Author/s:
Gunewardene, N;Ma, Y;Lam, P;Wagstaff, S;Cortez-Jugo, C;Hu, Y;Caruso, F;Richardson, RT;Wise, AK

Title:
Developing the supraparticle technology for round window-mediated drug administration into the cochlea

Date:
2023-09-01

Citation:
Gunewardene, N., Ma, Y., Lam, P., Wagstaff, S., Cortez-Jugo, C., Hu, Y., Caruso, F., Richardson, R. T. & Wise, A. K. (2023). Developing the supraparticle technology for round window-mediated drug administration into the cochlea. Journal of Controlled Release, 361, pp.621-635. https://doi.org/10.1016/j.jconrel.2023.08.016.

Persistent Link:
https://hdl.handle.net/11343/337021

Journal of Controlled Release

Developing the supraparticle technology for round window-mediated drug administration into the cochlea

--Manuscript Draft--

Manuscript Number:	COREL-D-23-01727R2
Article Type:	Research paper
Keywords:	Hearing loss, Drug delivery, Neurotrophin-3, Pharmacokinetics, Supraparticles
Corresponding Author:	Niliksha Gunewardene, Ph.D. East Melbourne, VIC AUSTRALIA
First Author:	Niliksha Gunewardene, PhD
Order of Authors:	Niliksha Gunewardene, PhD
	Yutian Ma
	Patrick Lam
	Sherryl Wagstaff
	Christina Cortez-Jugo
	Yingjie Hu
	Frank Caruso
	Rachael Richardson
	Andrew Wise
Abstract:	The semi-permeable round window membrane (RWM) is the gateway to the cochlea. Although the RWM is considered a minimally invasive and clinically accepted route for localised drug delivery to the cochlea, overcoming this barrier is challenging, hindering development of effective therapies for hearing loss. Neurotrophin 3 (NT3) is an emerging treatment option for hearing loss, but its therapeutic effect relies on sustained delivery across the RWM into the cochlea. Silica supraparticles (SPs) are drug delivery carriers capable of providing long-term NT3 delivery in the cochlea, when injected directly into the guinea pig cochlea. However, for clinical translation, a RWM delivery approach is desirable. Here, we aimed to test approaches to improve the longevity and biodistribution of NT3 inside the cochlea after RWM implantation of SPs in guinea pigs and cats. Three approaches were tested (i) coating the SPs to slow drug release (ii) improving the retention of SPs on the RWM using a clinically approved gel formulation and (iii) permeabilising the RWM with hyaluronic acid. A radioactive tracer (iodine 125: 125I) tagged to NT3 (125I NT3) was loaded into the SPs to characterise drug pharmacokinetics in vitro and in vivo. The neurotrophin-loaded SPs were coated using a chitosan and alginate layer-by-layer coating strategy, named as '(Chi/Alg)SPs', to promote long term drug release. The guinea pigs were implanted with 5x 125I NT3 loaded (Chi/Alg) SPs on the RWM, while cats were implanted with 30x (Chi/Alg) SPs. A cohort of animals were also implanted with SPs (controls). We found that the NT3 loaded (Chi/Alg)SPs exhibited a more linear release profile compared to NT3 loaded SPs alone. The 125I NT3 loaded (Chi/Alg)SPs in fibrin sealant had efficient drug loading (~5 µg of NT3 loaded per SP that weights ~50 µg) and elution capacities (~49% over one month) in vitro. Compared to the SPs in fibrin sealant, the (Chi/Alg)SPs in fibrin sealant had a significantly slower 125I NT3 drug release profile over the first 7 days in vitro (~12% for (Chi/Alg) SPs in fibrin sealant vs ~43% for SPs in fibrin sealant). One-month post-implantation of (Chi/Alg) SPs, gamma count measurements revealed an average of 0.3 µg NT3 remained in the guinea pig cochlea, while for the cat, 1.3 µg remained. Histological analysis of cochlear tissue revealed presence of a 125I NT3 signal localised in the basilar membrane of the lower basal turn in some cochleae after 4 weeks in guinea pigs and 8 weeks in cats. Comparatively, and in contrast to the in vitro release data, implantation of the SPs presented better NT3 retention and distribution inside the cochlea in both the guinea pigs and cats. No significant difference in drug entry was observed upon acute

	treatment of the RWM with hyaluronic acid. Collectively, our findings indicate that SPs and (Chi/Alg)SPs can facilitate drug transfer across the RWM, with detectable levels inside the cat cochlea even after 8 weeks with the intracochlear approach. This is the first study to examine neurotrophin pharmacokinetics in the cochlea for such an extended period of times these two animal species. Whilst promising, we note that outcomes between animals were variable, and opposing results were found between in vitro and in vivo release studies. These findings have important clinical ramifications, emphasising the need to understand the physical properties and mechanics of this complex barrier in parallel with the development of therapies for hearing loss.

Developing the supraparticle technology for round window-mediated drug administration into the cochlea

Niliksha Gunewardene[1,2*#], Yutian Ma[1,4*], Patrick Lam[1], Sherryl Wagstaff[1], Christina Cortez-Jugo[4], Yingjie Hu[1,2,4], Frank Caruso[4], Rachael Richardson[1,2,3] and Andrew Wise[1,2#]

[1]Bionics Institute, East Melbourne, Victoria 3002, Australia.

[2]Department of Medical Bionics, The University of Melbourne, Fitzroy, Victoria 3065, Australia.

[3]Department of Surgery (Otolaryngology), University of Melbourne, The Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria 3002, Australia

[4]Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

[*]Co-first authors

[#]Corresponding authors

1. ABSTRACT

The semi-permeable round window membrane (RWM) is the gateway to the cochlea. Although the RWM is considered a minimally invasive and clinically accepted route for localised drug delivery to the cochlea, overcoming this barrier is challenging, hindering development of effective therapies for hearing loss. Neurotrophin 3 (NT3) is an emerging treatment option for hearing loss, but its therapeutic effect relies on sustained delivery across the RWM into the cochlea. Silica supraparticles (SPs) are drug delivery carriers capable of providing long-term NT3 delivery in the cochlea, when injected directly into the guinea pig cochlea. However, for clinical translation, a RWM delivery approach is desirable. Here, we aimed to test approaches to improve the longevity and biodistribution of NT3 inside the cochlea after RWM implantation of SPs in guinea pigs and cats. Three approaches were tested (i) coating the SPs to slow drug release (ii) improving the retention of SPs on the RWM using a clinically approved gel formulation and (iii) permeabilising the RWM with hyaluronic acid. A radioactive tracer (iodine 125: ^{125}I) tagged to NT3 (^{125}I NT3) was loaded into the SPs to characterise drug pharmacokinetics *in vitro* and *in vivo*. The neurotrophin-loaded SPs were coated using a chitosan and alginate layer-by-layer coating strategy, named as '(Chi/Alg)SPs', to promote long term drug release. The guinea pigs were implanted with 5x ^{125}I NT3 loaded (Chi/Alg) SPs on the RWM, while cats were implanted with 30x (Chi/Alg) SPs. A cohort of animals were also implanted with SPs (controls). We found that the NT3 loaded (Chi/Alg)SPs exhibited a more linear release profile compared to NT3 loaded SPs alone. The ^{125}I NT3 loaded (Chi/Alg)SPs in fibrin sealant had efficient drug loading (~5 µg of NT3 loaded per SP that weights ~50 µg) and elution capacities (~49% over one month) *in vitro*. Compared to the SPs in fibrin sealant, the (Chi/Alg)SPs in fibrin sealant had a significantly slower ^{125}I NT3 drug release profile over the first 7 days *in vitro* (~12% for (Chi/Alg) SPs in fibrin sealant vs ~43% for SPs in fibrin sealant). One-month post-implantation of (Chi/Alg) SPs, gamma count measurements revealed an average of 0.3 µg NT3 remained in the guinea pig cochlea, while for the cat, 1.3 µg remained. Histological analysis of cochlear tissue revealed presence of a ^{125}I NT3 signal localised in the basilar membrane of the lower basal turn in some cochleae after 4

weeks in guinea pigs and 8 weeks in cats. Comparatively, and in contrast to the *in vitro* release data, implantation of the SPs presented better NT3 retention and distribution inside the cochlea in both the guinea pigs and cats. No significant difference in drug entry was observed upon acute treatment of the RWM with hyaluronic acid. Collectively, our findings indicate that SPs and (Chi/Alg)SPs can facilitate drug transfer across the RWM, with detectable levels inside the cat cochlea even after 8 weeks with the intracochlear approach. This is the first study to examine neurotrophin pharmacokinetics in the cochlea for such an extended period of times these two animal species. Whilst promising, we note that outcomes between animals were variable, and opposing results were found between *in vitro* and *in vivo* release studies. These findings have important clinical ramifications, emphasising the need to understand the physical properties and mechanics of this complex barrier in parallel with the development of therapies for hearing loss.

2. INTRODUCTION

Sensorineural hearing loss is caused by the degeneration of the sensory pathway in the cochlea, including the hair cells, primary auditory neurons, and their synapses. Exposure to environmental noise, viral infections, inherited and autoimmune disorders, or the ageing process, can damage the sensory cells in the cochlea, resulting in hearing impairment. Currently there is no available treatment to repair or prevent the progression of hearing loss. Devices such as hearing aids and cochlear implants can assist with hearing, but they fail to address the underlying pathology of deafness or halt its progression. With the growing number of people affected by hearing loss, there is a large unmet need for a drug therapy that can restore the sensory cells of the cochlea and return lost function.

The inner ear is a bony structure comprised of the cochlea (hearing organ) and vestibular system (balance organs). Accessing the cochlea is challenging due to its small size, embedded location, and complex architecture. The cochlea is separated into three chambers filled with ionic fluids; perilymph and endolymph, that bathe the sensory hair cells that together with auditory neurons transduce auditory information from the cochlea to the brain. The semi-permeable membranes, the round and oval windows are the gateways into the cochlea from the middle ear. Effective therapy requires entry of bioactive drug into the cochlea at therapeutically relevant concentrations and durations.

There are three main local drug delivery routes to the cochlea, (i) systemic delivery, (ii) direct intracochlear delivery (e.g. injection into the scala tympani via cochleostomy or penetration of the round window membrane (RWM)) or (iii) intratympanic administration (diffusion of drug across the semi-permeable membranes). With the systemic approach, only a portion of the injected therapeutic drug (primarily small molecule therapeutics) crosses through the blood labyrinth barrier and reaches the cochlea thus limiting what can be delivered [1, 2]. Systemic delivery also carries the risk of side effects or dose-limiting toxicity. Direct drug delivery approaches into the cochlea carry the risk of damaging residual hearing due to the invasiveness of the surgery and fluid leakage, therefore, this approach would typically only be considered if the cochlea is already being accessed such as during cochlear implant surgery [3, 4]. The intratympanic administration routes are widely accepted as optimal for the treatment of inner ear disorders as there is minimal risk to residual hearing. For example, steroids such as dexamethasone or gentamicin are commonly injected via the intratympanic approach as a treatment for Meniere's disease or idiopathic sudden hearing loss. Although effective, variable outcomes due to reduced drug diffusion across the membranes have been reported. Of note, in the guinea pig cochlea, the basal turn concentrations of dexamethasone-phosphate or gentamicin was only 2.5% or 1.4% of the injected concentration, respectively over 1-3 hours post-administration [5, 6]. Furthermore, drugs with a large molecular weight, negative charge or hydrophilic properties have poor diffusion properties through the RWM [7].

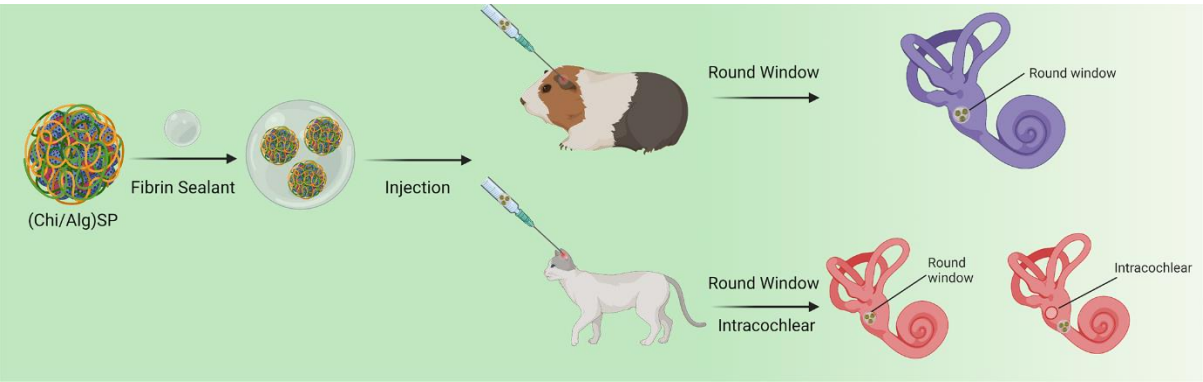
The RWM is a semi-permeable membrane consisting of three epithelial layers. The first, and, outer layer, facing the middle ear, consists of epithelial cuboidal cells that form tight junctions. The tight junctions limit the passage of most molecules that occurs via passive diffusion. The second layer is made of fibroblasts, collagen, and elastic fibres, which, contains blood and lymph vessels as well as nerve endings. The third layer consists of squamous flat inner epithelial cells and faces the scala tympani. Accumulating pharmacokinetic and functional evidence demonstrates insufficient protein transfer through the semi-permeable RWM, indicating its selective nature in substance entry to the cochlea. Factors such as molecule size, configuration, concentration, liposolubility, electrical charge, and thickness of the membrane influencing its permeability [7-9]. For instance, certain low molecular weight steroids such as dexamethasone or gentamicin easily penetrate across the RWM (MW ~400). However, with the larger molecules such as albumin (MW 70,000), the efficiency of drug entry via the RWM can be significantly lower [10]. Of note, a study focused on RWM delivery of recombinant neurotrophin (MW 29,355) to deafened animals revealed variability in hearing function improvements, with only 50% of the animals showing hearing recovery [11], suggesting that variability in drug transfer between animals. Despite the limitations in permeability of the RWM, it remains the most clinically relevant drug delivery route for reversing hearing loss. Therefore, there is a need to develop strategies to improve drug penetration across the RWM.

Neurotrophins have been implicated as an effective therapeutic option to treat hearing loss. They are naturally occurring proteins expressed by hair cells and supporting cells in the cochlea. It is well-established that neurotrophin 3 (NT3) improves the survival of sensory neurons and promotes the protection and/or regeneration of ribbon synapses [12-21]. Despite this evidence, a key challenge that has impeded clinical translation of neurotrophins, with a molecular weight of 27.3 kDa, is the lack of an effective approach for drug delivery into the cochlea in a sustained and consistent manner. We previously demonstrated that upon acute delivery of a bolus of radiolabelled neurotrophin 3 (^{125}I NT3) to guinea pig cochleae via direct intracochlear infusion, there was an exponential decrease in NT3 in the cochlea over time, with nearly complete clearance of NT3 in just 3 days [22]. There is a need to improve NT3 levels in the cochlea to gain long-term therapeutic benefit and optimise an intratympanic approach for safe drug delivery.

Several drug delivery approaches have been tested to improve neurotrophin delivery to the cochlea, including cell-based therapies, bisphosphonate agonists, mini-osmotic pumps, viral vectors, and nanoparticles, each with their own benefits and limits (reviewed in [23]). We have shown that the application of mesoporous silica supraparticles (SPs) is effective for NT3 delivery into the cochlea [24-28]. Supraparticles are spheroid structures formed from smaller colloidal nanoparticles that provide a platform for long-term controlled drug release. The major advantage of this system includes its potential to be tailored (size, porosity, and composition) to modify performance, including drug pharmacokinetics based on the treatment requirement. We previously showed that intracochlear delivery of SPs loaded with brain derived neurotrophic factor (BDNF), a neurotrophin closely related to NT3 with similar neuro-protective properties in the cochlea, to profoundly deafened guinea pigs led to a significant improvement in auditory neuron survival one-month post-treatment [26]. However, given the potential therapeutics effect of NT3 on ribbon synapse repair, we undertook a thorough characterisation of the pharmacokinetics of the SP-mediated NT3 delivery [24]. Using a TrkC cell line bioactivity test and auditory nerve survival culture assay, we previously confirmed that the SP-released drug had maintained bioactivity over an extended period *in vitro* (tested up to 28 days; [24]). Delivery of SPs to guinea pig cochleae using both the intracochlear and round window routes revealed efficient drug retention even after one-month post-implantation. With the intracochlear delivery approach, widespread drug distribution was observed throughout the cochlea. However, with

the RWM approach, drug distribution was limited and restricted to the RWM niche (outside the cochlea) and/or basal regions of the cochlea [24].

Given our previous data showing variability in the amount of drug entering the cochlea via the RWM using supraparticles, we hypothesised that this could be attributed to several factors: the particles being in limited contact with the RWM, restricted permeability of the RWM minimising the amount of NT3 crossing the membrane and/or a burst-release elution profile that may cause rapid drug clearance. The aim of this project was to address these hypotheses and develop approaches such as coating the SPs to slow down their drug release were tested to improve the longevity and biodistribution of NT3 inside the cochlea after RWM implantation of SPs in guinea pigs and cats (Scheme 1).



Scheme 1. Schematic illustration of the concept of the manuscript.

3. EXPERIMENTAL METHODS SECTION

Supraparticle manufacturing process. Primary silica particles ranging in diameter up to about 800 nm were synthesised using a modified version of a previously published protocol [27]. Specifically, 1.1 g of Cetyltrimethlyammonium bromide (CTAB) was first dissolved in 50 mL of Milli-Q water followed by adding 4.3g of Poly(acrylic acid) (PAA, Mw ~250 kDa, 35 wt % solution in water) at room temperature (i.e., ~22 °C) under vigorous stirring until a clear solution appears. After 20 min, 3.5 mL of ammonium hydroxide solution (28–30%) was added to the above suspension and stirred vigorously for 20 min until a milky suspension was obtained. Then, 4.46 mL of Tetraethyl orthosilicate (TEOS) was added with vigorous stirring for 15 min. Subsequently, the mixture was placed into a Teflon-sealed autoclave at 90 °C for 48 h. The silica particles were removed from the autoclave and washed with Milli-Q water and ethanol twice, followed by drying at 80 °C overnight. Finally, the primary silica particles were placed in a chamber furnace (Tetlow, Australia) and calcined at 550 °C for 30 h to remove the organic materials. The silica SPs (SPs) were produced by electrospraying. Briefly, primary silica particles (80 mg) were added to 2 mL of aqueous alginate solution (30 mg/mL in Milli-Q water). Then, the suspension was ultra-sonicated by an ultrasonic processor with a microtip probe (Qsonica, CT, USA) at an output amplitude of 30% for 40 s and sonicated further in an ultrasonic cleaner (Branson, USA) for 1 h to disperse the primary silica particles evenly within the alginate solution. After sonication, the solution was poured into a syringe, which was positioned in a syringe pump at a constant flow rate of 8 mL h. With an applied voltage of 13 kV, alginate/primary silica particle droplets were electrosprayed into a reservoir of 1% w/v of aqueous $CaCl_2$ to crosslink the alginate. The distance between the end of the nozzle and the surface of the $CaCl_2$ solution was set to 10 cm. Subsequently, the alginate/silica SPs were collected directly from the CaCl2 bath using a strainer and washed extensively in Milli-Q water (four times). Finally, to obtain SPs, alginate/silica SPs were calcinated at 650 °C for 30 h to remove the organic materials (i.e. alginate).

Synthesis of chitosan-alginate supraparticles. The preparation procedure of layer by layer-coated supraparticles using chitosan and alginate ((Chi/Alg)SPs) is shown in Figure 1a. Briefly, SPs were sterilised with 100 µL of 80% (v/v) ethanol at ~22 °C for 4 h, followed by rinsing with sterile Milli-Q water for six times. Chitosan (50 µL of 0.5 wt%) dissolved in 1 wt% acetic acid containing 0.15 M NaCl with pH adjusted to 5.5 was added to the SPs. After 15 min of incubation, the supernatant was aspirated, and the SPs were washed with Milli-Q water 3 times. Then, chitosan-coated SPs were incubated with 50 µL of 0.5 wt% of alginate dissolved in 1 wt% of acetic acid containing 0.15 M NaCl with pH adjusted to 5.5 for 15 min in a rotating mixer. Finally, the supernatant containing excess alginate was aspirated and the particles washed with Milli-Q water 3 times. The alternate deposition of chitosan and alginate was repeated twice.

FITC-labelled chitosan (FITC-chitosan) was synthesized based on the protocol below.Typically, 5 mg of FITC was dissolved in 10 mL of methanol and mixed with 210.1 mg of chitosan in 1 wt% of acetic acid. Then, the pH was adjusted to 7 using NaOH and acetic acid. The solution was stirred in the dark at room temperature (~22 °C) for 24 h, then dialysed using SnakeSkin dialysis tubing with 3.5k molecular weight cutoff (Thermo Scientific) against 0.15 M NaCl, followed by Milli-Q water for 3 days. Finally, the dialysed FITC-chitosan was lyophilised and stored in the fridge until use.

