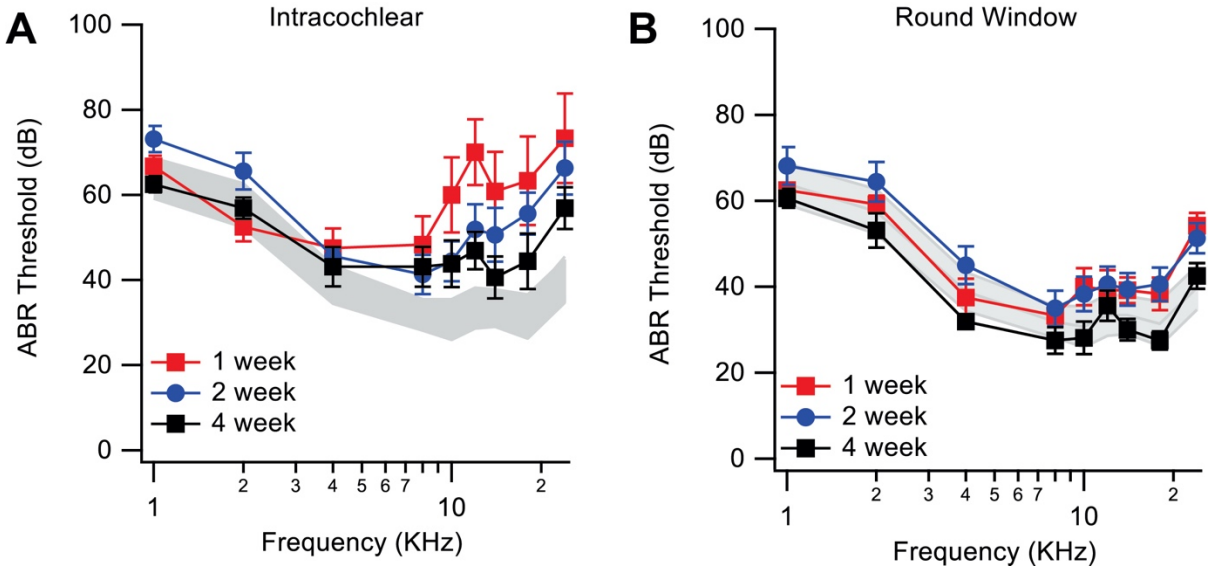



**Figure 6: Impact of surgery on hearing function.** Auditory brainstem response (ABR) thresholds for tone pip acoustic stimuli was measured pre and post-surgery. The grey region represents the pre-implant thresholds obtained with a 99% confidence interval across all groups. (A) For the intracochlear delivery, one $^{125}$I-NT3 loaded SP was delivered into the scala tympani. Three cohorts were examined, 1-week treatment (n=6 cochleae), 2-week treatment (n=8 cochleae), and 4-week treatment (n=8 cochleae). (B) For the round window delivery, five $^{125}$INT3 loaded SPs were implanted. Three cohorts were examined, 1-week (n=6 cochleae), 2-week (n=8 cochleae), and 4-week (n=8 cochleae) treatments. Data presented as mean $\pm$ standard deviation.

### *3.9 Limited spread of $^{125}$INT3 into other tissues*

Distribution of $^{125}$I NT3 was also examined in non-cochlear tissues including kidney, liver, intestine, heart, inferior colliculus, cochlear nucleus and ventricles using gamma counts. Compared to background levels (30cpm), the average $^{125}$INT3 signal was not significantly different to background levels in all the organs analysed (Figure 7A and B). There was very limited, or no distribution of the $^{125}$INT3 in any of the organs analysed using either delivery approaches. Of note, with the round window approach, the tissue surrounding the cochlear regions including the semi-circular canal and bulla had detectable levels of $^{125}$INT3 (1.6% and 0.9% of the injected dose), but still considerably lower than the cochlea (an average of 16% of the injected dose across all the time points analysed).