Characterisation of supraparticles. Scanning electron microscopy (SEM) images of SPs and (Chi/Alg)SPs were taken using a Philips XL30 field-emission scanning electron microscope (Philips, Netherlands) at an operating voltage of 5 kV. The samples were prepared by placing them onto conductive carbon tape followed by sputter coating with a 20 nm gold coating on the samples. SEM–energy-dispersive X-ray spectroscopy (SEM-EDX) mapping of (Chi/Alg)SPs was taken with a Philips XL30 field-emission scanning electron microscope (Philips, Netherlands). The thickness of the chitosan and alginate coating was characterised by quartz crystal microbalance with dissipation technique (QCM-D) (Kyushu Dentsu, Japan). Briefly, QCM chips were first cleaned with Piranha solution (3:1 mixture of sulfuric acid and 30% hydrogen peroxide) for 1 min followed by rinsing with copious amount of Milli-Q water and dried with a gentle stream of nitrogen. Chitosan and alginate layers were sequentially deposited on the clean gold surface at a constant flow rate of 200 µL min-1 for 15 min. After each layer deposition, the crystal was thoroughly washed with NaCl (0.15 M at 1 wt% acetic acid, pH 5.5) for 10 min. The changes in dissipation (ΔD) and frequency (ΔF) were monitored in real-time. The thickness of the chitosan-FITC and alginate coating on (Chi/Alg)SPs was also characterised using a confocal laser-scanning microscope (Leica, Germany) equipped with an argon laser. Fourier transform infrared (FTIR) spectra of SPs and (Chi/Alg)SPs were recorded on an FTIR spectrophotometer (Bruker, Australia). The mass of the chitosan/alginate coating was monitored by thermogravimetric analysis (TGA) (Netzsch, Australia). Briefly, SPs and (Chi/Alg)SPs were placed on separate crucibles and then loaded into the TGA instrument where the temperature was increased to 900 °C at a heating rate of 10 K/min.

Radiolabelling of BDNF and NT3. Neurotrophin-3 (NT3; Peprotech) was labelled with ^{125}I as reported previously [29]. Unbounded (free) ^{125}I was separated from the NT3 bound ^{125}I using Bio-Gel P-6DG columns (Bio-Rad). The ^{125}I labelled NT3 was transferred in phosphate buffer (including 0.25 wt% BSA and 0.1 wt% sodium azide), then purified using an Amicon Ultra-4 10K filtration unit. The specific activity of the ^{125}I NT3 was within the range of 39 to 51.6 µCi/µg, with 73 to 93% incorporation. The desired concentration of ^{125}I NT3 (1 mg/ mL in Milli-Q water) was adjusted by mixing unlabelled NT3 and ^{125}I NT3 with the volume ratio 1:1. We previously showed using western blots and an auditory nerve survival assay that iodination of NT3 does not impact the integrity and bioactivity of the protein before and after loading the protein into SPs [24, 30].

In Vitro Loading and Release Studies. For SP characterisation studies using only the native (unlabelled) protein, 40 µL of 1.0 mg/mL NT3 or BDNF was mixed with four sterilised SPs. For SP characterisation studies using radiolabelled NT3 (^{125}I NT3), 14 µL of ^{125}I NT3 (stock 1.1-5 µg/ml) was mixed with 14 µL of 2.0 mg/mL NT3 in a total volume of 28 µL with four sterilised SPs. After incubation for 3 days, the supernatant was removed, and the SPs were placed in separate 1.7 mL microcentrifuge tubes (one SP per tube). ^{125}I NT3 loaded SPs were coated with chitosan and alginate as described above to monitor the release from (Chi/Alg)SPs. Then, 100 µL of phosphate buffered saline (PBS; pH 7.4) for NT3 - loaded (Chi/Alg)SPs, artificial perilymph without glucose for ^{125}I NT3 loaded (Chi/Alg)SPs was added to each tube and incubated at 37 °C. At defined time intervals (3, 7, 14, 21, 28 days), 95 µL of the supernatant was collected and replaced with 95 µL of fresh PBS. The amount of ^{125}I NT3 loading and released from SPs and (Chi/Alg)SPs were quantified using a Perkin Elmer WIZARD automatic gamma counter with 1 min measurement time. The amount of BDNF loading and released from SPs and (Chi/Alg)SPs were quantified using a Human BDNF ELISA kit (Abcam) according to manufacturer's instructions. Briefly, 50 µL of diluted BDNF loading or released samples was added to the pre-coated ELISA plates followed by adding 50 µL of the antibody cocktail into each well. After 1 h incubation at room temperature, the plate was washed three times with 1x wash buffer. Finally, 100 µL of TMB substrate was added for color development for 10 min followed by adding 100 µL of TMB stop solution to stop the reaction. The concentration of BDNF was quantified using a Microplate reader with the 450 nm absorbance wavelength.

The amount of NT3 loading and released from SPs and (Chi/Alg)SPs were quantified using a NT3 Human ELISA kit (Thermofisher Scientific) according to manufacturer's instructions. Briefly, 100 µL of diluted NT3 loading or released samples was added to the pre-coated ELISA plates for 2.5 h incubation at room temperature. Then, the solution was discarded and washed four times with 1x wash buffer. Subsequently, 100 µL of diluted biotin conjugate was added and incubated for 1 h at room temperature. Then, the solution was discarded and washed four times with 1x wash buffer. After that, 100 µL of diluted Streptavidin-HRP was added followed by 45 min incubation at room temperature. Finally, 100 µL of TMB substrate was added for color development for 30 min followed by adding 50 µL of TMB stop solution to stop the reaction. The concentration of NT3 was quantified using a Microplate reader with the 450 nm absorbance wavelength.

Experimental animals. In this study, the use of cats and guinea pigs were approved by the St Vincent's Hospital (Melbourne) Animal Ethics Committee (18-393AB; Table 1). The study follows the Guidelines to Promote the Wellbeing of Animals used Scientific Purposes (2013), the Prevention of Cruelty to Animals Amendment Act (2015) and the NHMRC Code for Care and Use of Animals for Scientific purposes (2013).

Table 1: Study groups: Number of guinea pigs and cats used for each experimental condition

	Guinea pigs				**Cats**			
	Fibrin	N=	Timepoints	RW/IC	Fibrin	N=	Timepoints	RW/IC
SPs	✗	Data published (Gunewardene et al., 2022)			✓	N=4	2 weeks	RW
					✓	N=1	4 weeks	IC
SPs + HA	✓	N=1	4 hr	RW				
	✓	N=4	3D	RW				
(Chi/Alg)SPs	✓	N=4	2 weeks	RW	✓	N=1	4 weeks	IC
	✓	N=6	4 weeks	RW	✓	N=1	6 weeks	IC
					✓	N=1	8 weeks	IC
					✓	N=1	4 weeks	RW
					✓	N=1	6 weeks	RW
					✓	N=2	8 weeks	RW

Neurotrophin loading and SP coating for in vivo studies. Each SP was loaded in a solution containing 7 µg of NT3 per SP over a period of 3 days at room temperature. Neurotrophin stocks were prepared by diluting 100 µg of NT3 with 50 µl of Milli Q water to obtain a stock concentration of 2 mg/ml. ^{125}I NT3 was added to the native (unlabelled) NT3 at a 1:1 volume ratio. For the round window groups, SPs were loaded in batches of 5 SPs in a solution containing 17.5 µl of NT3 (stock 2 mg/ml) and ^{125}I NT3 (stock 1.1-5 µg/ml) in a total volume of 35 µl. For the intracochlear groups, SPs were loaded in batches of 6 SPs in a solution containing 21 µl of NT3 (stock 2 mg/ml) and 1.1-5 µg/ml of ^{125}I NT3 in a total volume of 42 µl. After the 3-day incubation with neurotrophin, the supernatant was removed, and the ^{125}I NT3 loaded SPs were coated with with two bilayers of 0.5 wt% chitosan and alginate as described above. Then, the (Chi/Alg)SPs were washed three times with Milli-Q water and gamma measurements taken with the (Chi/Alg)SPs in Milli-Q water to determine neurotrophin loading per SP.

Implantation of supraparticles to guinea pigs and cats. Before the surgery, the (Chi/Alg)SPs were embedded in 10 µL of clinically approved gelation system (Tisseel, Baxter) consisting of thrombin and fibronectin. The gel (called "fibrin sealant") can form a small pellet containing the loaded (Chi/Alg) SPs, which can be easily placed on the RWM to secure the fibrin sealant in place by a surgical applicator. Both the guinea pigs and cats were bilaterally implanted with SPs or (Chi/Alg)SPs.

Guinea pig implantation- The guinea pigs were anaesthetised with isoflurane. The animal's vitals were carefully monitored and documented throughout the duration of the surgery and temperature maintained within a normal range using a heat blanket. A post-auricular incision was used to expose the bulla. For the RWM surgeries, a hole was made in the bulla to expose the round window and 4-5 SPs were placed on the membrane, followed by sealing the bulla with dental cement. For the hyaluronic acid (HA) experiments, 4 mg/ml HA (hyaluronic acid sodium salt from rooster comb; Sigma H5388; Sigma, St. Louis, MO) was diluted in sterile saline. The concentration of HA used was based on previous cochlear *in vivo* studies [31]. The HA treated cochlea was randomly selected across the animals – right versus left ear (the contralateral ear was used as the control). Gelfoam was immersed in HA and then used to coat the membrane for up to 30 minutes prior to placement of the SPs on the RWM.

Cat implantation - Prior to SP administration, the cats were anesthetised with a combination of Ketamine (5-10mg/kg) and Medetomidine (10µg/kg) intramuscularly. The anaesthesia was then maintained with a mixture of inhalant isoflurane and oxygen. The animal's vitals were carefully monitored and documented throughout the duration of the surgery and temperature maintained within a normal range using a heat blanket. A post-auricular incision was performed to separate underlying muscle on the ventral bulla, followed by a #14 diamond burr to open the bulla, exposing the basal turn of the cochlea. After visualisation of the basal turn of the cochlea, 30 loaded (Chi/Alg) SPs or SPs were embedded in fibrin sealant and placed onto RWM, followed by sealing of the bulla with dental cement. For intracochlear injection, six (Chi/Alg) SPs embedded in fibrin sealant were injected into the basal turn of the cochlea through a small incision made in the RWM. A small piece of muscle was placed over the RWM incision and the bulla was closed with dental cement.

Tissue extraction and histology. At the conclusion of the treatment period, the guinea pigs and cats were and intracardially perfused with 0.9% NaCl (37 ◦C) followed by 10% Neutral Buffered Formalin (10% NBF; 4 ◦C). The cochleae from both the guinea pigs and cats were dissected for gamma count

measurement. Organs including brain, liver, intestine, kidney, and semi-circular canal were also separated for the measurement of radioactivity to assess off-target activity. Following gamma count measurements, the cochleae were 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 1.5 weeks of decalcification for guinea pigs and 2 weeks for cats, the cochleae were cryoprotected in 15% and 30% sucrose solution. They were then oriented and frozen in Tissue-Tek O.C.T. cryosectioning compound (Sakura, Japan). The cochleae were sectioned at 12 μm using a CM 1900 UV cryostat (Leica, Germany) at − 22 ◦C in the modiolar plane and mounted onto Superfrost-Plus slides (Menzel-Glaser, Braunschweig, Germany). The slides were stored at -20°C until ready for autoradiography analyses.

Autoradiography and imaging. We used two autoradiography analysis methods- film and emulsion techniques to assess the drug distribution in the cochlea. The specifics of the analyses were previously published [24, 25]. Briefly for the film analysis, standards were prepared to obtain a NT3 concentration gradient. They were prepared using Whatman filter paper disks (5 mm diameter), with each disk numbered from 1 to 10. A 1:2 serial dilution of ^{125}I NT3 was prepared, and each disk treated with 5 μL of the sample, disk 1was the most concentrated. The slides containing cochlear sections 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) and 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. The freehand tool was used to draw insets around 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 obtained. The background levels were subtracted from measurements obtained at the regions of interest.

Physiology testing (Auditory brainstem responses). Auditory brainstem responses (ABRs) were recorded prior to surgical implantation of the SPs using techniques described previously (ref). Auditory thresholds were measured for tone pip acoustic stimuli at a range of frequencies (1kHz to 24kHz) and intensities (0 dB to 100 dB sound pressure level).

Data analysis. The amount of NT3 loaded and eluted by the SPs is expressed respectively as a percentage of the concentration of the loading solution and percentage of the loaded NT3 into the SPs. The amount of ^{125}I NT3 retained in the cochlea is expressed as a percentage of the dose injected (e.g., the amount of NT loaded into the SPs). The data is presented as individual points or as means ± standard deviation or error of mean. 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 tests as appropriate. Autoradiographic film analysis was performed in ImageJ with MATLAB used for image visualisation. The emulsion coated slides were analysed using ImageJ with background subtracted and data expressed as normalized relative integrated density [15]. The specifics of the analyses were previously published [24, 25].

Minimum Information Reporting in Bio–Nano Experimental Literature (MIRIBEL). The studies conducted herein, including material characterisation, biological characterisation and experimental details, conform to the MIRIBEL reporting standard for bio–nano research [32], and include a companion checklist of these components in the Supporting Information.

4. RESULTS

Characterisation of the coated (Chi/Alg)SPs

To control the release of loaded protein from supraparticles, SPs were coated with alternating layers of chitosan and alginate. Chitosan and alginate are two polysaccharides used previously in layer-by-layer applications due to their biocompatibility and electrostatically interacting properties. The carboxyl group from alginate gives it a net negative charge, which allows interaction with the amine groups on positively charged chitosan [33]. Figure 1a demonstrates the formulation of (Chi/Alg)SPs. The surface structure of SPs following layer-by-layer coating with chitosan and alginate was characterized by scanning electron microscopy (SEM). At low resolution, the morphology of the SPs and (Chi/Alg)SPs appear similar (Figure 1b and c), but at higher magnification, distinct surface morphology differences can be observed (Figure 1d and e), indicative of the successful coating of the SPs with the chitosan/alginate film. SEM-EDX elemental mapping confirmed the presence of silicon (Si), oxygen (O), calcium (Ca) and nitrogen (N) uniformly distributed on the surface of (Chi/Alg)SPs (Figure 1f).

Fourier transform infrared (FTIR) spectroscopy of the uncoated SPs confirmed the presence of Si–O–Si bonds with stretching vibrations of Si–O–Si observed at 1065 cm^{-1}, bending vibrations of O–Si–O observed at 801 cm^{-1} and rocking vibrations of Si–O–Si observed at 452 cm^{-1} (Figure 1g, black curve). The coated (Chi/Alg)SPs displayed a comparable FTIR spectrum (Figure 1g, blue curve). The additional transmittance band appearing at 3355 cm^{-1} corresponds to the stretching vibration of O–H. The peak at 1600 cm^{-1} is assigned to the bending of N–H. The peak at 1408 cm^{-1} corresponds to the C–N stretching mode of the acrylamide group.

Quartz crystal microgravimetry with dissipation (QCM-D) was performed to monitor the chitosan and alginate build-up. QCM-D measures the change in frequency of an oscillating piezoelectric crystal, with a decrease in frequency indicating surface deposition. Multilayers of chitosan and alginate were deposited on a clean QCM substrate and the stepwise decrease in the frequency (ΔF) monitored by QCM-D indicated the gradual growth of a chitosan/alginate multilayer film (Figure 1h). The dissipation (ΔD) values increased with time, suggesting that the chitosan and alginate coating is soft, not rigid [34].

To quantify the mass of chitosan/alginate coating deposited, thermogravimetric analysis (TGA) was performed. Chitosan and alginate are two organic components that decompose at high temperatures. Thermogravimetric analysis (TGA) showed that one bilayer coating of chitosan/alginate accounted for ~4% of the weight (~2 µg considering the weight of each SP is ~0.05 mg) of one SP (Figure 1i). In addition, SEM images of (Chi/Alg)SPs cross-sections (Figure S1) and confocal laser scanning microscopy images (Figure S2) of fluorescein isothiocyanate (FITC) labelled chitosan and alginate coated (Chi/Alg)SPs also confirmed the presence of a chitosan/alginate coating on the surface of SPs but not within the supraparticles.

Neurotrophin loading and elution profile of the coated (Chi/Alg)SPs in vitro

To evaluate the release profile of neurotrophins, native brain derived growth factor (BDNF) and NT3 were loaded into the SPs followed by coating with 0.5 wt% of chitosan and alginate with a bi-layer coating. The concentration of the loading and released neurotrophins was characterized by ELISA. The results showed that there was an average of ~6.75 µg of BDNF and ~7.39 µg of NT3 loaded in each SP. Figure 1j and k show that the *in vitro* neurotrophins release can be extended to ~28 days with an average of ~49% of BDNF and 52% of NT3 release from (Chi/Alg)SPs compared to an average of ~63% of BDNF and ~54% of NT3 released from SPs over 28 days. Notably, there was only an average of ~18% of BDNF and ~21% NT3 released from (Chi/Alg)SPs, which is lower than an

average of ~48% of BDNF and ~35% of NT3 released in the first 7 days, which further indicated that the coating was able to extend the release profile of the SPs.

A radiolabelling NT3 technique, previously used to characterize the pharmacokinetics and distribution of NT3 in the cochlea [29, 30], was employed to characterize the loading and release *in vitro* NT3. During loading and coating, the percentage of the radioactivity detected in the washing solution, coating solution and the loading tube was low (<10%), which suggests minimal drug loss during the post-coating steps (Figure S3). Before the coating of chitosan and alginate, the ^{125}I NT3 was first loaded into uncoated SPs with 5 µg, which was ~70% of the total ^{125}I NT3 loading amount (Figure 1l, m). For the *in vivo* experiments, we used a clinically approved fibrin sealant (Tisseel, Baxter, USA) that permits the SPs to be encapsulated within a gel matrix for ease of placement of multiple SPs on the RWM of the cochlea or intracochlear implantation. The percentage cumulative release of ^{125}I NT3 from uncoated SPs and (Chi/Alg)SPs in fibrin sealant experiment was performed in artificial perilymph without glucose (Figure 1n). A linear release profile was observed from (Chi/Alg)SPs in fibrin sealant compared to the SPs in fibrin sealant. In addition, for uncoated SPs in fibrin sealant, a cumulative release of ~43% of the loaded ^{125}I NT3 was observed in the first 7 days. The coated (Chi/Alg)SPs in fibrin sealant had a significantly lower cumulative drug release profile in the first 7 days, with only ~12%of ^{125}I NT3 released ($p < 0.001$ from unpaired T test). After 28 days, there was an average of ~49% of ^{125}I NT3 released from (Chi/Alg)SPs in fibrin sealant, which was lower, but not statistically significant than the uncoated SPs in fibrin sealant with an average of ~62% of ^{125}I NT3 released over 28 days ($p = 0.14$ from unpaired T test). These results indicate that chitosan, alginate and fibrin form a firm matrix that restricts the burst-release drug profile observed in standard SPs, thus supporting a more linear and sustained drug release profile that can extend the potential treatment duration using this technology.

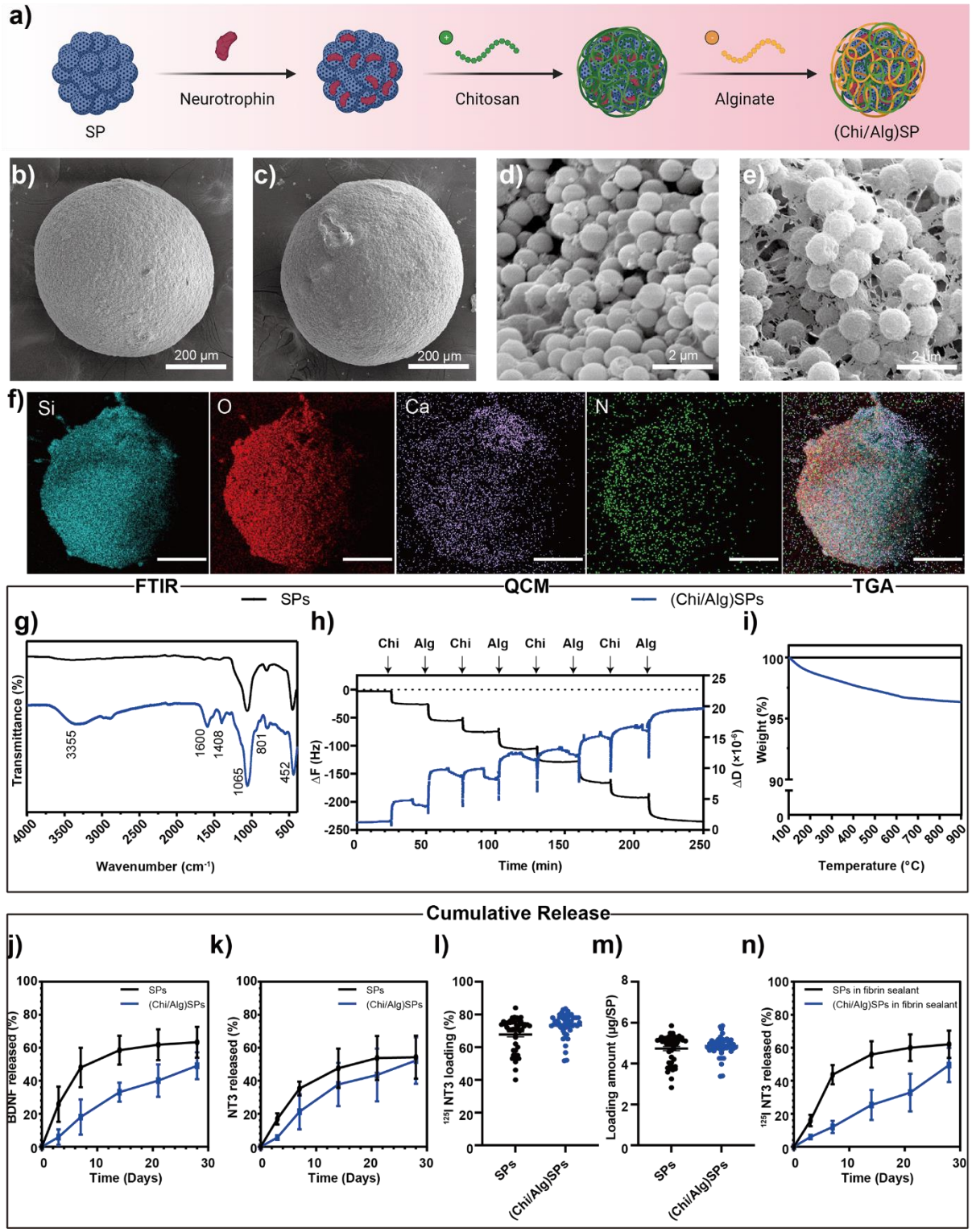


Figure 1: (a) Schematic illustration of the formulation of (Chi/Alg)SPs. SEM image of a (b) SP and (c) (Chi/Alg)SP. High-resolution SEM image displaying the surface of (d) a SP and (e) a (Chi/Alg)SP. (f) Elemental distribution of a (Chi/Alg)SP. Scale bars = 200 µm (g) FTIR spectra of SPs and (Chi/Alg)SPs. (h) Frequency and dissipation changes monitored by QCM-D for the layer-by-layer assembly of chitosan and alginate. (i) Thermogravimetric analysis (TGA) of SPs and (Chi/Alg)SPs showing the weight percentage of chitosan/alginate bi-layer coating. (j) *In vitro* cumulative release profiles of (j) BDNF and (k) NT3 from SPs and (Chi/Alg)SPs. ^{125}I NT3 loading (l)

percentage and (m) amount in SPs and (Chi/Alg)SPs. (n) *In vitro* cumulative release profiles of ^{125}I NT3 from SPs and (Chi/Alg)SPs in fibrin sealant.

Pharmacokinetic properties of (Chi/Alg)SPs and SPs after RWM delivery in guinea pigs

A cohort of guinea pigs were bilaterally implanted with (Chi/Alg)SPs incorporating the fibrin sealant. The animals were implanted with five ^{125}I NT3 loaded (Chi/Alg)SPs on the RWM. The animals were terminated after 14 (n=4) and 28 (n=6) days post-surgery. Whole-cochlear gamma counts were obtained to measure the amount of NT3 that diffused into the cochlea as well as the amount remaining in the SP. Here, it needs to be noted that whole cochlear gamma count measurements reveal the total amount of drug available, both on the RWM (outside the cochlea) and inside the cochlea. Autoradiography analyses of cochlear sections were then performed to assess intracochlear drug biodistribution.

Whole-cochlear gamma counts revealed that the amount of NT3 retained in both the cochlea and (Chi/Alg)SPs present on the RWM at 2 weeks averaged 0.4 μg ± 0.16 μg (~2.7% of the loaded NT3), and at 4 weeks it was 0.38 μg ± 0.17 μg, (~2.1% of the loaded NT3; Figure 2a, b). Despite the slower release profile of (Chi/Alg)SPs, this level of NT3 retention in the cochlea is significantly lower compared to our previously published data using uncoated SPs, in which NT3 retention in both the cochlea and particles averaged at 2 weeks was 4.3 ± 1.1 μg (~16.6% of the loaded NT3) and at 4 weeks was 2.3 ± 0.2 μg, (~5.8% of the loaded NT3; [24]) ($p < 0.0001$ at both time points analysed, n=4 per timepoint; 2-way ANOVA with Tukey's multiple comparison test).

Autoradiography analyses revealed a strong ^{125}I signal from (Chi/Alg)SPs within the round window niche external to the cochlea (Figure 2c), but also on internal structures of the cochlea, indicating low-level diffusion of the NT3 through the RWM. Comparison of the ^{125}I integrated densities between the basal turn segments indicated that the diffusion of NT3 did not extend beyond the lower section of the basal turn. However, a clear ^{125}I signal was detected in the basilar membrane, situated just below the hair cells after treatment with both the SPs and (Chi/Alg)SPs (Figure 2d). For the (Chi/Alg)SPs, the ^{125}I signal in the basilar membrane of the lower basal turn of the cochlea was 4-fold higher at 2 weeks, than the integrated density after 4 weeks of implantation, but this was not statistically significant (14D- 0.03 ± 0.025 and 28D- 0.007 ± 0.005 a.u; T test; p=0.3; Figure 2e). In comparison to our previously published data using SPs, there was no statistically significant difference in the amount of signal on the basilar membrane with the (Chi/Alg)SPs (p=0.99 at 2 weeks and p=0.99 at 4 weeks, 2-way ANOVA with Tukey's multiple comparison test , n=4 per timepoint; [24]).

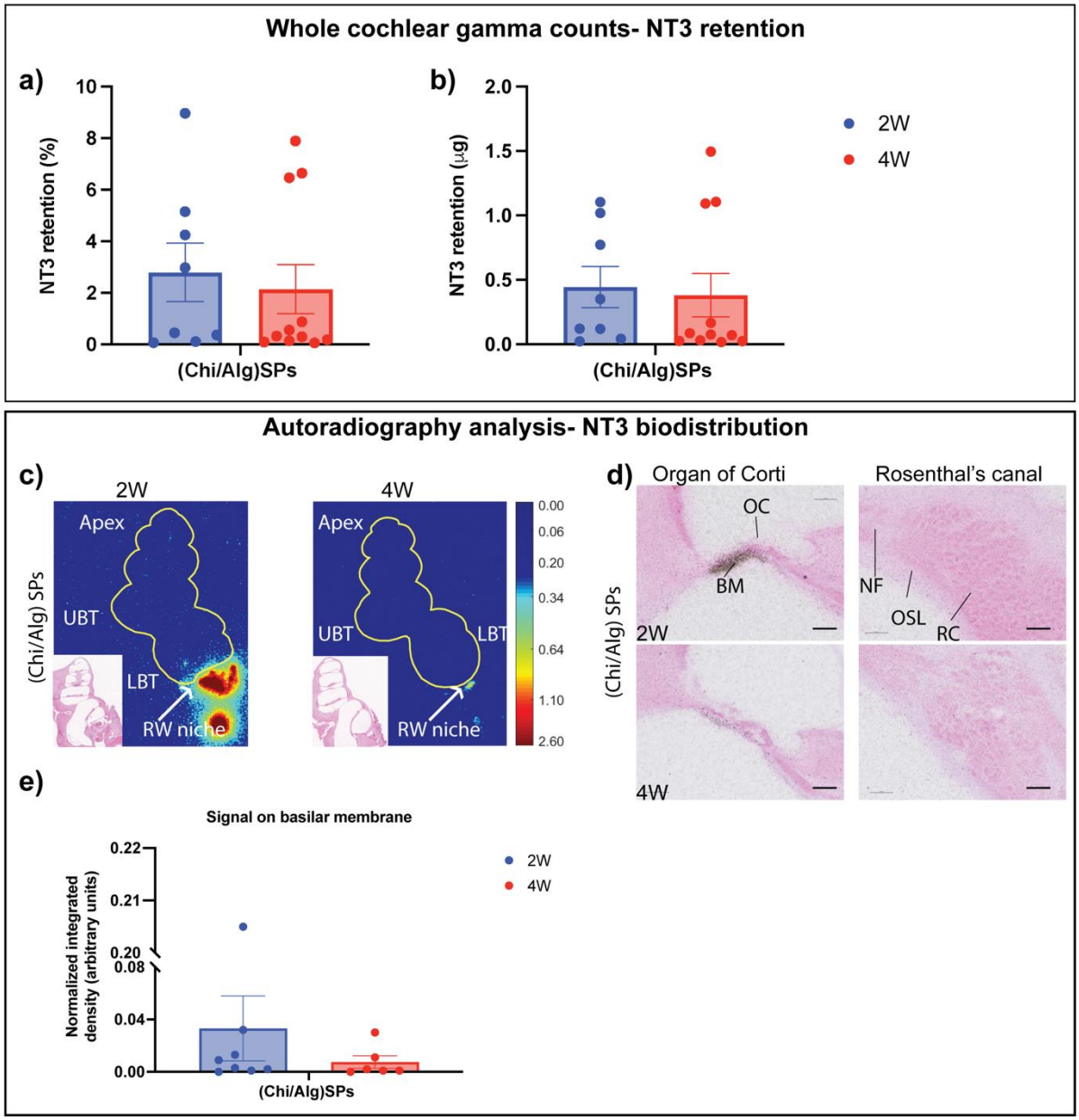


Figure 2: Guinea pig implantations-retention of NT3 after delivery of (Chi/Alg)SPs on to the RWM. The percentage (a) and amount of NT3 (b) retained was calculated from the gamma counts of the administered (Chi/Alg)SPs and whole cochleae post-surgery. (c) Film-based analysis of whole-cochlear sections with MATLAB image processing showed strong ^{125}I signal in (Chi/Alg)SPs within the RWM niche. Insets show H&E stained sections of the processed images. (d) High magnification images of H&E stained autoradiographs of the cochlear lower basal turn showing representative images of the organ of Corti region (OC) and Rosenthal's canal (RC). Scale bars = 50 µm. Autoradiography analyses of ^{125}I signal density in the (**e**) basilar membrane at day 14 and 28. Data presented as mean ± standard errors of mean (SEM). Day 14 (n=4) and 28 (n=6; 12 cochleae).

Pharmacokinetic properties of (Chi/Alg)SPs after RWM delivery in cats

The properties of the RWM differ between species, in terms of their thickness and permeability [4, 7]. Therefore, we also tested the use of (Chi/Alg)SPs in cats as the larger cat cochlea is more anatomically similar to the human cochlea than rodent cochlea. It has a similar RWM thickness [9] and cross sectional area in the basal turn (2.0mm^2) as the human cochlea [35]. Here, the animals were

bilaterally implanted with 30x ^{125}I NT3 loaded (Chi/Alg)SPs via the RWM approach (Figure 3a). The animals were terminated after 4-, 6- and 8-weeks post-surgery. We also included an additional cohort of cats implanted with ^{125}I NT3 loaded SPs that were not coated with Chi/Alg. These animals were terminated after 2 weeks. Of note, only a small cohort of cats were used in these experiments and limited statistical tests could be performed.

Whole-cochlear gamma counts revealed that after 4-, 6- and 8-weeks post-surgery of the (Chi/Alg)SPs, less than 1.3 ± 1.29 μg (<1% at 28 days) of the loaded NT3 was detected in the cochlea (Figure 3b, c). In comparison, cat implantations with the SPs had an average of 54 ± 11.4 μg, corresponding to 37% of drug remaining in the cochlea after the shorter 2-week treatment duration (Figure 3b, c).

Analysis of whole cochlear sections from cat cochleae revealed limited intracochlear spread of the drug. In some sections, a strong ^{125}I signal from (Chi/Alg)SPs was detected within the round window niche external to the cochlea (Figure 3d). Of note, in one cochlea treated with Chi/Alg)SPs for 8 weeks, a strong ^{125}I NT3 signal was detected at the RWM niche and a very weak signal at the basilar membrane.

Quantification of the ^{125}I integrated densities in the emulsion-stained cochlear sections revealed a small, but detectable signal in some treated cochleae throughout the regions of interest. However, with increased post-surgery time, a significant decline in the ^{125}I NT3 signal was observed.

In the basilar membrane, the ^{125}I signal in the lower basal turn after 8 weeks was 8-fold lower than the integrated density after 4 weeks with the (Chi/Alg)SPs (4W - 0.005 ± 0.004 and 8W - 0.0005 ± 0.0005 a.u; Figure 3d). There was also variability between the cochleae in terms of the levels of ^{125}I NT3 signal on the basilar membrane with the SPs after 2 weeks, the range of integrated densities was 0.001 to 0.018 ± 0.002 a.u (Figure 3d and e). We were unable to detect any signal in Rosenthal's canal in any of the cats analysed with both the SPs and (Chi/Alg)SPs. Collectively, the data suggests that while the SPs show better NT3 retention (compared to (Chi/Alg)SPs), significant variability in drug biodistribution is present with both the standard and coated particles *in vivo*.

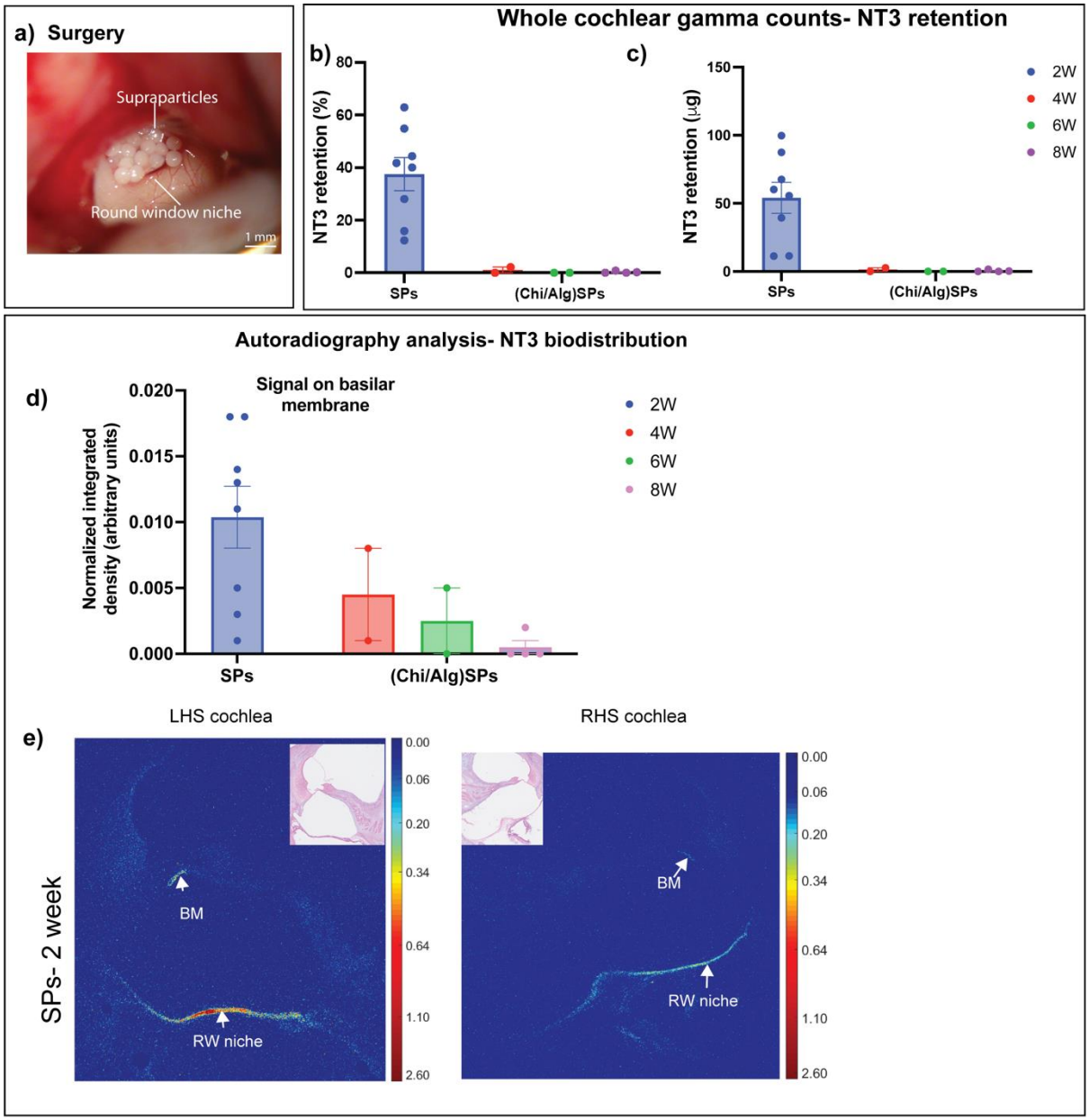


Figure 3: Cat implantations- retention of NT3 after delivery of (Chi/Alg)SPs on to the RWM. (a) Surgical image of the round window of a cat cochlea implanted with SPs. (b) The percentage and (c) amount of NT3 retained was calculated from the gamma counts of the administered (Chi/Alg)SPs and gamma counts of the whole cochleae post-surgery. Autoradiography analysis of ^{125}I signal density in the (d) basilar membrane at 2, 4, 6 and 8 weeks. (e) Film-based analysis of cochlea sections with MATLAB image processing showed strong ^{125}I signal in SPs within the RWM niche and basilar membrane. Insets show H&E stained sections of the processed images. Basilar membrane (BM). Data presented as mean ± standard errors of mean (SEM).

Pharmacokinetic properties of (Chi/Alg)SPs after intracochlear delivery in cats

Given the variable biodistribution of drug using the RWM approach, we tested (Chi/Alg)SPs using the intracochlear approach in cats in order to assess the capacity of SP for sustained delivery NT3 to the intracochlear environment. Six ^{125}I NT3 loaded (Chi/Alg)SPs were bilaterally implanted into the cochlear fluids of cat cochleae (Figure 4a). The animals were terminated after 4, 6 and 8 weeks. We

also bilaterally implanted one cat with ^{125}I NT3 loaded SPs (uncoated). This animal was terminated after 4 weeks.

With the (Chi/Alg)SPs, whole-cochlear gamma counts revealed that after 4 weeks, some ^{125}I NT3 was detectable in the cochlea, corresponding to 0.2 ± 0.19 µg (~0.66% at 4 weeks), however after 6 and 8 weeks post-implantation, the levels were much lower (Figure 4b and c). With the uncoated SPs, there was a slightly higher amount of ^{125}I NT3 remaining in the cochlea, corresponding to 0.9 ± 0.4 µg (~2.5% at 4 weeks).

Importantly, analysis of cochlear sections showed evidence of NT3 remaining in the cochlea after 8 weeks of intracochlear delivery of (Chi/Alg)SPs, albeit a weak ^{125}I signal on the basilar membrane (Figure 4d), while a weak ^{125}I signal was particularly evident in the basilar membrane at 4 weeks (Figure 4e). This result indicates that the SPs are able to deliver NT3 over a long duration (up to 2 months) when delivered to the intracochlear environment. Quantification of the ^{125}I integrated densities in the emulsion-stained cochlear sections revealed a decrease in the levels of ^{125}I NT3 with increased post-surgery time. In the basilar membrane, the ^{125}I signal in the lower basal turn after 8 weeks was 3-fold lower than the integrated density after 4 weeks (4 weeks - 0.1 ± 0.047 and 8 weeks - 0.004 ± 0.008 a.u; Figure 4f). With the SPs, the signal on the basilar membrane was weak, but still slightly higher than with the (Chi/Alg)SPs after 4 weeks (0.16 ± 0.027 a.u).