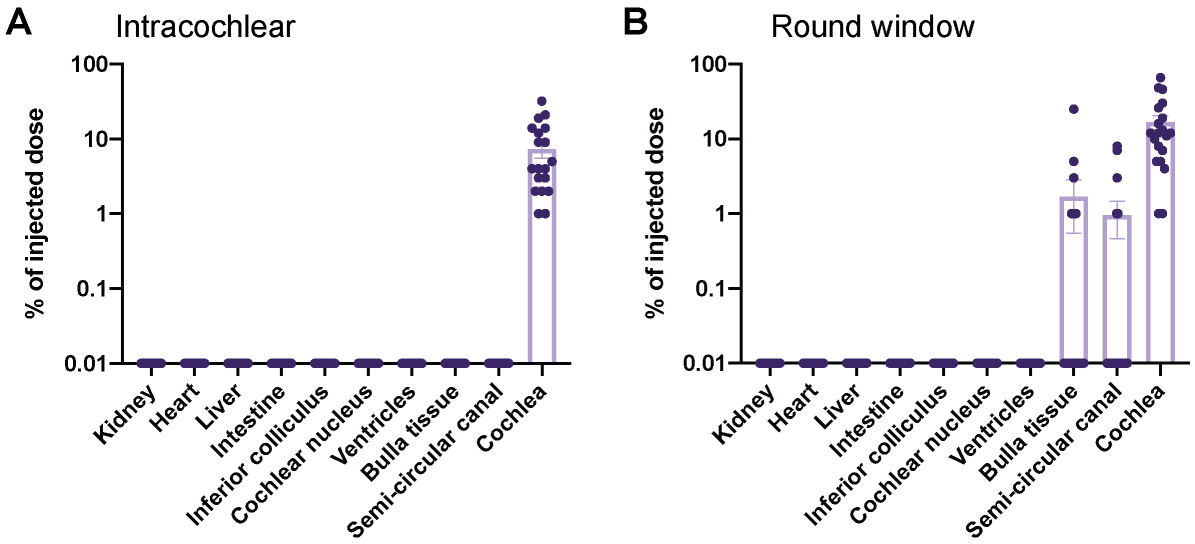



**Figure 7: Dispersion of $^{125}$INT3 into non-target tissue.** Gamma counts were obtained from multiple tissue from animals implanted with SPs using the intracochlear approach (A) or round window (B) approaches. The percentage retaining in the tissue was calculated relative to the injected dose. Data presented as mean ± standard error of mean.

## 4 Discussion

The lack of effective drug delivery strategies is one of the challenges impeding clinical translation of inner ear therapeutic treatments for hearing loss. The use of SPs for drug delivery is advantageous as they can be tailored to have high drug loading capacities, extended elution properties and maintain drug bioactivity to extend effective drug half-life [22, 23]. Here we aimed to characterize the pharmacokinetics of SP-mediated neurotrophin delivery and impact of surgical delivery in the normal hearing guinea pig. We show that SPs support neurotrophin availability and biodistribution in the cochlea for one month. Intracochlear delivery of just one SP resulted in significant drug distribution throughout all the cochlear turns but led to an elevation of hearing thresholds at high frequencies in the region of the cochleostomy and location of implantation. Whereas, following round window delivery of NT3 via SPs, lower intracochlear NT3 levels were measured in the basal cochlear region only whilst the impact of surgical placement of SPs on hearing thresholds was minimal. We also demonstrate that the $^{125}$I signal is retained within the cochlea with minimal spread to other tissue using both the intracochlear and round window approaches indicating a good safety profile for off target drug effects.

A major advantage of SPs is their capacity to be tailored to obtain the optimal size, shape, surface properties and pore size; factors that contribute to the drug loading and release kinetics. Furthermore, the pores of the SPs serve to immobilize the loaded protein, thus protecting the drug from denaturing and maintaining its bioactivity *in vivo* [7]. This is particularly important for neurotrophins, as they have a rapid half-life and protein denaturation over time has always been a hurdle when attempting to deliver neurotrophins using mini-pumps *in vivo* [8-10]. We have previously completed an extensive characterisation of the SPs to obtain the optimal manufacturing processes and performance parameters to ensure efficient neurotrophin loading and release kinetics [5-7]. We demonstrated using a lysozyme assay (fluorescently labelled model drug) and ELISA (for BDNF) that SPs have the capacity to load significant amounts of drug and support sustained drug release over 150 days. As radiolabelling the neurotrophin does not impact the protein