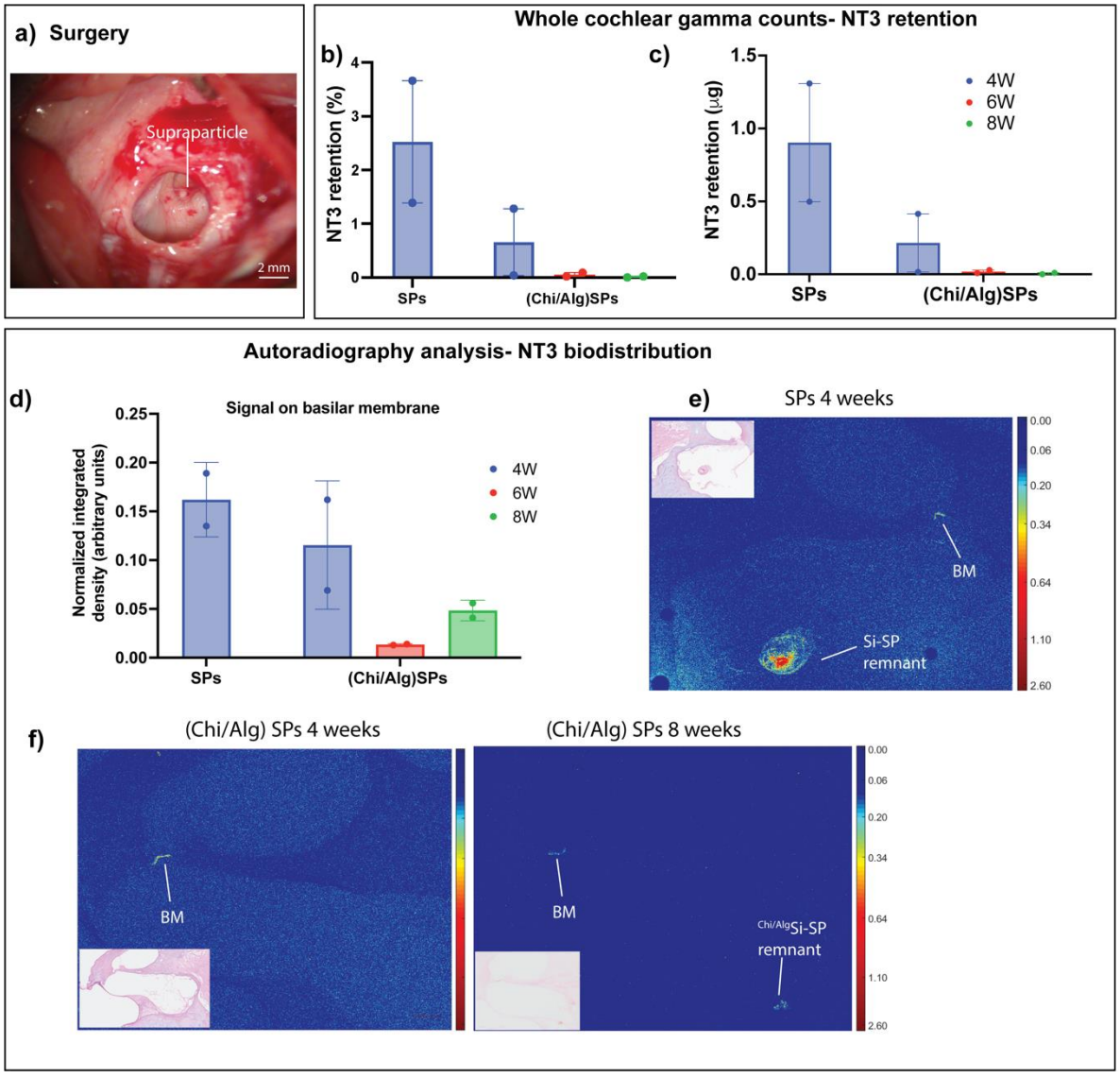


Figure 4: Cat implantations- retention of NT3 after intracochlear delivery of (Chi/Alg)SPs. (a) Surgical image of intracochlear implantation of the SPs via a small incision in the RWM. A SP can be observed on the inside of the RWM. (b) The percentage and (c) amount of NT3 retained was calculated from the gamma counts of the administered (Chi/Alg)SPs and gamma counts of the whole cochleae post-surgery. Autoradiography analyses of ^{125}I signal density in the (d) basilar membrane at 4, 6 and 8 weeks. (e) Film-based analysis of whole-cochlea sections with MATLAB image processing showed strong ^{125}I signal in SPs in the basilar membrane after 4 weeks. (f) For the (Chi/Alg)SPs, signal in the basilar membrane was detected at 4 and 8 weeks. Basilar membrane (BM). Data presented as mean ± standard error of mean (SEM).

Hearing function changes following implantation of (Chi/Alg)SPs in cats

To establish the safety of the surgical approach to deliver (Chi/Alg)SPs in fibrin sealant to the RWM as well as that of the intracochlear approach, hearing thresholds were measured before and after treatment. Auditory brainstem responses (ABRs) were recorded prior to surgical implantation of the SPs using techniques described previously. Auditory thresholds were measured for tone pip acoustic stimuli at a range of frequencies.

(Chi/Alg)SPs containing ^{125}I NT3 were delivered to the RWM (n=30 SPs in each cat cochlea). After 8 weeks, the ABRs was measured. The RWM approach had minimal impact on normal functional hearing (Figure 5). With the intracochlear delivery (n=6 SPs in each cat cochlea), post treatment thresholds were elevated in the high frequency region (≥ 12kHz) indicative of hearing loss (Figure 5).

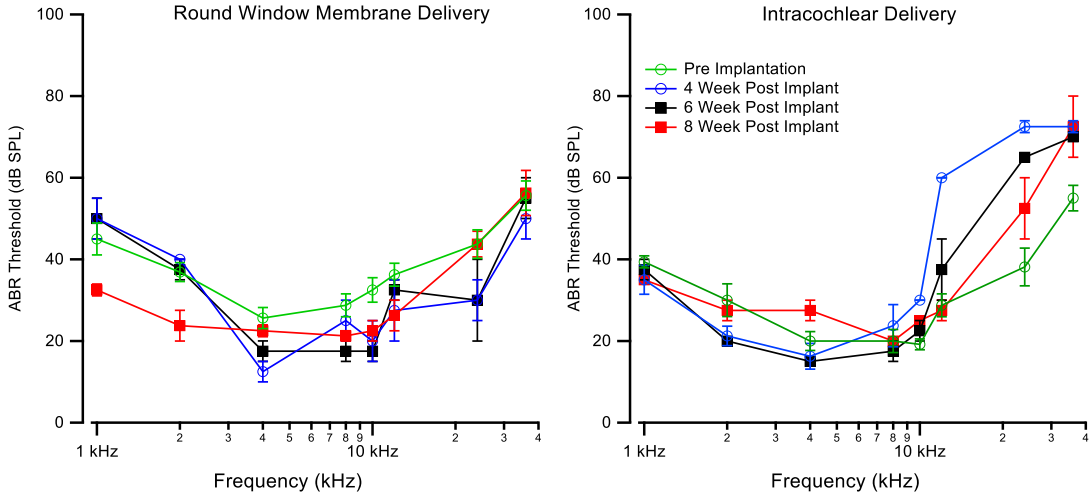


Figure 5. Cat auditory brainstem response (ABR) thresholds for tone pip acoustic stimuli for Round Window delivery and Intracochlear delivery. Average (±SEM) thresholds are shown prior to implantation and after 4-, 6- and 8-weeks post bilateral implantation of the ^{125}I NT3 loaded SPs. Round window, 4 (n=2), 6 (n=2) and 8 weeks (n=4 cochleae). Intracochlear, 4 (n=4), 6 (n=2) and 8 weeks (n=2 cochleae).

Impact of hyaluronic acid coating of the RWM in NT3 levels inside the guinea pig cochlea

Hyaluronic acid (HA) has previously shown to improve drug delivery across the RWM [31, 36]. We hypothesised that HA would increase RWM permeability and promote SP-released NT3 transfer into the cochlea. Therefore, we initially tested this approach in guinea pigs using standard SPs. To implant SPs onto the RWM, on one ear, HA was applied on the RWM for 30 minutes using gelfoam prior to the SPs (n= 4-5) being placed on the membrane. The contralateral ear was not treated with HA (SPs

only; control). The animals were terminated at 4 hours and 3-days post-implantation. Whole cochlear gamma counts were obtained to measure the total drug availability (inside the cochlea and external to the cochlea) at each time point. There was a decline in the amount of available NT3 after 3 days of treatment, averaging 72% (~21 µg) after 4 hours and 28% (~10 µg) at day 3 (Figure 6a and b). However, there was no statistically significant difference in the amount of available NT3 after treatment of the RWM with HA, compared to cochleae that were not pre-treated with HA (p=0.98, unpaired T test).

Autoradiography analysis of cochlear sections revealed a strong ^{125}I signal from the SPs on the basilar membrane (Fig 6d) indicating intracochlear delivery of NT3 following RWM placement of the NT3-SPs. To determine the extent of drug entry into the cochlea, we compared the radiolabel signal on the basilar membrane of the cochleae treated with the RW SPs and HA+RW SPs on day 3. There was no statistically significant difference in the amount of NT3 signal in the basilar membrane after HA treatment compared to no HA treatment (p=0.45, unpaired T test) indicating that HA was not effective in promoting NT3 transfer across the RWM.

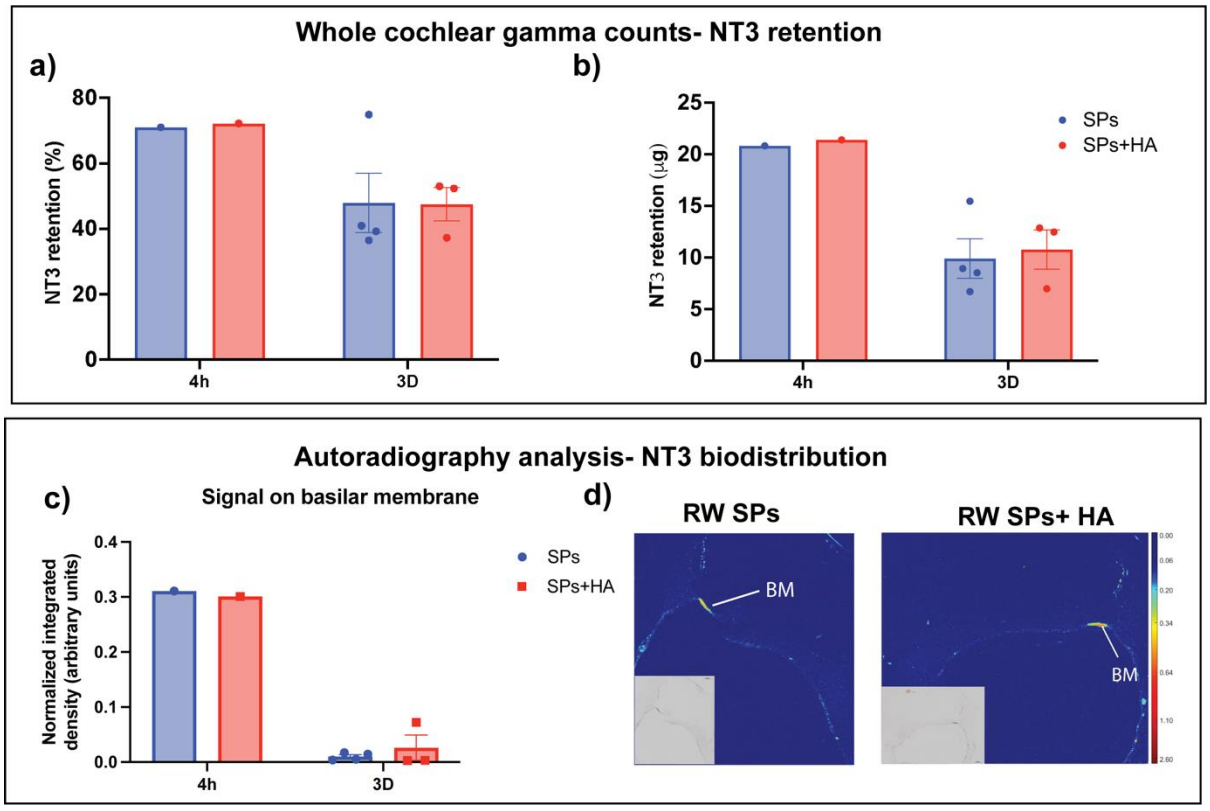


Figure 6: Impact of hyaluronic acid coating of the RWM in NT3 levels inside the guinea pig cochlea. (a) The percentage and (b) amount of NT3 retained was calculated from the gamma counts of the administered SPs and gamma counts of the whole cochleae post-surgery with and without hyaluronic acid (HA) treatment. The animals were bilaterally implanted- one ear with the RW SPs + HA and the other ear only the RW SPs (control) (c) Autoradiography analyses of ^{125}I signal density in the basilar membrane 4 hours post-treatment with and without HA. (d) High magnification images of H&E-stained cochlear lower basal turns showing representative images of the organ of Corti region (OC) containing the basilar membrane (BM) and Rosenthal's canal (RC) in the left (RW SP+HA-treated) and right ear (RW SP) of the same animal. Scale bars= 50 µm. Data presented as mean ± standard errors of mean (SEM). Day 1 (n=1) and 3 (n=4).

Table 2: Data summary of guinea pig and cat supraparticle RWM implantations. Round window membrane (RWM), basilar membrane (BM).

	Treatment	Timepoint	No. of cochleae	SP detected	Signal on RWM	Signal on BM	% of cochleae with signal on BM
Guinea pigs	SPs	4 hr	N=1	1/1	1/1	1/1	100%
		3D	N=4	4/4	4/4	2/4	50%
	SPs + HA	4 hr	N=1	1/1	1/1	1/1	100%
		3D	N=4	4/4	3/4	1/4	25%
	(Chi/Alg)SP	2 weeks	N=8	8/8	4/8	2/8	25%
		4 weeks	N=12	12/12	1/12	1/12	8.3%
Cats	SPs	2 weeks	N=8	8/8	7/8	5/8	62.5%
	(Chi/Alg)SP	4 weeks	N=2	2/2	1/2	1/2	50%
		6 weeks	N=2	0/2	0/2	0/2	0%
		8 weeks	N=4	2/4	1/4	1/4	25%

5. DISCUSSION

There is a need to improve intratympanic administration of neurotrophins to the cochlea as a treatment for hearing loss. The application of SPs holds promise as a vehicle for transferring high drug load capacities via the RWM, with maintained bioactivity over an extended period [24]. We previously showed that SP delivery of ^{125}I NT3 using the RWM approach led to some accumulation of NT3 within the RWM niche and the basal region of the guinea pig cochlea [24]. The signal was detected in the cochlea for up to one month. While these results were promising, further optimisation of the SP delivery approach could improve the levels of drug transfer across the RWM for clinical translation of this therapy. To improve the capacity to deliver NT3 over longer periods of time, in this study, we developed a coating technique that reduces NT3 elution over the first few weeks to extend the elution profile. We show using two animal species that the uncoated SPs and coated (Chi/Alg)SPs can be used to effective release NT3 for transfer across the RWM, but with low efficiency and variability between animals. Encouragingly, with the intracochlear approach, a weak ^{125}I NT3 signal was detected inside the cochlea even after 2 months post-treatment with (Chi/Alg)SPs, thus supporting the long term drug deliverability of the system.

The coated (Chi/Alg)SPs combined with fibrin sealant improved drug elution in vitro but not in vivo.

The coated (Chi/Alg)SPs reduced the burst-release profile of the SPs at the early elution timepoints. When combined with the fibrin sealant the (Chi/Alg)SPs had a sustained drug elution profile *in vitro*. To evaluate *in vitro* elution, the (Chi/Alg)SPs were immersed in PBS buffer with the buffer collected at various time points. Immersing the loaded SPs in PBS leads to disruption of the electrostatic interactions established between the positively charged neurotrophins and negatively charged silica SPs, prompting drug release *in vitro*. In both guinea pigs and cats, RWM implantation of (Chi/Alg)SPs led to a progressive decrease in ^{125}I NT3 levels, but still detectable at the latest timepoints analysed (0.38 µg for guinea pig cochlea after 4 weeks; ~2.1% and 0.46 µg for cat cochlea after 8 weeks; ~0.27%). Although this was promising, the uncoated SPs performed significantly better with the guinea pig cochlea retaining an average of ~2.3 µg (corresponding to ~5%) of drug after 4 weeks, while for the cats, an average of ~54 µg (corresponding to ~37%) of drug was remaining in the

cochlea after 2 weeks. These findings contrasted with our hypothesis whereby coated SPs were expected to exhibit greater NT3 retention over time therefore enabling improved drug delivery at later timepoints (weeks to months) than that achievable with uncoated SPs. While there are several obvious differences between *in vitro* and *in vivo* environments, another potential factor could be the chemistry of the coated SPs reducing capacity for the (Chi/Alg)SPs to interact with the RWM and/or increasing the propensity for immunoclearance of the (Chi/Alg)SPs from the middle ear [37]. Of note, previous studies that applied chitosan coated nanoparticles for cochlear viral transfection and neomycin delivery reported inconsistent transfection *in vitro* and *in vivo* [38, 39]. They attributed this variability to chitosan causing a transient increase in thickness of the RWM, impacting substance transfer [39]. We also have some preliminary data suggesting that the elution profile of NT3 *in vivo* is faster compared to elution *in vitro* (unpublished). Collectively, although chitosan is accepted as safe and effective for drug delivery in other systems, investigation of the mechanism underlying the discrepant results in the cochlea is required.

Given that there are many factors that contribute to the efficiency of NT3 delivery across the RWM (e.g. residence time, middle ear clearance, local tissue response, RWM permeability), we used intracochlear delivery to examine the ability of the (Chi/Alg)SPs to deliver drug over time without the influence of the above factors. In terms of clinical applicability, the intracochlear delivery route is most suited when the cochlea is already being accessed during cochlear implant surgery and NT3 can be applied to improve auditory neuron survival and patient outcomes [12-21]. We previously showed that intracochlear delivery of SPs carrying neurotrophin significantly improved auditory neuron survival in deafened guinea pigs [26], providing strong evidence that sufficient bioactive neurotrophin is released from the particles. We also showed that after intracochlear SPs implantation to guinea pigs, there was widespread drug distribution inside the cochlea, with an average of ~0.18 μg (corresponding to ~3.6%) of ^{125}I NT3 remaining after two weeks [24]. SPs implantation into cat cochleae led to accumulation of the ^{125}I NT3 signal at the basilar membrane of the lower basal turn, with up to ~0.9 μg (corresponding to ~2.5%) of drug remaining in the cochlea after four weeks. However, with the (Chi/Alg)SPs , there was much less NT3 within the cochlea at the same timepoint, nearly a 4.5 fold lower level of drug retained, with only ~0.2 μg (corresponding to ~0.66%) of ^{125}I NT3 detected. Nevertheless, in one of the cat cochleae treated with (Chi-Alg)SPs, accumulation of ^{125}I NT3 was detected on the basilar membrane even after 8 weeks indicating that the delivery system is capable of long-term delivery of a therapeutic molecule into the inner ear environment. Although only a small cohort of cats were used in this experiment this data indicates that there was no benefit of coating SPs with Chi/Alg to improve NT3 delivery into the cochlea over time. There are several potential reasons for the lower NT3 retention in (Chi/Alg)SPs *in vivo*. Firstly, the cochlear perilymph fluid is comprised of a complex chemical composition [40], that may interact with the (Chi/Alg)SPs and prompt its degradation. For instance, the presence of Ca^{2+} may react with the alginate and prompt erosion of the chitosan/alginate network. Secondly, flow of the perilymph fluid (albeit very slow) inside the cochlea may also replenish the ions the SPs are exposed to, thus further accelerating the eroding process of the (Chi/Alg) coating. Finally, the degradation of the (Chi/Alg)SPs is also influenced by properties of the polymers including molecular weight, viscosity, stiffness [41, 42].

To evaluate the functional changes of (Chi/Alg)SPs -mediated neurotrophin delivery to normal hearing cats, hearing function was tested. The delivery of SPs to the RWM had minimal effect on hearing function across the frequencies analysed. The intracochlear approach showed some hearing loss at the higher frequencies after surgery. The hearing loss was mostly likely caused by penetration of the RWM, surgical trauma from drilling the bulla and/or the presence of the SPs in the scala tympani. These findings reiterate the notion that the RWM approach is the safest route for drug

delivery and warrants further study into developing approaches to improve drug transfer across the RWM.

In our previous study, we showed that delivery of SPs using both the intracochlear and round window route promoted drug retention in the guinea pig cochlea even after one-month post-implantation [24]. In addition, we showed that the intracochlear delivery approach led to widespread drug distribution throughout the cochlea. While there was some variability in drug distribution with the RWM approach, across nearly all the animals tested, ^{125}I NT3 was detected in the basal region of the cochlea and within the RWM niche. Although the use of biocompatible coatings was effective in modifying drug release characteristics in *in vitro* elution studies, this was not observed when tested *in vivo*. These results highlight the importance of *in vivo* validation studies to test drug pharmacokinetics, whereby a more complex biological environment can have an impact. Nevertheless, we conclude based on our previous and current findings that the SPs are a viable approach to effectively deliver neurotrophins to the cochlea for extended durations.

No statistically significant difference in ^{125}I NT3 distribution after acute treatment of the RWM with HA and SPs.

HA is a viscous, high-molecular-weight polysaccharide that is broadly expressed in epithelial, connective, and neural tissue. It is approved by the United States Food and Drug Administration for use in cosmetic and ophthalmic surgeries or as a substrate for drug delivery. In the cochlea, both pre-clinical and clinical data indicate that intratympanic administration of steroids such as dexamethasone with HA produces a higher concentration of drug in the perilymph and restores auditory function (compared to dexamethasone alone; [43, 44]). The mechanism of HA activity in the cochlea is unknown, but it has been hypothesised that it increases the osmotic pressure above the RWM, improves membrane permeability, and/or promotes long-term contact of a drug/substance to the RWM due to its high viscosity promoting drug crossing across the RWM [31].

Given the clinical relevance of HA, we tested the potential for HA to improve the permeability of SP released NT3 across the guinea pig RWM. Here, the membrane was acutely treated with HA using gelfoam (~30 min) prior to SP implantation and analysed 4 hours and 3 days post-treatment. However, we found that there was no statistically significant difference in the retention of ^{125}I NT3 inside the cochlea after 3 days based on the whole cochlear gamma counts and autoradiography analyses. Other approaches to partially digest and permeabilize the RWM include chemicals such as sodium carprate [31, 45, 46], ultrasound microbubble disruption [47], creation of a micro-perforations [48], and/or incorporation of the Si-SPs in hydrogels to improve retention on the RWM [49]. Testing these approaches to improve diffusion of SP-released NT3 across the RWM warrants further investigation.

Understanding the mechanisms of drug transfer across the RWM is needed to advance clinical translation of hearing therapeutics

Several factors may have contributed to the low permeability of the RMW to ^{125}I NT3. In this study, the outer layer of the RWM is directly exposed to the SPs and the released ^{125}I NT3. The first factor that may have impacted drug transfer is the residence time of the SPs on the RWM [50]. Our data suggests that residence time may be a factor related to the level of drug inside the cochlea. In cases where there was an absence of SPs located on the RWM at the time of termination there was a higher propensity for the intracochlear signal to be reduced or absent (Table 2). Secondly, considerable evidence has revealed that the modality of transfer across the RWM varies based on the drug or substance placed, which remains unknown for the SPs and NT3. For example, the molecule may diffuse through the cytoplasm of the cells of the RWM (e.g., exotoxin), transverse via pinocytotic vesicles (e.g. cationic ferritin) or in some cases enter through different channels in between cells in

the outer epithelium [7, 8]. As the drug enters the second layer of the RWM, there is a possibility that the residing immune cells may phagocytize the substance and/or cause the drug to penetrate blood or lymph vessels in this layer before entering the perilymph contributing to the variable outcomes between animals. Thirdly, permeability of the RWM to a molecule may be impacted by the size, configuration, concentration, liposolubility and electrical charge of the molecule. Of note, NT3 is a large molecule with a size of 27.3 kDa and a positive charge. Finally, the thickness and condition of the RWM also plays a key role in drug transfer. Of note, the thickness of the RWM varies between species, whereby for guinea pigs, the thickness 10-14 μm, for cats its 20-40 μm and for humans its ~70 μm [7-9]. There is also the potential that with age or progression of hearing loss, there are structural changes to the RWM, which may further impact the mechanics of drug transfer across the membrane [51]. Although relevant to the clinical population, this may not have been a contributing factor in this study as age-matched normal hearing animals were used. Therefore, the passage of substances through the RWM is not a mere mechanical passage through an inert three-layered wall but represents a more dynamic biological mechanism whereby every substance that interacts with the RWM is dealt with in a different manner. As such, delivery strategies that overcome the RWM barrier to achieve effective long term drug treatment are likely to progress the development of clinically effective therapeutic treatments for hearing impairment.

Given the complexity and multitude of factors impacting transfer of small molecules, macromolecules, and viruses across the RWM, the development of *in vitro* or *ex vivo* RWM models will help to improve our understanding of drug transfer across the RWM [52-55]. Of note, a recent study developed and characterised an easy-to-build ex vivo porcine RWM model [56]. The porcine RWM is a similar thickness and size to human. They demonstrated the mechanics of the passage of the non-salt form of dexamethasone (Dex), a drug known to be highly permeable across the RWM, and the low-permeability non-salt form of Dex conjugated with fluorescein (DexF). Furthermore, the impact of permeabilization of the RWM on DexF passage using saponin and collagenase was presented. We believe the integration of models such as the above combined with computational modelling and ultimately *in vivo* testing, will be paramount for studying and optimising the passage of SP-released NT3 through the RWM to the cochlea.

6. CONCLUSIONS

Intratympanic administration of a therapeutic drug is the most favoured route for drug delivery across the RWM and into the cochlea as there is minimal risk to residual hearing. We previously reported that silica supraparticles (SPs) are an efficient modality for the delivery of bioactive NT3 into the cochlea. However, the effectiveness of SPs in releasing drug reduces over time both *in vitro* and *in vivo*. In this study, we developed a novel coating strategy to extend the drug release capacity of SPs by reducing the amount of drug eluted within the first few weeks. While the coated particles significantly improved the longevity of drug elution *in vitro*, no benefit of the coated particles was observed inside the cochlea, with elution potentially occurring much faster *in vivo*. We also noted considerable variability between animals in the amount of drug entering the cochlea. This may be attributed to the permeability of the RWM being highly variable between animals with up to 10-fold differences in drug adsorption across the round window [57]. We believe there is a need to understand the interaction and mechanism of the SP released NT3 across the RWM. Current studies are underway to develop novel approaches to improve drug entry across the RWM using SPs and evaluate the functional outcomes of the treatment in deaf animal models.

7. CREDIT AUTHOR STATEMENT

Niliksha Gunewardene: conceptualisation, methodology, formal analysis, investigation, data curation, writing-original draft, visualisation. Yutian Ma: conceptualisation, methodology, formal analysis, investigation, data curation, writing-original draft, visualisation. Patrick Lam: conceptualisation, methodology, formal analysis, visualization. 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.

8. ACKNOWLEDGEMENTS

This work was funded with support from the Department of Defence USA, Hearing Restoration Research Program, Translational Research Award W81XWH1810276, The Passe and Williams Foundation, the Australian National Health and Medical Research Council (GNT 1142910). F.C. acknowledges the award of a National Health and Medical Research Council Leadership Fellowship (GNT2016732). The Bionics Institute acknowledges the support it receives from the Victorian Government, Australia, through its Operational Infrastructure Support Program. The authors would like to thank Trung Nguyen, Donna Tuari, James Firth, Sayward Barone, Brianna Flynn, Alex Thompson, Ella Trang and Caitlin Singleton from the Bionics Institute, Melbourne, Australia, for technical assistance, Patricia WM Ho from St Vincent's Institute, Melbourne, Australia for the use of the gamma counter and David Casley from ProSearch International, Melbourne, Australia, for technical assistance with protein radiolabelling. Zhixing Lin from University of Melbourne, Australia, for technical assistance with SEM-EDX.

9. DATA AVAILABILITY

Source data are available upon request from the corresponding author.

10. 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.

REFERENCES

[1] N. Inamura, A.N. Salt, Permeability changes of the blood-labyrinth barrier measured in vivo during experimental treatments, Hear Res, 61 (1992) 12-18.
[2] K. Mizutari, M. Fujioka, M. Hosoya, N. Bramhall, H.J. Okano, H. Okano, A.S. Edge, Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma, Neuron, 77 (2013) 58-69.
[3] L.P. Rybak, A. Dhukhwa, D. Mukherjea, V. Ramkumar, Local Drug Delivery for Prevention of Hearing Loss, Front Cell Neurosci, 13 (2019) 300.
[4] C.R. Anderson, C. Xie, M.P. Su, M. Garcia, H. Blackshaw, A.G.M. Schilder, Local Delivery of Therapeutics to the Inner Ear: The State of the Science, Front Cell Neurosci, 13 (2019) 418.
[5] A.A. Mikulec, S.K. Plontke, J.J. Hartsock, A.N. Salt, Entry of substances into perilymph through the bone of the otic capsule after intratympanic applications in guinea pigs: implications for local drug delivery in humans, Otol Neurotol, 30 (2009) 131-138.
[6] S.K. Plontke, R. Mynatt, R.M. Gill, S. Borgmann, A.N. Salt, Concentration gradient along the scala tympani after local application of gentamicin to the round window membrane, Laryngoscope, 117 (2007) 1191-1198.

[7] M.V. Goycoolea, Clinical aspects of round window membrane permeability under normal and pathological conditions, Acta Otolaryngol, 121 (2001) 437-447.
[8] M.V. Goycoolea, D. Muchow, G.C. Martinez, P.B. Aguila, H.G. Goycoolea, C.V. Goycoolea, P. Schachern, W. Knight, Permeability of the human round-window membrane to cationic ferritin, Arch Otolaryngol Head Neck Surg, 114 (1988) 1247-1251.
[9] M.V. Goycoolea, A.M. Carpenter, D. Muchow, Ultrastructural studies of the round-window membrane of the cat, Arch Otolaryngol Head Neck Surg, 113 (1987) 617-624.
[10] Y. Hamaguchi, T. Morizono, S.K. Juhn, Round window membrane permeability to human serum albumin in antigen-induced otitis media, Am J Otolaryngol, 9 (1988) 34-40.
[11] J. Suzuki, G. Corfas, M.C. Liberman, Round-window delivery of neurotrophin 3 regenerates cochlear synapses after acoustic overexposure, Sci Rep, 6 (2016) 24907.
[12] P. Ernfors, M.L. Duan, W.M. ElShamy, B. Canlon, Protection of auditory neurons from aminoglycoside toxicity by neurotrophin-3, Nat Med, 2 (1996) 463-467.
[13] S.L. McGuinness, R.K. Shepherd, Exogenous BDNF rescues rat spiral ganglion neurons in vivo, Otol Neurotol, 26 (2005) 1064-1072.
[14] A.K. Wise, R. Richardson, J. Hardman, G. Clark, S. O'Leary, Resprouting and survival of guinea pig cochlear neurons in response to the administration of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3, J Comp Neurol, 487 (2005) 147-165.
[15] A.J. Evans, B.C. Thompson, G.G. Wallace, R. Millard, S.J. O'Leary, G.M. Clark, R.K. Shepherd, R.T. Richardson, Promoting neurite outgrowth from spiral ganglion neuron explants using polypyrrole/BDNF-coated electrodes, J Biomed Mater Res A, 91 (2009) 241-250.
[16] L.N. Gillespie, G.M. Clark, P.L. Marzella, Delayed neurotrophin treatment supports auditory neuron survival in deaf guinea pigs, Neuroreport, 15 (2004) 1121-1125.
[17] T.G. Landry, A.K. Wise, J.B. Fallon, R.K. Shepherd, Spiral ganglion neuron survival and function in the deafened cochlea following chronic neurotrophic treatment, Hearing research, 282 (2011) 303-313.
[18] R.K. Shepherd, A. Coco, S.B. Epp, Neurotrophins and electrical stimulation for protection and repair of spiral ganglion neurons following sensorineural hearing loss, Hear Res, 242 (2008) 100-109.
[19] A.K. Wise, R. Richardson, J. Hardman, G. Clark, S. O'Leary, Resprouting and survival of guinea pig cochlear neurons in response to the administration of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3, The Journal of comparative neurology, 487 (2005) 147-165.
[20] R. Glueckert, M. Bitsche, J.M. Miller, Y. Zhu, D.M. Prieskorn, R.A. Altschuler, A. Schrott-Fischer, Deafferentation-associated changes in afferent and efferent processes in the guinea pig cochlea and afferent regeneration with chronic intrascalar brain-derived neurotrophic factor and acidic fibroblast growth factor, J Comp Neurol, 507 (2008) 1602-1621.
[21] P.A. Leake, G.T. Hradek, A.M. Hetherington, O. Stakhovskaya, Brain-derived neurotrophic factor promotes cochlear spiral ganglion cell survival and function in deafened, developing cats, The Journal of comparative neurology, 519 (2011) 1526-1545.
[22] R.T. Richardson, Q.Y. Hu, F. Shi, T. Nguyen, J.B. Fallon, B.O. Flynn, A.K. Wise, Pharmacokinetics and tissue distribution of neurotrophin 3 after intracochlear delivery, J Control Release, 299 (2019) 53-63.
[23] Y. Ma, A.K. Wise, R.K. Shepherd, R.T. Richardson, New molecular therapies for the treatment of hearing loss, Pharmacol Ther, 200 (2019) 190-209.
[24] N. Gunewardene, P. Lam, Y. Ma, F. Caruso, S. Wagstaff, R.T. Richardson, A.K. Wise, Pharmacokinetics and biodistribution of supraparticle-delivered neurotrophin 3 in the guinea pig cochlea, J Control Release, 342 (2022) 295-307.
[25] P. Lam, N. Gunewardene, Y. Ma, F. Caruso, T. Nguyen, B. Flynn, A.K. Wise, R.T. Richardson, A radiolabeled drug tracing method to study neurotrophin-3 retention and distribution in the cochlea after nano-based local delivery, MethodsX, 7 (2020) 101078.
[26] A.K. Wise, J. Tan, Y. Wang, F. Caruso, R.K. Shepherd, Improved Auditory Nerve Survival with Nanoengineered Supraparticles for Neurotrophin Delivery into the Deafened Cochlea, PLoS One, 11 (2016) e0164867.
[27] Y. Ma, M. Bjornmalm, A.K. Wise, C. Cortez-Jugo, E. Revalor, Y. Ju, O.M. Feeney, R.T. Richardson, E. Hanssen, R.K. Shepherd, C.J.H. Porter, F. Caruso, Gel-Mediated Electrospray

Assembly of Silica Supraparticles for Sustained Drug Delivery, ACS Appl Mater Interfaces, 10 (2018) 31019-31031.
[28] Y. Ma, C. Cortez-Jugo, J. Li, Z. Lin, R.T. Richardson, Y. Han, J. Zhou, M. Bjornmalm, O.M. Feeney, Q.Z. Zhong, C.J.H. Porter, A.K. Wise, F. Caruso, Engineering Biocoatings To Prolong Drug Release from Supraparticles, Biomacromolecules, 20 (2019) 3425-3434.
[29] R.T. Richardson, Q.-Y. Hu, F. Shi, T. Nguyen, J.B. Fallon, B.O. Flynn, A.K. Wise, Pharmacokinetics and tissue distribution of neurotrophin 3 after intracochlear delivery, Journal of Controlled Release, 299 (2019) 53-63.
[30] R.T. Richardson, A. Wise, S. O'Leary, J. Hardman, D. Casley, G. Clark, Tracing neurotrophin-3 diffusion and uptake in the guinea pig cochlea, Hear Res, 198 (2004) 25-35.
[31] S.B. Shibata, S.R. Cortez, J.A. Wiler, D.L. Swiderski, Y. Raphael, Hyaluronic acid enhances gene delivery into the cochlea, Hum Gene Ther, 23 (2012) 302-310.
[32] M. Faria, M. Björnmalm, K.J. Thurecht, S.J. Kent, R.G. Parton, M. Kavallaris, A.P. Johnston, J.J. Gooding, S.R. Corrie, B.J. Boyd, Minimum information reporting in bio–nano experimental literature, Nature nanotechnology, 13 (2018) 777-785.
[33] J. Hatami, S.G. Silva, M.B. Oliveira, R.R. Costa, R.L. Reis, J.F. Mano, Multilayered films produced by layer-by-layer assembly of chitosan and alginate as a potential platform for the formation of human adipose-derived stem cell aggregates, Polymers, 9 (2017) 440.
[34] N.-J. Cho, K.K. Kanazawa, J.S. Glenn, C.W. Frank, Employing two different quartz crystal microbalance models to study changes in viscoelastic behavior upon transformation of lipid vesicles to a bilayer on a gold surface, Analytical chemistry, 79 (2007) 7027-7035.
[35] S. Hatsushika, R.K. Shepherd, Y.C. Tong, G.M. Clark, S. Funasaka, Dimensions of the scala tympani in the human and cat with reference to cochlear implants, Ann Otol Rhinol Laryngol, 99 (1990) 871-876.
[36] Y.J. Hwang, M. Park, M.K. Park, J.H. Lee, S.H. Oh, M.W. Suh, High-Molecular-Weight Hyaluronic Acid Vehicle Can Deliver Gadolinium Into the Cochlea at a Higher Concentration for a Longer Duration: A 9.4-T Magnetic Resonance Imaging Study, Front Neurol, 12 (2021) 650884.
[37] K. Paunovska, C.D. Sago, C.M. Monaco, W.H. Hudson, M.G. Castro, T.G. Rudoltz, S. Kalathoor, D.A. Vanover, P.J. Santangelo, R. Ahmed, A.V. Bryksin, J.E. Dahlman, A Direct Comparison of in Vitro and in Vivo Nucleic Acid Delivery Mediated by Hundreds of Nanoparticles Reveals a Weak Correlation, Nano Lett, 18 (2018) 2148-2157.
[38] A. Saber, S.P. Strand, M. Ulfendahl, Use of the biodegradable polymer chitosan as a vehicle for applying drugs to the inner ear, Eur J Pharm Sci, 39 (2010) 110-115.
[39] A. Saber, C. Gont, S. Dash-Wagh, M. Kirkegaard, S. Strand, M. Ulfendahl, Assessment of in Vitro and in Vivo Transfection Efficiency of the Biodegradable
Polymer Chitosan in the Inner Ear, Journal of International Advanced Otology, 6 (2010) 307-315.
[40] J.C. Palmer, M.S. Lord, J.L. Pinyon, A.K. Wise, N.H. Lovell, P.M. Carter, Y.L. Enke, G.D. Housley, R.A. Green, Comparing perilymph proteomes across species, Laryngoscope, 128 (2018) E47-E52.
[41] E. Axpe, M.L. Oyen, Applications of alginate-based bioinks in 3D bioprinting, International journal of molecular sciences, 17 (2016) 1976.
[42] T. Boontheekul, H.-J. Kong, D.J. Mooney, Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution, Biomaterials, 26 (2005) 2455-2465.
[43] S.S. Chandrasekhar, R.Y. Rubinstein, J.A. Kwartler, M. Gatz, P.E. Connelly, E. Huang, S. Baredes, Dexamethasone pharmacokinetics in the inner ear: comparison of route of administration and use of facilitating agents, Otolaryngol Head Neck Surg, 122 (2000) 521-528.
[44] O.A. Selivanova, H. Gouveris, A. Victor, R.G. Amedee, W. Mann, Intratympanic dexamethasone and hyaluronic acid in patients with low-frequency and Meniere's-associated sudden sensorineural hearing loss, Otol Neurotol, 26 (2005) 890-895.
[45] H. Wang, R. Murphy, D. Taaffe, S. Yin, L. Xia, W.W. Hauswirth, M. Bance, G.S. Robertson, J. Wang, Efficient cochlear gene transfection in guinea-pigs with adeno-associated viral vectors by partial digestion of round window membrane, Gene Ther, 19 (2012) 255-263.
[46] W. Li, J.J. Hartsock, C. Dai, A.N. Salt, Permeation Enhancers for Intratympanically-applied Drugs Studied Using Fluorescent Dexamethasone as a Marker, Otol Neurotol, 39 (2018) 639-647.

[47] Y.C. Lin, C.P. Shih, H.C. Chen, Y.L. Chou, H.K. Sytwu, M.C. Fang, Y.Y. Lin, C.Y. Kuo, H.H. Su, C.L. Hung, H.K. Chen, C.H. Wang, Ultrasound Microbubble-Facilitated Inner Ear Delivery of Gold Nanoparticles Involves Transient Disruption of the Tight Junction Barrier in the Round Window Membrane, Front Pharmacol, 12 (2021) 689032.
[48] C.M. Kelso, H. Watanabe, J.M. Wazen, T. Bucher, Z.J. Qian, E.S. Olson, J.W. Kysar, A.K. Lalwani, Microperforations significantly enhance diffusion across round window membrane, Otol Neurotol, 36 (2015) 694-700.
[49] C. Honeder, C. Zhu, H. Schopper, J.C. Gausterer, M. Walter, L.D. Landegger, N. Saidov, D. Riss, R. Plasenzotti, F. Gabor, C. Arnoldner, Effects of sustained release dexamethasone hydrogels in hearing preservation cochlear implantation, Hear Res, 341 (2016) 43-49.
[50] A.N. Salt, D.B. Sirjani, J.J. Hartsock, R.M. Gill, S.K. Plontke, Marker retention in the cochlea following injections through the round window membrane, Hear Res, 232 (2007) 78-86.
[51] S. Jiang, T.W. Seale, R.Z. Gan, Morphological changes in the round window membrane associated with Haemophilus influenzae-induced acute otitis media in the chinchilla, Int J Pediatr Otorhinolaryngol, 88 (2016) 74-81.
[52] R. Singh, B. Birru, J.G.S. Veit, E.M. Arrigali, M.A. Serban, Development and Characterization of an In Vitro Round Window Membrane Model for Drug Permeability Evaluations, Pharmaceuticals (Basel), 15 (2022).
[53] J.J. Sun, Y. Liu, W.J. Kong, P. Jiang, W. Jiang, In vitro permeability of round window membrane to transforming dexamethasone with delivery vehicles--a dosage estimation, Chin Med J (Engl), 120 (2007) 2284-2289.
[54] M.C. Witte, J.L. Kasperbauer, Round window membrane permeability to transforming growth factor-alpha: an in vitro study, Otolaryngol Head Neck Surg, 123 (2000) 91-96.
[55] L.A. Lundman, L. Holmquist, D. Bagger-Sjoback, Round window membrane permeability. An in vitro model, Acta Otolaryngol, 104 (1987) 472-480.
[56] A. Moatti, D. Silkstone, T. Martin, K. Abbey, K.A. Hutson, D.C. Fitzpatrick, C.J. Zdanski, A.G. Cheng, F.S. Ligler, A. Greenbaum, Assessment of drug permeability through an ex vivo porcine round window membrane model, iScience, 26 (2023) 106789.
[57] H. Hahn, B. Kammerer, A. DiMauro, A.N. Salt, S.K. Plontke, Cochlear microdialysis for quantification of dexamethasone and fluorescein entry into scala tympani during round window administration, Hear Res, 212 (2006) 236-244.