integrity and drug bioactivity [11, 12], we employed this technique to characterise the integrity and bioactivity of SP-released neurotrophin. This method permits a relatively faster and easier method to measure drug concentrations. We demonstrate that the SPs can load an average of 5.3 µg per SP that had an average size of 50 µg (0.11 µg NT3/ µg of SP). Based on our loading parameters (1:1 volume ratio of native 1 mg/ml to radiolabel 1 µg /mL NT3), we estimate that the fraction of radiolabeled protein amounts to 0.1% of the loaded protein. In addition, we show that the SPs support sustained neurotrophin release, with nearly 70% drug released after 1 month. Importantly, from a series of *in vitro* experiments employed to confirm the integrity and bioactivity of the released drug, we found that the SPs were effective at maintaining drug bioactivity over 1 month time period, comparable to the bioactivity of freshly prepared neurotrophin. It needs to be noted here that this data refers to the bioactivity of the drug *in vitro*. Unfortunately, there currently is no available assay to measure neurotrophin bioactivity *in vivo*, apart from perilymph sampling. However, this method only provides an indication of drug concentration, as measured by ELISA which determines protein integrity but not necessarily the bioactivity. As we believe an *in vivo* bioactivity assessment will be an important test of this technology, we are currently actively investigating methods to test drug bioactivity *in vivo*. Nevertheless, based on our *in vitro* results and our previous *in vivo* findings showing significant auditory neuron survival upon delivery of SP-loaded neurotrophins [4], we believe that our delivery system is effective at maintaining the integrity and bioactivity of neurotrophin.

Selecting the route of surgical access to the inner ear is perhaps the most vital aspect of drug delivery. Both the intracochlear and round window delivery approaches are well-established experimental and clinical routes for direct surgical access to the inner ear. The intratympanic route is an alternate approach, whereby access to the round window membrane and the oval window at the stapes can be used for substance entry into the perilymph. Our study revealed declining neurotrophin levels using both the intracochlear and round window approaches with increased post-surgery time. However, compared to a bolus $^{125}$INT3 injection, where 1% of the injected NT3 was retained after 3 days [15], SP-mediated NT3 delivery led to availability of 56% and 30% of the loaded drug using the intracochlear and round window approaches respectively. Furthermore, 28 days post-surgery, 3.6% and 10% of the loaded drug remained in the intracochlear and round window-implanted cochleae respectively. The amount remaining *in vivo* differed considerably between our *in vitro* elution data that showed 33% of drug remaining in the SPs after 28 days. This discrepancy may be attributed to the dynamic *in vivo* environment that may have caused accelerated elution of the protein compared to the *in vitro* technique of collecting the SP-released eluate from a tube (a closed system; [29]). For instance, in the intact, sealed guinea pig cochlea, the rate of fluid flow is extremely low (1.6 nl/min), however upon perforation of the cochlea, there can be a significant increase in the rate of fluid flow (1 µL /min; [30]). Another contributing factor may be the flow rate of CSF entry via the cochlear aqueduct, which in a normal, sealed cochlea is very low (30 nl/min), but still amounts to ~2 µL per hour- approximately half the volume of the scala tympani [29, 31]. These dynamics are likely contributors to the rapid elution of the protein *in vivo*. Furthermore, it needs to be noted that the cochlea gamma counts include the drug within the particles as well as the released drug, and for the round window implanted animals the $^{125}$I signal is measured within and outside (round window niche) the cochlea. These findings support the potential for SPs to maintain drug availability and levels within the cochlea for extended durations.

Autoradiography images revealed that the intracochlear-treated cochlea had widespread drug distribution across all the cochlear turns, with a base-to-apical gradient favouring the base. Here, it needs to be noted that this analysis did not include the signal from the particle and is

a measurement of the released $^{125}$INT3. With increased post-surgery time, the $^{125}$I signal was restricted to the basal turn but was still detectable after 28 days. Comparatively, the round window approach showed lower drug absorption into the inner ear, with the $^{125}$I signal restricted to the basal turn and round window niche. However, even after 28 days, a weak $^{125}$I signal was detectable indicating that round window NT3 delivery via SPs is effective at delivering NT3 to the intracochlear environment. In addition to the presence of the round window membrane as the major barrier to drug delivery to the cochlea the residence time of the drug on the round window membrane will influence the efficiency of $^{125}$INT3 delivery to the inner ear [32]. Furthermore, the permeability of the round window membrane is highly variable between animals with up to 10-fold differences in drug adsorption across the round window [33], which may account for some of the variability observed in our gamma counts and autoradiography analyses. Our findings agree with other studies showing reduced percentages of drugs entering the inner ear by drug injection to the round window niche. Basal turn concentrations reaching means of only 2.5% of the administered gentamicin concentration or 1.4% of the dexamethasone-phosphate concentration have been reported [34, 35]. Noting that these analyses were done by perilymph sampling 1-3 h post-administration, our findings showing a detectable $^{125}$I signal at day 28 in the round window implanted animals implicate the potential for SPs to improve long-term drug bioavailability across the round window membrane.