Developing the supraparticle technology for round window-mediated drug administration into the cochlea

Niliksha Gunewardene[1,2*#], Yutian Ma[1,4*], Patrick Lam[1], Sherryl Wagstaff[1], Christina Cortez-Jugo[4], Yingjie Hu[1,2,4], Frank Caruso[4], Rachael Richardson[1,2,3] and Andrew Wise[1,2#]

[1]Bionics Institute, East Melbourne, Victoria 3002, Australia.

[2]Department of Medical Bionics, The University of Melbourne, Fitzroy, Victoria 3065, Australia.

[3]Department of Surgery (Otolaryngology), University of Melbourne, The Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria 3002, Australia

[4]Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

[*]Co-first authors

[#]Corresponding authors

1. ABSTRACT

The semi-permeable round window membrane (RWM) is the gateway to the cochlea. Although the RWM is considered a minimally invasive and clinically accepted route for localised drug delivery to the cochlea, overcoming this barrier is challenging, hindering development of effective therapies for hearing loss. Neurotrophin 3 (NT3) is an emerging treatment option for hearing loss, but its therapeutic effect relies on sustained delivery across the RWM into the cochlea. Silica supraparticles (SPs) are drug delivery carriers capable of providing long-term NT3 delivery in the cochlea, when injected directly into the guinea pig cochlea. However, for clinical translation, a RWM delivery approach is desirable. Here, we aimed to test approaches to improve the longevity and biodistribution of NT3 inside the cochlea after RWM implantation of SPs in guinea pigs and cats. Three approaches were tested (i) coating the SPs to slow drug release (ii) improving the retention of SPs on the RWM using a clinically approved gel formulation and (iii) permeabilising the RWM with hyaluronic acid. A radioactive tracer (iodine 125: ^{125}I) tagged to NT3 (^{125}I NT3) was loaded into the SPs to characterise drug pharmacokinetics *in vitro* and *in vivo*. The neurotrophin-loaded SPs were coated using a chitosan and alginate layer-by-layer coating strategy, named as '(Chi/Alg)SPs', to promote long term drug release. The guinea pigs were implanted with 5x ^{125}I NT3 loaded (Chi/Alg) SPs on the RWM, while cats were implanted with 30x (Chi/Alg) SPs. A cohort of animals were also implanted with SPs (controls). We found that the NT3 loaded (Chi/Alg)SPs exhibited a more linear release profile compared to NT3 loaded SPs alone. The ^{125}I NT3 loaded (Chi/Alg)SPs in fibrin sealant had efficient drug loading (~5 µg of NT3 loaded per SP that weights ~50 µg) and elution capacities (~49% over one month) *in vitro*. Compared to the SPs in fibrin sealant, the (Chi/Alg)SPs in fibrin sealant had a significantly slower ^{125}I NT3 drug release profile over the first 7 days *in vitro* (~12% for (Chi/Alg) SPs in fibrin sealant vs ~43% for SPs in fibrin sealant). One-month post-implantation of (Chi/Alg) SPs, gamma count measurements revealed an average of 0.3 µg NT3 remained in the guinea pig cochlea, while for the cat, 1.3 µg remained. Histological analysis of cochlear tissue revealed presence of a ^{125}I NT3 signal localised in the basilar membrane of the lower basal turn in some cochleae after 4

weeks in guinea pigs and 8 weeks in cats. Comparatively, and in contrast to the *in vitro* release data, implantation of the SPs presented better NT3 retention and distribution inside the cochlea in both the guinea pigs and cats. No significant difference in drug entry was observed upon acute treatment of the RWM with hyaluronic acid. Collectively, our findings indicate that SPs and (Chi/Alg)SPs can facilitate drug transfer across the RWM, with detectable levels inside the cat cochlea even after 8 weeks with the intracochlear approach. This is the first study to examine neurotrophin pharmacokinetics in the cochlea for such an extended period of times these two animal species. Whilst promising, we note that outcomes between animals were variable, and opposing results were found between *in vitro* and *in vivo* release studies. These findings have important clinical ramifications, emphasising the need to understand the physical properties and mechanics of this complex barrier in parallel with the development of therapies for hearing loss.

2. INTRODUCTION

Sensorineural hearing loss is caused by the degeneration of the sensory pathway in the cochlea, including the hair cells, primary auditory neurons, and their synapses. Exposure to environmental noise, viral infections, inherited and autoimmune disorders, or the ageing process, can damage the sensory cells in the cochlea, resulting in hearing impairment. Currently there is no available treatment to repair or prevent the progression of hearing loss. Devices such as hearing aids and cochlear implants can assist with hearing, but they fail to address the underlying pathology of deafness or halt its progression. With the growing number of people affected by hearing loss, there is a large unmet need for a drug therapy that can restore the sensory cells of the cochlea and return lost function.

The inner ear is a bony structure comprised of the cochlea (hearing organ) and vestibular system (balance organs). Accessing the cochlea is challenging due to its small size, embedded location, and complex architecture. The cochlea is separated into three chambers filled with ionic fluids; perilymph and endolymph, that bathe the sensory hair cells that together with auditory neurons transduce auditory information from the cochlea to the brain. The semi-permeable membranes, the round and oval windows are the gateways into the cochlea from the middle ear. Effective therapy requires entry of bioactive drug into the cochlea at therapeutically relevant concentrations and durations.

There are three main local drug delivery routes to the cochlea, (i) systemic delivery, (ii) direct intracochlear delivery (e.g. injection into the scala tympani via cochleostomy or penetration of the round window membrane (RWM)) or (iii) intratympanic administration (diffusion of drug across the semi-permeable membranes). With the systemic approach, only a portion of the injected therapeutic drug (primarily small molecule therapeutics) crosses through the blood labyrinth barrier and reaches the cochlea thus limiting what can be delivered [1, 2]. Systemic delivery also carries the risk of side effects or dose-limiting toxicity. Direct drug delivery approaches into the cochlea carry the risk of damaging residual hearing due to the invasiveness of the surgery and fluid leakage, therefore, this approach would typically only be considered if the cochlea is already being accessed such as during cochlear implant surgery [3, 4]. The intratympanic administration routes are widely accepted as optimal for the treatment of inner ear disorders as there is minimal risk to residual hearing. For example, steroids such as dexamethasone or gentamicin are commonly injected via the intratympanic approach as a treatment for Meniere's disease or idiopathic sudden hearing loss. Although effective, variable outcomes due to reduced drug diffusion across the membranes have been reported. Of note, in the guinea pig cochlea, the basal turn concentrations of dexamethasone-phosphate or gentamicin was only 2.5% or 1.4% of the injected concentration, respectively over 1-3 hours post-administration [5, 6]. Furthermore, drugs with a large molecular weight, negative charge or hydrophilic properties have poor diffusion properties through the RWM [7].

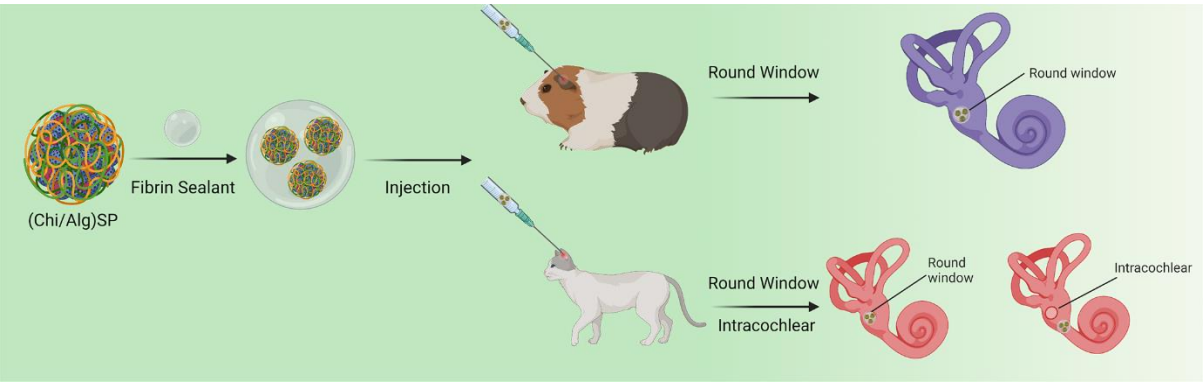
The RWM is a semi-permeable membrane consisting of three epithelial layers. The first, and, outer layer, facing the middle ear, consists of epithelial cuboidal cells that form tight junctions. The tight junctions limit the passage of most molecules that occurs via passive diffusion. The second layer is made of fibroblasts, collagen, and elastic fibres, which, contains blood and lymph vessels as well as nerve endings. The third layer consists of squamous flat inner epithelial cells and faces the scala tympani. Accumulating pharmacokinetic and functional evidence demonstrates insufficient protein transfer through the semi-permeable RWM, indicating its selective nature in substance entry to the cochlea. Factors such as molecule size, configuration, concentration, liposolubility, electrical charge, and thickness of the membrane influencing its permeability [7-9]. For instance, certain low molecular weight steroids such as dexamethasone or gentamicin easily penetrate across the RWM (MW ~400). However, with the larger molecules such as albumin (MW 70,000), the efficiency of drug entry via the RWM can be significantly lower [10]. Of note, a study focused on RWM delivery of recombinant neurotrophin (MW 29,355) to deafened animals revealed variability in hearing function improvements, with only 50% of the animals showing hearing recovery [11], suggesting that variability in drug transfer between animals. Despite the limitations in permeability of the RWM, it remains the most clinically relevant drug delivery route for reversing hearing loss. Therefore, there is a need to develop strategies to improve drug penetration across the RWM.

Neurotrophins have been implicated as an effective therapeutic option to treat hearing loss. They are naturally occurring proteins expressed by hair cells and supporting cells in the cochlea. It is well-established that neurotrophin 3 (NT3) improves the survival of sensory neurons and promotes the protection and/or regeneration of ribbon synapses [12-21]. Despite this evidence, a key challenge that has impeded clinical translation of neurotrophins, with a molecular weight of 27.3 kDa, is the lack of an effective approach for drug delivery into the cochlea in a sustained and consistent manner. We previously demonstrated that upon acute delivery of a bolus of radiolabelled neurotrophin 3 (^{125}I NT3) to guinea pig cochleae via direct intracochlear infusion, there was an exponential decrease in NT3 in the cochlea over time, with nearly complete clearance of NT3 in just 3 days [22]. There is a need to improve NT3 levels in the cochlea to gain long-term therapeutic benefit and optimise an intratympanic approach for safe drug delivery.

Several drug delivery approaches have been tested to improve neurotrophin delivery to the cochlea, including cell-based therapies, bisphosphonate agonists, mini-osmotic pumps, viral vectors, and nanoparticles, each with their own benefits and limits (reviewed in [23]). We have shown that the application of mesoporous silica supraparticles (SPs) is effective for NT3 delivery into the cochlea [24-28]. Supraparticles are spheroid structures formed from smaller colloidal nanoparticles that provide a platform for long-term controlled drug release. The major advantage of this system includes its potential to be tailored (size, porosity, and composition) to modify performance, including drug pharmacokinetics based on the treatment requirement. We previously showed that intracochlear delivery of SPs loaded with brain derived neurotrophic factor (BDNF), a neurotrophin closely related to NT3 with similar neuro-protective properties in the cochlea, to profoundly deafened guinea pigs led to a significant improvement in auditory neuron survival one-month post-treatment [26]. However, given the potential therapeutics effect of NT3 on ribbon synapse repair, we undertook a thorough characterisation of the pharmacokinetics of the SP-mediated NT3 delivery [24]. Using a TrkC cell line bioactivity test and auditory nerve survival culture assay, we previously confirmed that the SP-released drug had maintained bioactivity over an extended period *in vitro* (tested up to 28 days; [24]). Delivery of SPs to guinea pig cochleae using both the intracochlear and round window routes revealed efficient drug retention even after one-month post-implantation. With the intracochlear delivery approach, widespread drug distribution was observed throughout the cochlea. However, with

the RWM approach, drug distribution was limited and restricted to the RWM niche (outside the cochlea) and/or basal regions of the cochlea [24].

Given our previous data showing variability in the amount of drug entering the cochlea via the RWM using supraparticles, we hypothesised that this could be attributed to several factors: the particles being in limited contact with the RWM, restricted permeability of the RWM minimising the amount of NT3 crossing the membrane and/or a burst-release elution profile that may cause rapid drug clearance. The aim of this project was to address these hypotheses and develop approaches such as coating the SPs to slow down their drug release were tested to improve the longevity and biodistribution of NT3 inside the cochlea after RWM implantation of SPs in guinea pigs and cats (Scheme 1).



Scheme 1. Schematic illustration of the concept of the manuscript.

3. EXPERIMENTAL METHODS SECTION

Supraparticle manufacturing process. Primary silica particles ranging in diameter up to about 800 nm were synthesised using a modified version of a previously published protocol [27]. Specifically, 1.1 g of Cetyltrimethlyammonium bromide (CTAB) was first dissolved in 50 mL of Milli-Q water followed by adding 4.3g of Poly(acrylic acid) (PAA, Mw ~250 kDa, 35 wt % solution in water) at room temperature (i.e., ~22 °C) under vigorous stirring until a clear solution appears. After 20 min, 3.5 mL of ammonium hydroxide solution (28–30%) was added to the above suspension and stirred vigorously for 20 min until a milky suspension was obtained. Then, 4.46 mL of Tetraethyl orthosilicate (TEOS) was added with vigorous stirring for 15 min. Subsequently, the mixture was placed into a Teflon-sealed autoclave at 90 °C for 48 h. The silica particles were removed from the autoclave and washed with Milli-Q water and ethanol twice, followed by drying at 80 °C overnight. Finally, the primary silica particles were placed in a chamber furnace (Tetlow, Australia) and calcined at 550 °C for 30 h to remove the organic materials. The silica SPs (SPs) were produced by electrospraying. Briefly, primary silica particles (80 mg) were added to 2 mL of aqueous alginate solution (30 mg/mL in Milli-Q water). Then, the suspension was ultra-sonicated by an ultrasonic processor with a microtip probe (Qsonica, CT, USA) at an output amplitude of 30% for 40 s and sonicated further in an ultrasonic cleaner (Branson, USA) for 1 h to disperse the primary silica particles evenly within the alginate solution. After sonication, the solution was poured into a syringe, which was positioned in a syringe pump at a constant flow rate of 8 mL h. With an applied voltage of 13 kV, alginate/primary silica particle droplets were electrosprayed into a reservoir of 1% w/v of aqueous $CaCl_2$ to crosslink the alginate. The distance between the end of the nozzle and the surface of the $CaCl_2$ solution was set to 10 cm. Subsequently, the alginate/silica SPs were collected directly from the CaCl2 bath using a strainer and washed extensively in Milli-Q water (four times). Finally, to obtain SPs, alginate/silica SPs were calcinated at 650 °C for 30 h to remove the organic materials (i.e. alginate).

Synthesis of chitosan-alginate supraparticles. The preparation procedure of layer by layer-coated supraparticles using chitosan and alginate ((Chi/Alg)SPs) is shown in Figure 1a. Briefly, SPs were sterilised with 100 µL of 80% (v/v) ethanol at ~22 °C for 4 h, followed by rinsing with sterile Milli-Q water for six times. Chitosan (50 µL of 0.5 wt%) dissolved in 1 wt% acetic acid containing 0.15 M NaCl with pH adjusted to 5.5 was added to the SPs. After 15 min of incubation, the supernatant was aspirated, and the SPs were washed with Milli-Q water 3 times. Then, chitosan-coated SPs were incubated with 50 µL of 0.5 wt% of alginate dissolved in 1 wt% of acetic acid containing 0.15 M NaCl with pH adjusted to 5.5 for 15 min in a rotating mixer. Finally, the supernatant containing excess alginate was aspirated and the particles washed with Milli-Q water 3 times. The alternate deposition of chitosan and alginate was repeated twice.

FITC-labelled chitosan (FITC-chitosan) was synthesized based on the protocol below.Typically, 5 mg of FITC was dissolved in 10 mL of methanol and mixed with 210.1 mg of chitosan in 1 wt% of acetic acid. Then, the pH was adjusted to 7 using NaOH and acetic acid. The solution was stirred in the dark at room temperature (~22 °C) for 24 h, then dialysed using SnakeSkin dialysis tubing with 3.5k molecular weight cutoff (Thermo Scientific) against 0.15 M NaCl, followed by Milli-Q water for 3 days. Finally, the dialysed FITC-chitosan was lyophilised and stored in the fridge until use.

Characterisation of supraparticles. Scanning electron microscopy (SEM) images of SPs and (Chi/Alg)SPs were taken using a Philips XL30 field-emission scanning electron microscope (Philips, Netherlands) at an operating voltage of 5 kV. The samples were prepared by placing them onto conductive carbon tape followed by sputter coating with a 20 nm gold coating on the samples. SEM–energy-dispersive X-ray spectroscopy (SEM-EDX) mapping of (Chi/Alg)SPs was taken with a Philips XL30 field-emission scanning electron microscope (Philips, Netherlands). The thickness of the chitosan and alginate coating was characterised by quartz crystal microbalance with dissipation technique (QCM-D) (Kyushu Dentsu, Japan). Briefly, QCM chips were first cleaned with Piranha solution (3:1 mixture of sulfuric acid and 30% hydrogen peroxide) for 1 min followed by rinsing with copious amount of Milli-Q water and dried with a gentle stream of nitrogen. Chitosan and alginate layers were sequentially deposited on the clean gold surface at a constant flow rate of 200 µL min-1 for 15 min. After each layer deposition, the crystal was thoroughly washed with NaCl (0.15 M at 1 wt% acetic acid, pH 5.5) for 10 min. The changes in dissipation (ΔD) and frequency (ΔF) were monitored in real-time. The thickness of the chitosan-FITC and alginate coating on (Chi/Alg)SPs was also characterised using a confocal laser-scanning microscope (Leica, Germany) equipped with an argon laser. Fourier transform infrared (FTIR) spectra of SPs and (Chi/Alg)SPs were recorded on an FTIR spectrophotometer (Bruker, Australia). The mass of the chitosan/alginate coating was monitored by thermogravimetric analysis (TGA) (Netzsch, Australia). Briefly, SPs and (Chi/Alg)SPs were placed on separate crucibles and then loaded into the TGA instrument where the temperature was increased to 900 °C at a heating rate of 10 K/min.

Radiolabelling of BDNF and NT3. Neurotrophin-3 (NT3; Peprotech) was labelled with ^{125}I as reported previously [29]. Unbounded (free) ^{125}I was separated from the NT3 bound ^{125}I using Bio-Gel P-6DG columns (Bio-Rad). The ^{125}I labelled NT3 was transferred in phosphate buffer (including 0.25 wt% BSA and 0.1 wt% sodium azide), then purified using an Amicon Ultra-4 10K filtration unit. The specific activity of the ^{125}I NT3 was within the range of 39 to 51.6 µCi/µg, with 73 to 93% incorporation. The desired concentration of ^{125}I NT3 (1 mg/ mL in Milli-Q water) was adjusted by mixing unlabelled NT3 and ^{125}I NT3 with the volume ratio 1:1. We previously showed using western blots and an auditory nerve survival assay that iodination of NT3 does not impact the integrity and bioactivity of the protein before and after loading the protein into SPs [24, 30].

In Vitro Loading and Release Studies. For SP characterisation studies using only the native (unlabelled) protein, 40 µL of 1.0 mg/mL NT3 or BDNF was mixed with four sterilised SPs. For SP characterisation studies using radiolabelled NT3 (^{125}I NT3), 14 µL of ^{125}I NT3 (stock 1.1-5 µg/ml) was mixed with 14 µL of 2.0 mg/mL NT3 in a total volume of 28 µL with four sterilised SPs. After incubation for 3 days, the supernatant was removed, and the SPs were placed in separate 1.7 mL microcentrifuge tubes (one SP per tube). ^{125}I NT3 loaded SPs were coated with chitosan and alginate as described above to monitor the release from (Chi/Alg)SPs. Then, 100 µL of phosphate buffered saline (PBS; pH 7.4) for NT3 - loaded (Chi/Alg)SPs, artificial perilymph without glucose for ^{125}I NT3 loaded (Chi/Alg)SPs was added to each tube and incubated at 37 °C. At defined time intervals (3, 7, 14, 21, 28 days), 95 µL of the supernatant was collected and replaced with 95 µL of fresh PBS. The amount of ^{125}I NT3 loading and released from SPs and (Chi/Alg)SPs were quantified using a Perkin Elmer WIZARD automatic gamma counter with 1 min measurement time. The amount of BDNF loading and released from SPs and (Chi/Alg)SPs were quantified using a Human BDNF ELISA kit (Abcam) according to manufacturer's instructions. Briefly, 50 µL of diluted BDNF loading or released samples was added to the pre-coated ELISA plates followed by adding 50 µL of the antibody cocktail into each well. After 1 h incubation at room temperature, the plate was washed three times with 1x wash buffer. Finally, 100 µL of TMB substrate was added for color development for 10 min followed by adding 100 µL of TMB stop solution to stop the reaction. The concentration of BDNF was quantified using a Microplate reader with the 450 nm absorbance wavelength.

The amount of NT3 loading and released from SPs and (Chi/Alg)SPs were quantified using a NT3 Human ELISA kit (Thermofisher Scientific) according to manufacturer's instructions. Briefly, 100 µL of diluted NT3 loading or released samples was added to the pre-coated ELISA plates for 2.5 h incubation at room temperature. Then, the solution was discarded and washed four times with 1x wash buffer. Subsequently, 100 µL of diluted biotin conjugate was added and incubated for 1 h at room temperature. Then, the solution was discarded and washed four times with 1x wash buffer. After that, 100 µL of diluted Streptavidin-HRP was added followed by 45 min incubation at room temperature. Finally, 100 µL of TMB substrate was added for color development for 30 min followed by adding 50 µL of TMB stop solution to stop the reaction. The concentration of NT3 was quantified using a Microplate reader with the 450 nm absorbance wavelength.

Experimental animals. In this study, the use of cats and guinea pigs were approved by the St Vincent's Hospital (Melbourne) Animal Ethics Committee (18-393AB; Table 1). The study follows the Guidelines to Promote the Wellbeing of Animals used Scientific Purposes (2013), the Prevention of Cruelty to Animals Amendment Act (2015) and the NHMRC Code for Care and Use of Animals for Scientific purposes (2013).

Table 1: Study groups: Number of guinea pigs and cats used for each experimental condition

	Guinea pigs				**Cats**			
	Fibrin	N=	Timepoints	RW/IC	Fibrin	N=	Timepoints	RW/IC
SPs	✗	Data published (Gunewardene et al., 2022)			✓	N=4	2 weeks	RW
					✓	N=1	4 weeks	IC
SPs + HA	✓	N=1	4 hr	RW				
	✓	N=4	3D	RW				
(Chi/Alg)SPs	✓	N=4	2 weeks	RW	✓	N=1	4 weeks	IC
	✓	N=6	4 weeks	RW	✓	N=1	6 weeks	IC
					✓	N=1	8 weeks	IC
					✓	N=1	4 weeks	RW
					✓	N=1	6 weeks	RW
					✓	N=2	8 weeks	RW

Neurotrophin loading and SP coating for in vivo studies. Each SP was loaded in a solution containing 7 µg of NT3 per SP over a period of 3 days at room temperature. Neurotrophin stocks were prepared by diluting 100 µg of NT3 with 50 µl of Milli Q water to obtain a stock concentration of 2 mg/ml. ^{125}I NT3 was added to the native (unlabelled) NT3 at a 1:1 volume ratio. For the round window groups, SPs were loaded in batches of 5 SPs in a solution containing 17.5 µl of NT3 (stock 2 mg/ml) and ^{125}I NT3 (stock 1.1-5 µg/ml) in a total volume of 35 µl. For the intracochlear groups, SPs were loaded in batches of 6 SPs in a solution containing 21 µl of NT3 (stock 2 mg/ml) and 1.1-5 µg/ml of ^{125}I NT3 in a total volume of 42 µl. After the 3-day incubation with neurotrophin, the supernatant was removed, and the ^{125}I NT3 loaded SPs were coated with with two bilayers of 0.5 wt% chitosan and alginate as described above. Then, the (Chi/Alg)SPs were washed three times with Milli-Q water and gamma measurements taken with the (Chi/Alg)SPs in Milli-Q water to determine neurotrophin loading per SP.

Implantation of supraparticles to guinea pigs and cats. Before the surgery, the (Chi/Alg)SPs were embedded in 10 µL of clinically approved gelation system (Tisseel, Baxter) consisting of thrombin and fibronectin. The gel (called "fibrin sealant") can form a small pellet containing the loaded (Chi/Alg) SPs, which can be easily placed on the RWM to secure the fibrin sealant in place by a surgical applicator. Both the guinea pigs and cats were bilaterally implanted with SPs or (Chi/Alg)SPs.

Guinea pig implantation- The guinea pigs were anaesthetised with isoflurane. The animal's vitals were carefully monitored and documented throughout the duration of the surgery and temperature maintained within a normal range using a heat blanket. A post-auricular incision was used to expose the bulla. For the RWM surgeries, a hole was made in the bulla to expose the round window and 4-5 SPs were placed on the membrane, followed by sealing the bulla with dental cement. For the hyaluronic acid (HA) experiments, 4 mg/ml HA (hyaluronic acid sodium salt from rooster comb; Sigma H5388; Sigma, St. Louis, MO) was diluted in sterile saline. The concentration of HA used was based on previous cochlear *in vivo* studies [31]. The HA treated cochlea was randomly selected across the animals – right versus left ear (the contralateral ear was used as the control). Gelfoam was immersed in HA and then used to coat the membrane for up to 30 minutes prior to placement of the SPs on the RWM.

Cat implantation - Prior to SP administration, the cats were anesthetised with a combination of Ketamine (5-10mg/kg) and Medetomidine (10µg/kg) intramuscularly. The anaesthesia was then maintained with a mixture of inhalant isoflurane and oxygen. The animal's vitals were carefully monitored and documented throughout the duration of the surgery and temperature maintained within a normal range using a heat blanket. A post-auricular incision was performed to separate underlying muscle on the ventral bulla, followed by a #14 diamond burr to open the bulla, exposing the basal turn of the cochlea. After visualisation of the basal turn of the cochlea, 30 loaded (Chi/Alg) SPs or SPs were embedded in fibrin sealant and placed onto RWM, followed by sealing of the bulla with dental cement. For intracochlear injection, six (Chi/Alg) SPs embedded in fibrin sealant were injected into the basal turn of the cochlea through a small incision made in the RWM. A small piece of muscle was placed over the RWM incision and the bulla was closed with dental cement.

Tissue extraction and histology. At the conclusion of the treatment period, the guinea pigs and cats were and intracardially perfused with 0.9% NaCl (37 ◦C) followed by 10% Neutral Buffered Formalin (10% NBF; 4 ◦C). The cochleae from both the guinea pigs and cats were dissected for gamma count

measurement. Organs including brain, liver, intestine, kidney, and semi-circular canal were also separated for the measurement of radioactivity to assess off-target activity. Following gamma count measurements, the cochleae were 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 1.5 weeks of decalcification for guinea pigs and 2 weeks for cats, the cochleae were cryoprotected in 15% and 30% sucrose solution. They were then oriented and frozen in Tissue-Tek O.C.T. cryosectioning compound (Sakura, Japan). The cochleae were sectioned at 12 μm using a CM 1900 UV cryostat (Leica, Germany) at − 22 ◦C in the modiolar plane and mounted onto Superfrost-Plus slides (Menzel-Glaser, Braunschweig, Germany). The slides were stored at -20°C until ready for autoradiography analyses.

Autoradiography and imaging. We used two autoradiography analysis methods- film and emulsion techniques to assess the drug distribution in the cochlea. The specifics of the analyses were previously published [24, 25]. Briefly for the film analysis, standards were prepared to obtain a NT3 concentration gradient. They were prepared using Whatman filter paper disks (5 mm diameter), with each disk numbered from 1 to 10. A 1:2 serial dilution of ^{125}I NT3 was prepared, and each disk treated with 5 μL of the sample, disk 1was the most concentrated. The slides containing cochlear sections 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) and 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. The freehand tool was used to draw insets around 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 obtained. The background levels were subtracted from measurements obtained at the regions of interest.

Physiology testing (Auditory brainstem responses). Auditory brainstem responses (ABRs) were recorded prior to surgical implantation of the SPs using techniques described previously (ref). Auditory thresholds were measured for tone pip acoustic stimuli at a range of frequencies (1kHz to 24kHz) and intensities (0 dB to 100 dB sound pressure level).

Data analysis. The amount of NT3 loaded and eluted by the SPs is expressed respectively as a percentage of the concentration of the loading solution and percentage of the loaded NT3 into the SPs. The amount of ^{125}I NT3 retained in the cochlea is expressed as a percentage of the dose injected (e.g., the amount of NT loaded into the SPs). The data is presented as individual points or as means ± standard deviation or error of mean. 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 tests as appropriate. Autoradiographic film analysis was performed in ImageJ with MATLAB used for image visualisation. The emulsion coated slides were analysed using ImageJ with background subtracted and data expressed as normalized relative integrated density [15]. The specifics of the analyses were previously published [24, 25].

Minimum Information Reporting in Bio–Nano Experimental Literature (MIRIBEL). The studies conducted herein, including material characterisation, biological characterisation and experimental details, conform to the MIRIBEL reporting standard for bio–nano research [32], and include a companion checklist of these components in the Supporting Information.

4. RESULTS

Characterisation of the coated (Chi/Alg)SPs

To control the release of loaded protein from supraparticles, SPs were coated with alternating layers of chitosan and alginate. Chitosan and alginate are two polysaccharides used previously in layer-by-layer applications due to their biocompatibility and electrostatically interacting properties. The carboxyl group from alginate gives it a net negative charge, which allows interaction with the amine groups on positively charged chitosan [33]. Figure 1a demonstrates the formulation of (Chi/Alg)SPs. The surface structure of SPs following layer-by-layer coating with chitosan and alginate was characterized by scanning electron microscopy (SEM). At low resolution, the morphology of the SPs and (Chi/Alg)SPs appear similar (Figure 1b and c), but at higher magnification, distinct surface morphology differences can be observed (Figure 1d and e), indicative of the successful coating of the SPs with the chitosan/alginate film. SEM-EDX elemental mapping confirmed the presence of silicon (Si), oxygen (O), calcium (Ca) and nitrogen (N) uniformly distributed on the surface of (Chi/Alg)SPs (Figure 1f).

Fourier transform infrared (FTIR) spectroscopy of the uncoated SPs confirmed the presence of Si–O–Si bonds with stretching vibrations of Si–O–Si observed at 1065 cm^{-1}, bending vibrations of O–Si–O observed at 801 cm^{-1} and rocking vibrations of Si–O–Si observed at 452 cm^{-1} (Figure 1g, black curve). The coated (Chi/Alg)SPs displayed a comparable FTIR spectrum (Figure 1g, blue curve). The additional transmittance band appearing at 3355 cm^{-1} corresponds to the stretching vibration of O–H. The peak at 1600 cm^{-1} is assigned to the bending of N–H. The peak at 1408 cm^{-1} corresponds to the C–N stretching mode of the acrylamide group.

Quartz crystal microgravimetry with dissipation (QCM-D) was performed to monitor the chitosan and alginate build-up. QCM-D measures the change in frequency of an oscillating piezoelectric crystal, with a decrease in frequency indicating surface deposition. Multilayers of chitosan and alginate were deposited on a clean QCM substrate and the stepwise decrease in the frequency (ΔF) monitored by QCM-D indicated the gradual growth of a chitosan/alginate multilayer film (Figure 1h). The dissipation (ΔD) values increased with time, suggesting that the chitosan and alginate coating is soft, not rigid [34].

To quantify the mass of chitosan/alginate coating deposited, thermogravimetric analysis (TGA) was performed. Chitosan and alginate are two organic components that decompose at high temperatures. Thermogravimetric analysis (TGA) showed that one bilayer coating of chitosan/alginate accounted for ~4% of the weight (~2 µg considering the weight of each SP is ~0.05 mg) of one SP (Figure 1i). In addition, SEM images of (Chi/Alg)SPs cross-sections (Figure S1) and confocal laser scanning microscopy images (Figure S2) of fluorescein isothiocyanate (FITC) labelled chitosan and alginate coated (Chi/Alg)SPs also confirmed the presence of a chitosan/alginate coating on the surface of SPs but not within the supraparticles.

Neurotrophin loading and elution profile of the coated (Chi/Alg)SPs in vitro

To evaluate the release profile of neurotrophins, native brain derived growth factor (BDNF) and NT3 were loaded into the SPs followed by coating with 0.5 wt% of chitosan and alginate with a bi-layer coating. The concentration of the loading and released neurotrophins was characterized by ELISA. The results showed that there was an average of ~6.75 µg of BDNF and ~7.39 µg of NT3 loaded in each SP. Figure 1j and k show that the *in vitro* neurotrophins release can be extended to ~28 days with an average of ~49% of BDNF and 52% of NT3 release from (Chi/Alg)SPs compared to an average of ~63% of BDNF and ~54% of NT3 released from SPs over 28 days. Notably, there was only an average of ~18% of BDNF and ~21% NT3 released from (Chi/Alg)SPs, which is lower than an

average of ~48% of BDNF and ~35% of NT3 released in the first 7 days, which further indicated that the coating was able to extend the release profile of the SPs.

A radiolabelling NT3 technique, previously used to characterize the pharmacokinetics and distribution of NT3 in the cochlea [29, 30], was employed to characterize the loading and release *in vitro* NT3. During loading and coating, the percentage of the radioactivity detected in the washing solution, coating solution and the loading tube was low (<10%), which suggests minimal drug loss during the post-coating steps (Figure S3). Before the coating of chitosan and alginate, the ^{125}I NT3 was first loaded into uncoated SPs with 5 µg, which was ~70% of the total ^{125}I NT3 loading amount (Figure 1l, m). For the *in vivo* experiments, we used a clinically approved fibrin sealant (Tisseel, Baxter, USA) that permits the SPs to be encapsulated within a gel matrix for ease of placement of multiple SPs on the RWM of the cochlea or intracochlear implantation. The percentage cumulative release of ^{125}I NT3 from uncoated SPs and (Chi/Alg)SPs in fibrin sealant experiment was performed in artificial perilymph without glucose (Figure 1n). A linear release profile was observed from (Chi/Alg)SPs in fibrin sealant compared to the SPs in fibrin sealant. In addition, for uncoated SPs in fibrin sealant, a cumulative release of ~43% of the loaded ^{125}I NT3 was observed in the first 7 days. The coated (Chi/Alg)SPs in fibrin sealant had a significantly lower cumulative drug release profile in the first 7 days, with only ~12%of ^{125}I NT3 released ($p < 0.001$ from unpaired T test). After 28 days, there was an average of ~49% of ^{125}I NT3 released from (Chi/Alg)SPs in fibrin sealant, which was lower, but not statistically significant than the uncoated SPs in fibrin sealant with an average of ~62% of ^{125}I NT3 released over 28 days ($p = 0.14$ from unpaired T test). These results indicate that chitosan, alginate and fibrin form a firm matrix that restricts the burst-release drug profile observed in standard SPs, thus supporting a more linear and sustained drug release profile that can extend the potential treatment duration using this technology.

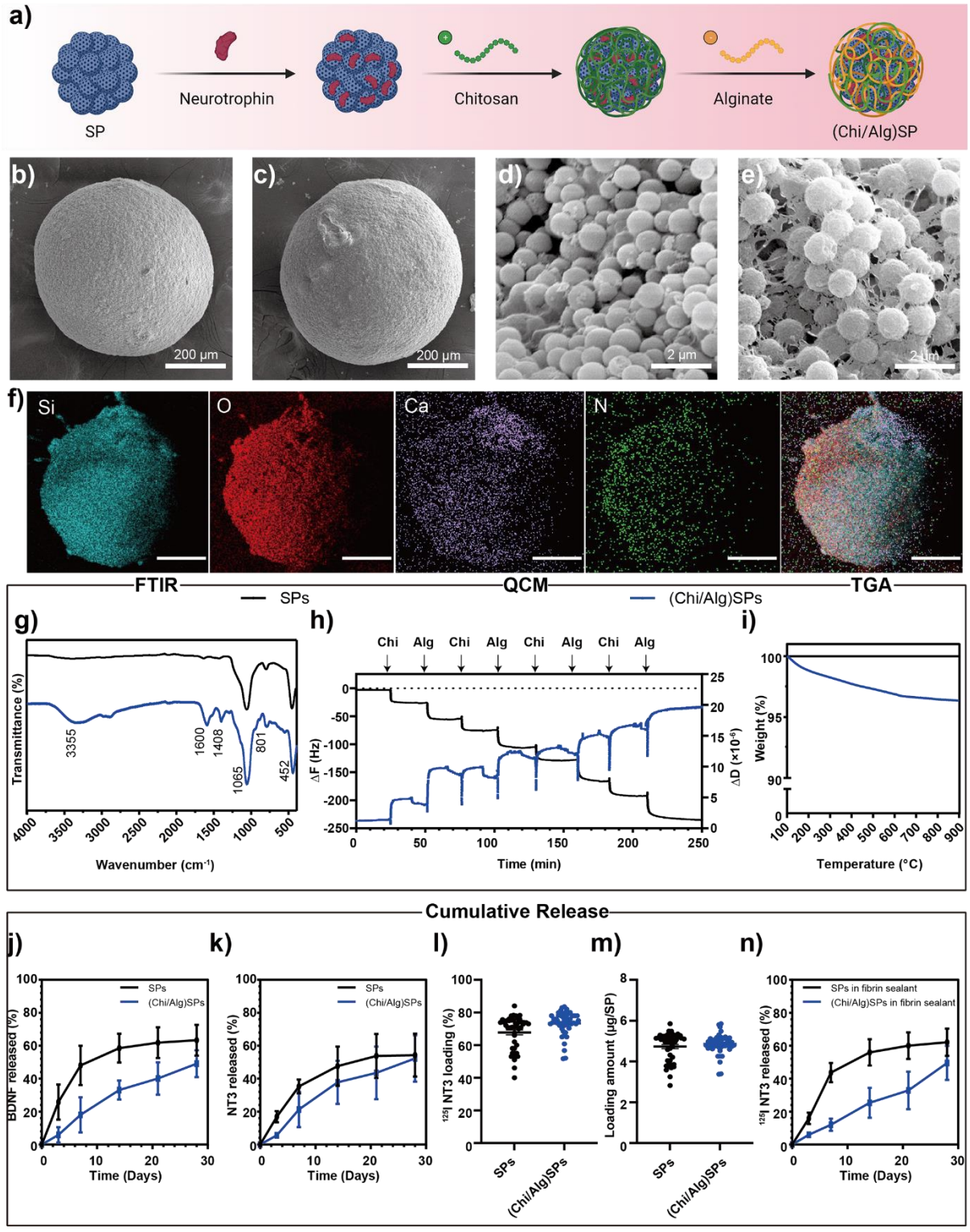


Figure 1: (a) Schematic illustration of the formulation of (Chi/Alg)SPs. SEM image of a (b) SP and (c) (Chi/Alg)SP. High-resolution SEM image displaying the surface of (d) a SP and (e) a (Chi/Alg)SP. (f) Elemental distribution of a (Chi/Alg)SP. Scale bars = 200 µm (g) FTIR spectra of SPs and (Chi/Alg)SPs. (h) Frequency and dissipation changes monitored by QCM-D for the layer-by-layer assembly of chitosan and alginate. (i) Thermogravimetric analysis (TGA) of SPs and (Chi/Alg)SPs showing the weight percentage of chitosan/alginate bi-layer coating. (j) *In vitro* cumulative release profiles of (j) BDNF and (k) NT3 from SPs and (Chi/Alg)SPs. ^{125}I NT3 loading (l)

percentage and (m) amount in SPs and (Chi/Alg)SPs. (n) *In vitro* cumulative release profiles of ^{125}I NT3 from SPs and (Chi/Alg)SPs in fibrin sealant.

Pharmacokinetic properties of (Chi/Alg)SPs and SPs after RWM delivery in guinea pigs

A cohort of guinea pigs were bilaterally implanted with (Chi/Alg)SPs incorporating the fibrin sealant. The animals were implanted with five ^{125}I NT3 loaded (Chi/Alg)SPs on the RWM. The animals were terminated after 14 (n=4) and 28 (n=6) days post-surgery. Whole-cochlear gamma counts were obtained to measure the amount of NT3 that diffused into the cochlea as well as the amount remaining in the SP. Here, it needs to be noted that whole cochlear gamma count measurements reveal the total amount of drug available, both on the RWM (outside the cochlea) and inside the cochlea. Autoradiography analyses of cochlear sections were then performed to assess intracochlear drug biodistribution.

Whole-cochlear gamma counts revealed that the amount of NT3 retained in both the cochlea and (Chi/Alg)SPs present on the RWM at 2 weeks averaged 0.4 μg ± 0.16 μg (~2.7% of the loaded NT3), and at 4 weeks it was 0.38 μg ± 0.17 μg, (~2.1% of the loaded NT3; Figure 2a, b). Despite the slower release profile of (Chi/Alg)SPs, this level of NT3 retention in the cochlea is significantly lower compared to our previously published data using uncoated SPs, in which NT3 retention in both the cochlea and particles averaged at 2 weeks was 4.3 ± 1.1 μg (~16.6% of the loaded NT3) and at 4 weeks was 2.3 ± 0.2 μg, (~5.8% of the loaded NT3; [24]) ($p < 0.0001$ at both time points analysed, n=4 per timepoint; 2-way ANOVA with Tukey's multiple comparison test).