Using both delivery approaches, we found that the $^{125}$INT3 signal passed from the perilymph to other cochlear tissue including the osseous spiral lamina, spiral ligament and spiral limbus. A strong $^{125}$INT3 signal was also detected in the basilar membrane across the time points analysed. This pattern of drug distribution has previously been reported in structures where the boundary layers are incomplete or lack tight junctions [15, 24, 36]. For instance, the basilar membrane is formed by cells that have incomplete tight junctions creating a compact labyrinth-like environment with a high collagen content, which may explain the slow clearance and accumulation of the neurotrophin to this region. The $^{125}$I signal was also detected in Rosenthal's canal, the region that houses the AN and their nerve fibres – the therapeutic target cells that express the Trk receptor for neurotrophin. The clear $^{125}$INT3 signal in the cell bodies in Rosenthal's canal containing both ANs and glial cells support findings from our previous study confirming the bioactivity of BDNF associated with improved AN survival [1].

SP-mediated neurotrophin delivery using the intracochlear approach showed significant hearing loss at the high frequencies after surgery. However, with increased time, there was some hearing recovery, with an average threshold shift of 13 dB SPL maintained at 4 weeks post-treatment in the mid to high-frequency regions. The round window approach had minimal effect on functional hearing across the frequencies analysed although an average 3.5 dB SPL threshold shift was observed after 28 days at the highest frequency measured (24 kHz). Although normal hearing animals were used in this study, the clinical indication targeted for this therapy would be people with high frequency hearing loss, thus the surgical delivery of the SP therapy is unlikely to cause additional loss of functional hearing in this population. The observed high frequency hearing loss with intracochlear delivery is most likely caused by surgical trauma from drilling the cochlear bulla and/or delivery of the SPs using the cannula. This loss of hearing function after a cochleostomy has been well-documented [37]. Carvalho et al., showed that intracochlear delivery of saline into the cochlea with a mini-osmotic pump caused a 10 dB SPL shift in hearing threshold 28 days post-surgery [37]. While it is possible that hearing function may return to pre-operative levels over a longer term, these findings support the application of these delivery routes for SP delivery. It needs to be noted here the functional measurements obtained were to assess the

impact of the surgical approach on hearing function. Current studies are underway to assess the functional impact of SP-released neurotrophin in deafened animal models.

Optimization of drug delivery approaches for the inner ear requires a pharmacokinetic technique that provides spatiotemporal information on drug concentration, distribution within and clearance from the target tissue. Other techniques such as serial perilymph sampling provides information on drug entry and elimination in cochlear fluids at set time points, however sampling of the fluid from either the cochlear apex or semi-circular canal means the drug can become bound to internal cochlear structures as the fluid is extracted. Furthermore, fluid sampling only enables evaluation of a drug that is suspended or dissolved into the cochlear perilymph whereas drug that might be bound to cochlear tissues is unavailable for extraction. Whilst the potential for fluid contamination from CSF entry can be mitigated with accurate sampling volumes there is likely to be some mixing of the CSF and cochlear fluids [38]. Furthermore, the assay does not provide information on the extent of drug uptake at the cellular level along the tonotopic axis of the cochlea. Other approaches such as micro CT imaging [39, 40], electrophysiological measurements [41], fluorescently labelled drug conjugates or tracers [42-44] have also been explored each with their own advantages and disadvantages. The radiolabelling technique permits quantification of drug levels and clearance in the intact cochlea (prior to fixing) using whole-cochlea gamma counts and visualization of drug distribution is possible with autoradiography techniques on cochlear sections [15, 24]. We previously showed that NT3 iodination does not impact the integrity or functionality of the protein and have analysed the retention and distribution of direct intracochlear injections of bolus $^{125}$INT3 over a period of time. [24]. Therefore, application of the radiolabelling technique proved to be a useful measure for obtaining quantitative measures of neurotrophin retention and biodistribution using SPs for this study. It needs to be noted here that the technique may not be applicable to all drug/substances and the half-life of $^{125}$I at 59.5 days restricts extended duration studies.

To assess the therapeutic potential of SPs for NT3 delivery, it is necessary to understand the biofate of SPs in the cochlea, noting here that their rate of degradation and elimination will differ based on their location of delivery. Tracking the fate of SPs in real-time *in vivo* is technically challenging. Generally, from nanoparticle studies, it has been determined that the size of the particle is directly correlated to their biofate, whereby smaller nanoparticles (20–30 nm) are rapidly excreted in the kidney by renal filtration, but larger nanoparticles (30-200 nm) have a longer persistence time in the bloodstream and are eliminated by phagocytosis [45]. The biofate of nanoparticles is routinely assessed using optical measures such as near infrared fluorescence imaging. While useful, this method permits examination of the signal from only the outer parts of the body [46, 47]. From our autoradiography analysis and visual observation of some extracted cochleae, we were able to detect intact SPs within the round window membrane niche even after one-month post-treatment. However, in some cases, no trace of the 125I signal or SPs could be detected in the cochlea. It is possible that SPs delivered to the round window may be moved down the adjacent Eustachian tube and transported into the gut for excretion. Conversely, SPs delivered to the cochlea may be excreted from the cochlear aqueduct and/or enter the vasculature /lymphatic system before being phagocytosed–these mechanisms are yet to be elucidated. Nevertheless, based on our examination of the 125I signal in other tissue, we did not detect the presence of the drug or SPs in any of the other organs analysed. We also performed a silica analysis using inductively coupled plasma mass spectrometry (ICP-MS) to assess the biodistribution of the SPs in off-target organs after their implantation in the cochlea. No traces of silica were detected in the organs analysed (unpublished data). Overall, we believe that the SPs have an extended residence time in the cochlea compared to nanoparticles due to their larger size (+/-

500 um in diameter). However, further testing is required to determine the mechanisms of their fate *in vivo*.

We envisage several potential applications of SP-mediated drug delivery using both the intracochlear and round window approaches. With the intracochlear SP delivery route, there was some loss of functional hearing in the high frequencies, but widespread cochlear drug distribution extending to the apical region. We expect this approach to be applicable for improving the performance of the cochlear implant, given the evidence that neurotrophin administration causes reduced excitation thresholds with a cochlear implant possibly due to improved nerve-electrode interface [7, 12]. Given the drug distribution in the apex, there may also be the potential to apply SPs to deliver factors that preserve residual hearing function at the lower frequencies. In regard to the round window approach, we observed restricted drug distribution (compared to the intracochlear route), but minimal loss of hearing function although normal hearing animals were used here. We envisage this therapy to be most applicable for treating cochlear neural degeneration and synaptopathy. Thus, the target population would be patients with high frequency hearing impairment. Collectively, our pharmacokinetic data showing effective drug retention, distribution, and physiological changes after SP delivery, will inform the applicability and potential for this technology to be translated to the clinic.

## 5 Conclusions

This study revealed that delivery of drug using SPs support continued, long-term availability and release of biologically active NT3 in the inner ear. Utilizing a novel radiolabelling method to assess drug pharmacokinetics in the cochlea, we show that delivery of SPs using both the intracochlear and round window route promotes drug retention in the cochlea even after one-month post-implantation. In addition, we found that intracochlear delivery approach caused widespread drug distribution throughout the cochlea, but some loss of hearing function at the high frequencies. Conversely, the round window approach showed minimum loss of hearing function, but drug distribution was restricted to the basal regions of the cochlea. With both delivery approaches, a clear $^{125}$INT3 signal was detected in Rosenthal's canal containing ANs, the target cell population of neurotrophins. These findings indicate that SPs are a viable approach to effectively deliver neurotrophins to the cochlea for extended durations.

## CRediT author statement

Niliksha Gunewardene: conceptualisation, methodology, formal analysis, investigation, data curation, writing-original draft, visualisation. Patrick Lam: conceptualisation, methodology, formal analysis, visualization. Yutian Ma: investigation. Sherryl Wagstaff: conceptualisation, methodology, investigation. Frank Caruso: conceptualisation, supervision. Rachael Richardson: conceptualisation, methodology, formal analysis, writing review and editing, supervision. Andrew Wise: conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing review and editing, visualisation, supervision, project administration, funding acquisition.

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**Data availability**

Source data are available upon request from the corresponding author.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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