Autoradiography analyses revealed a strong ^{125}I signal from (Chi/Alg)SPs within the round window niche external to the cochlea (Figure 2c), but also on internal structures of the cochlea, indicating low-level diffusion of the NT3 through the RWM. Comparison of the ^{125}I integrated densities between the basal turn segments indicated that the diffusion of NT3 did not extend beyond the lower section of the basal turn. However, a clear ^{125}I signal was detected in the basilar membrane, situated just below the hair cells after treatment with both the SPs and (Chi/Alg)SPs (Figure 2d). For the (Chi/Alg)SPs, the ^{125}I signal in the basilar membrane of the lower basal turn of the cochlea was 4-fold higher at 2 weeks, than the integrated density after 4 weeks of implantation, but this was not statistically significant (14D- 0.03 ± 0.025 and 28D- 0.007 ± 0.005 a.u; T test; p=0.3; Figure 2e). In comparison to our previously published data using SPs, there was no statistically significant difference in the amount of signal on the basilar membrane with the (Chi/Alg)SPs (p=0.99 at 2 weeks and p=0.99 at 4 weeks, 2-way ANOVA with Tukey's multiple comparison test , n=4 per timepoint; [24]).

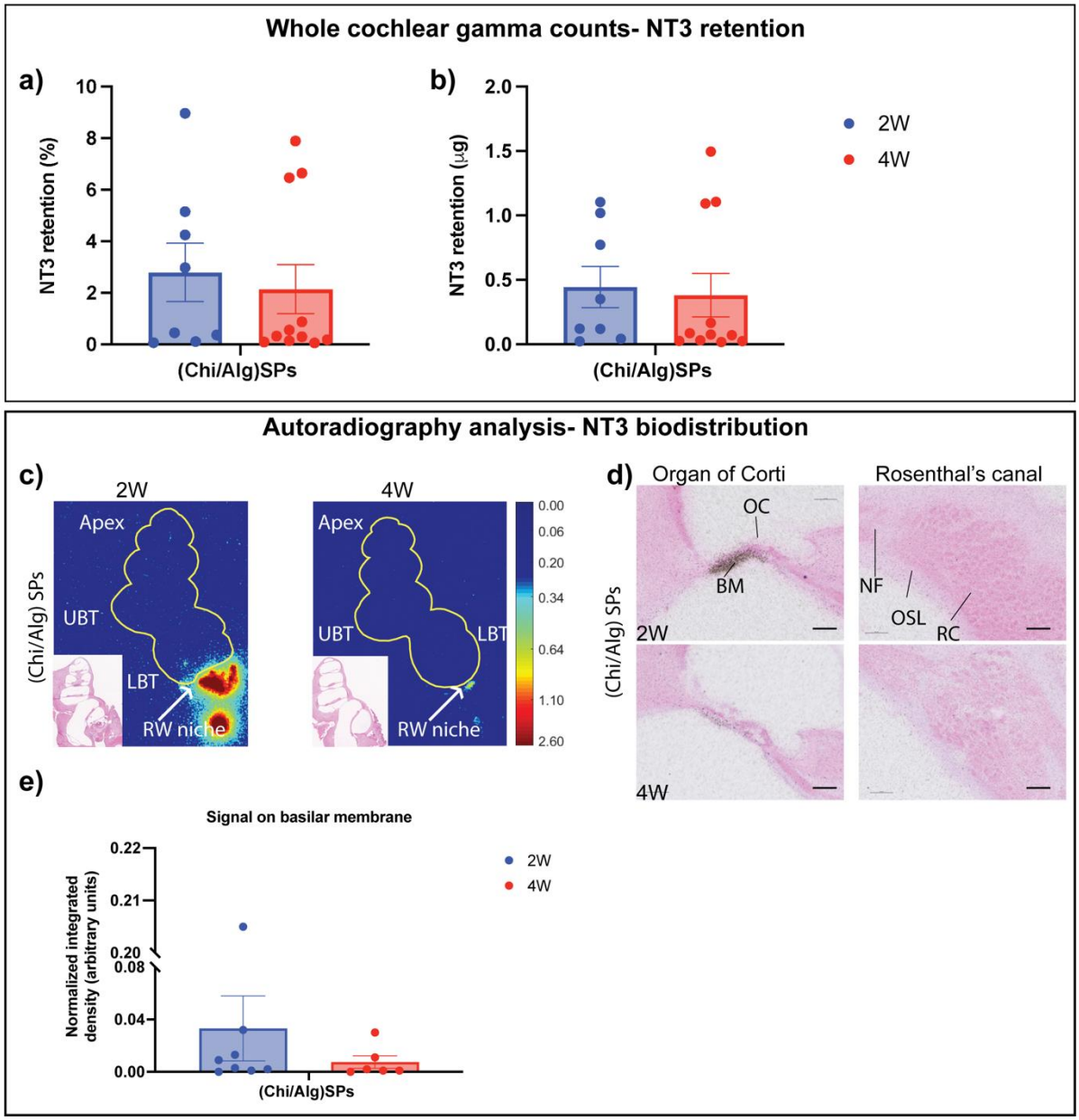


Figure 2: Guinea pig implantations-retention of NT3 after delivery of (Chi/Alg)SPs on to the RWM. The percentage (a) and amount of NT3 (b) retained was calculated from the gamma counts of the administered (Chi/Alg)SPs and whole cochleae post-surgery. (c) Film-based analysis of whole-cochlear sections with MATLAB image processing showed strong ^{125}I signal in (Chi/Alg)SPs within the RWM niche. Insets show H&E stained sections of the processed images. (d) High magnification images of H&E stained autoradiographs of the cochlear lower basal turn showing representative images of the organ of Corti region (OC) and Rosenthal's canal (RC). Scale bars = 50 µm. Autoradiography analyses of ^{125}I signal density in the (**e**) basilar membrane at day 14 and 28. Data presented as mean ± standard errors of mean (SEM). Day 14 (n=4) and 28 (n=6; 12 cochleae).

Pharmacokinetic properties of (Chi/Alg)SPs after RWM delivery in cats

The properties of the RWM differ between species, in terms of their thickness and permeability [4, 7]. Therefore, we also tested the use of (Chi/Alg)SPs in cats as the larger cat cochlea is more anatomically similar to the human cochlea than rodent cochlea. It has a similar RWM thickness [9] and cross sectional area in the basal turn (2.0mm^2) as the human cochlea [35]. Here, the animals were

bilaterally implanted with 30x ^{125}I NT3 loaded (Chi/Alg)SPs via the RWM approach (Figure 3a). The animals were terminated after 4-, 6- and 8-weeks post-surgery. We also included an additional cohort of cats implanted with ^{125}I NT3 loaded SPs that were not coated with Chi/Alg. These animals were terminated after 2 weeks. Of note, only a small cohort of cats were used in these experiments and limited statistical tests could be performed.

Whole-cochlear gamma counts revealed that after 4-, 6- and 8-weeks post-surgery of the (Chi/Alg)SPs, less than 1.3 ± 1.29 μg (<1% at 28 days) of the loaded NT3 was detected in the cochlea (Figure 3b, c). In comparison, cat implantations with the SPs had an average of 54 ± 11.4 μg, corresponding to 37% of drug remaining in the cochlea after the shorter 2-week treatment duration (Figure 3b, c).

Analysis of whole cochlear sections from cat cochleae revealed limited intracochlear spread of the drug. In some sections, a strong ^{125}I signal from (Chi/Alg)SPs was detected within the round window niche external to the cochlea (Figure 3d). Of note, in one cochlea treated with Chi/Alg)SPs for 8 weeks, a strong ^{125}I NT3 signal was detected at the RWM niche and a very weak signal at the basilar membrane.

Quantification of the ^{125}I integrated densities in the emulsion-stained cochlear sections revealed a small, but detectable signal in some treated cochleae throughout the regions of interest. However, with increased post-surgery time, a significant decline in the ^{125}I NT3 signal was observed.

In the basilar membrane, the ^{125}I signal in the lower basal turn after 8 weeks was 8-fold lower than the integrated density after 4 weeks with the (Chi/Alg)SPs (4W - 0.005 ± 0.004 and 8W - 0.0005 ± 0.0005 a.u; Figure 3d). There was also variability between the cochleae in terms of the levels of ^{125}I NT3 signal on the basilar membrane with the SPs after 2 weeks, the range of integrated densities was 0.001 to 0.018 ± 0.002 a.u (Figure 3d and e). We were unable to detect any signal in Rosenthal's canal in any of the cats analysed with both the SPs and (Chi/Alg)SPs. Collectively, the data suggests that while the SPs show better NT3 retention (compared to (Chi/Alg)SPs), significant variability in drug biodistribution is present with both the standard and coated particles *in vivo*.

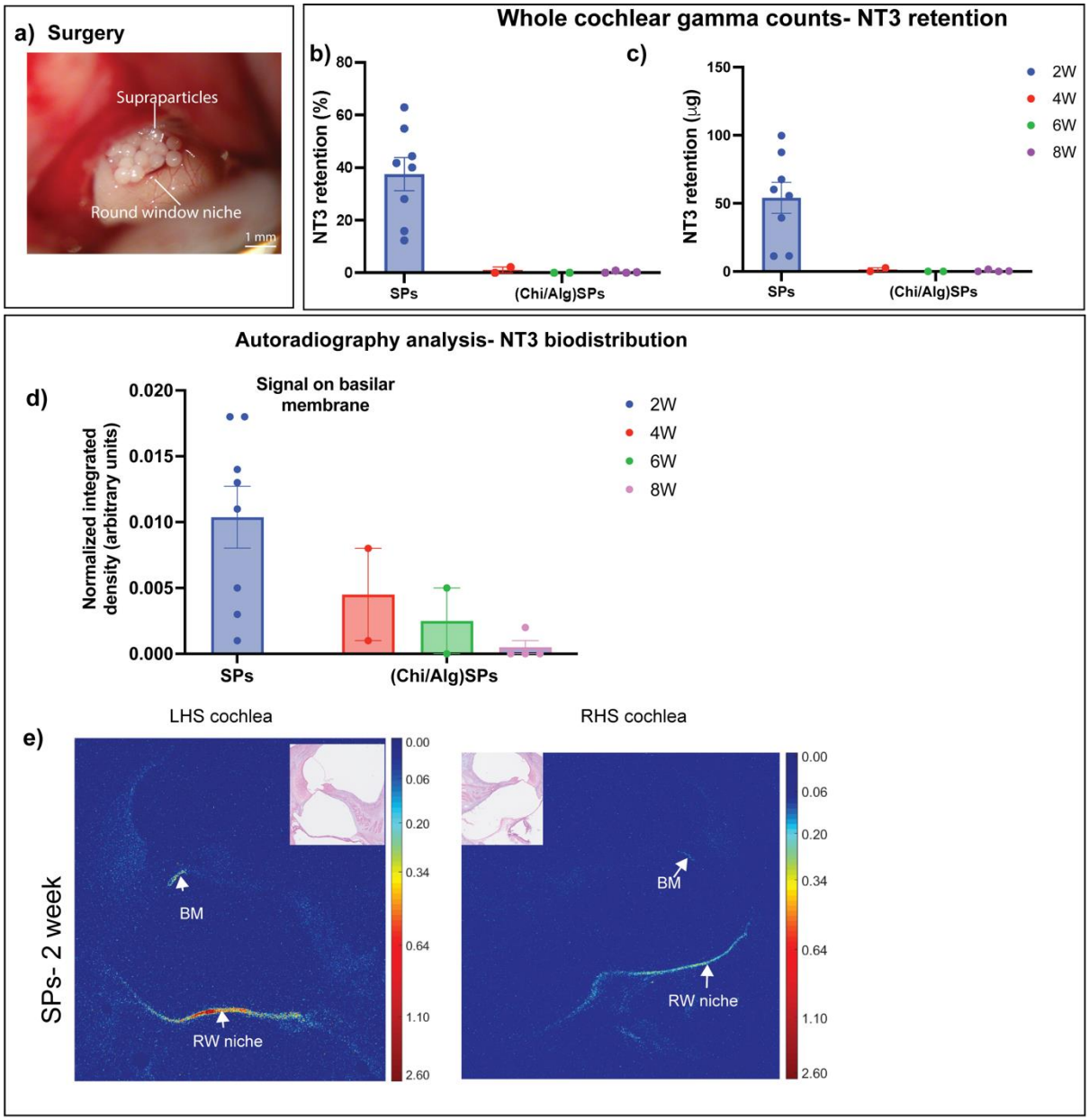


Figure 3: Cat implantations- retention of NT3 after delivery of (Chi/Alg)SPs on to the RWM. (a) Surgical image of the round window of a cat cochlea implanted with SPs. (b) The percentage and (c) amount of NT3 retained was calculated from the gamma counts of the administered (Chi/Alg)SPs and gamma counts of the whole cochleae post-surgery. Autoradiography analysis of ^{125}I signal density in the (d) basilar membrane at 2, 4, 6 and 8 weeks. (e) Film-based analysis of cochlea sections with MATLAB image processing showed strong ^{125}I signal in SPs within the RWM niche and basilar membrane. Insets show H&E stained sections of the processed images. Basilar membrane (BM). Data presented as mean ± standard errors of mean (SEM).

Pharmacokinetic properties of (Chi/Alg)SPs after intracochlear delivery in cats

Given the variable biodistribution of drug using the RWM approach, we tested (Chi/Alg)SPs using the intracochlear approach in cats in order to assess the capacity of SP for sustained delivery NT3 to the intracochlear environment. Six ^{125}I NT3 loaded (Chi/Alg)SPs were bilaterally implanted into the cochlear fluids of cat cochleae (Figure 4a). The animals were terminated after 4, 6 and 8 weeks. We

also bilaterally implanted one cat with ^{125}I NT3 loaded SPs (uncoated). This animal was terminated after 4 weeks.

With the (Chi/Alg)SPs, whole-cochlear gamma counts revealed that after 4 weeks, some ^{125}I NT3 was detectable in the cochlea, corresponding to 0.2 ± 0.19 µg (~0.66% at 4 weeks), however after 6 and 8 weeks post-implantation, the levels were much lower (Figure 4b and c). With the uncoated SPs, there was a slightly higher amount of ^{125}I NT3 remaining in the cochlea, corresponding to 0.9 ± 0.4 µg (~2.5% at 4 weeks).

Importantly, analysis of cochlear sections showed evidence of NT3 remaining in the cochlea after 8 weeks of intracochlear delivery of (Chi/Alg)SPs, albeit a weak ^{125}I signal on the basilar membrane (Figure 4d), while a weak ^{125}I signal was particularly evident in the basilar membrane at 4 weeks (Figure 4e). This result indicates that the SPs are able to deliver NT3 over a long duration (up to 2 months) when delivered to the intracochlear environment. Quantification of the ^{125}I integrated densities in the emulsion-stained cochlear sections revealed a decrease in the levels of ^{125}I NT3 with increased post-surgery time. In the basilar membrane, the ^{125}I signal in the lower basal turn after 8 weeks was 3-fold lower than the integrated density after 4 weeks (4 weeks - 0.1 ± 0.047 and 8 weeks - 0.004 ± 0.008 a.u; Figure 4f). With the SPs, the signal on the basilar membrane was weak, but still slightly higher than with the (Chi/Alg)SPs after 4 weeks (0.16 ± 0.027 a.u).

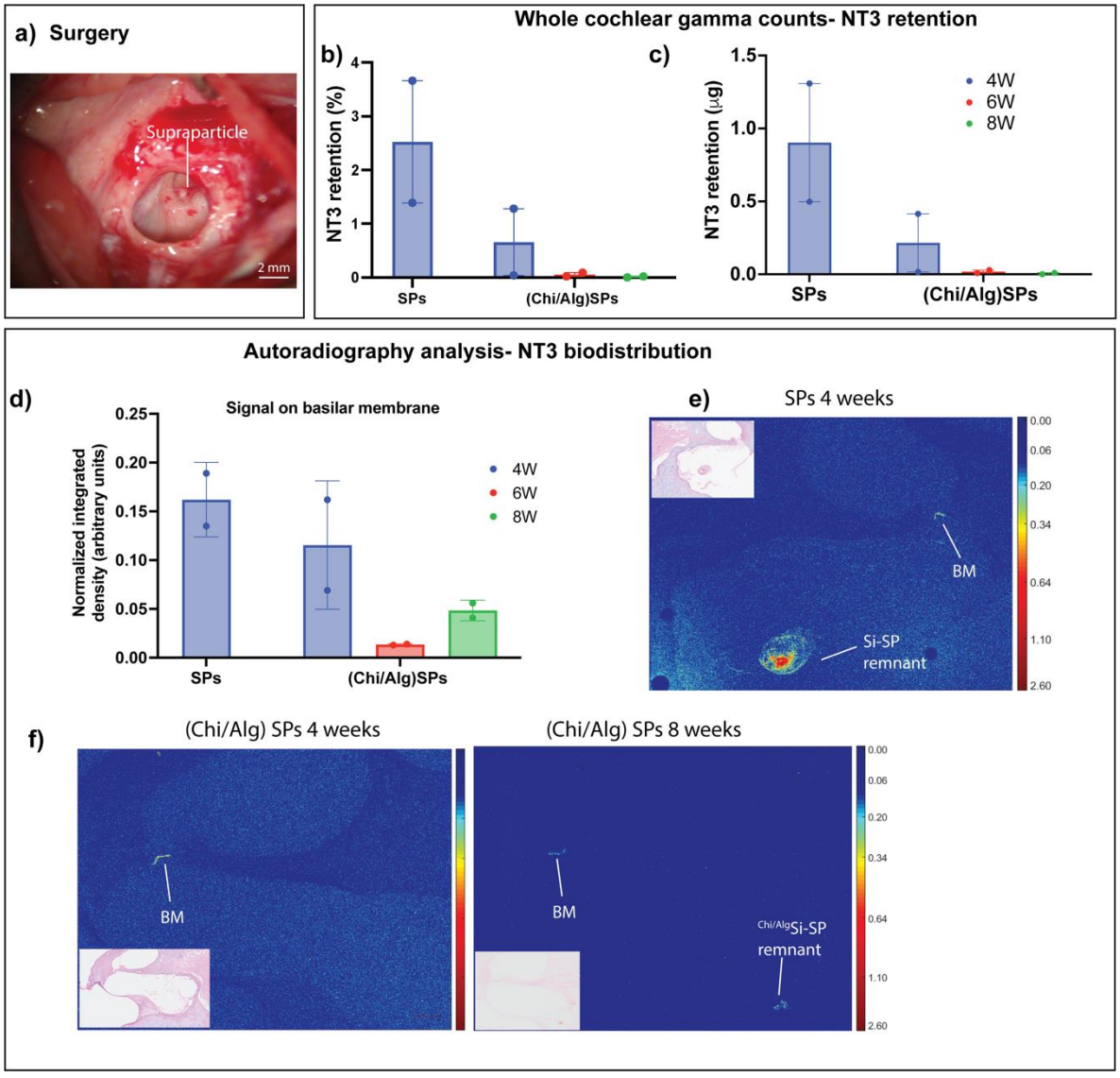


Figure 4: Cat implantations- retention of NT3 after intracochlear delivery of (Chi/Alg)SPs. (a) Surgical image of intracochlear implantation of the SPs via a small incision in the RWM. A SP can be observed on the inside of the RWM. (b) The percentage and (c) amount of NT3 retained was calculated from the gamma counts of the administered (Chi/Alg)SPs and gamma counts of the whole cochleae post-surgery. Autoradiography analyses of ^{125}I signal density in the (d) basilar membrane at 4, 6 and 8 weeks. (e) Film-based analysis of whole-cochlea sections with MATLAB image processing showed strong ^{125}I signal in SPs in the basilar membrane after 4 weeks. (f) For the (Chi/Alg)SPs, signal in the basilar membrane was detected at 4 and 8 weeks. Basilar membrane (BM). Data presented as mean ± standard error of mean (SEM).

Hearing function changes following implantation of (Chi/Alg)SPs in cats

To establish the safety of the surgical approach to deliver (Chi/Alg)SPs in fibrin sealant to the RWM as well as that of the intracochlear approach, hearing thresholds were measured before and after treatment. Auditory brainstem responses (ABRs) were recorded prior to surgical implantation of the SPs using techniques described previously. Auditory thresholds were measured for tone pip acoustic stimuli at a range of frequencies.

(Chi/Alg)SPs containing ^{125}I NT3 were delivered to the RWM (n=30 SPs in each cat cochlea). After 8 weeks, the ABRs was measured. The RWM approach had minimal impact on normal functional hearing (Figure 5). With the intracochlear delivery (n=6 SPs in each cat cochlea), post treatment thresholds were elevated in the high frequency region (≥ 12kHz) indicative of hearing loss (Figure 5).

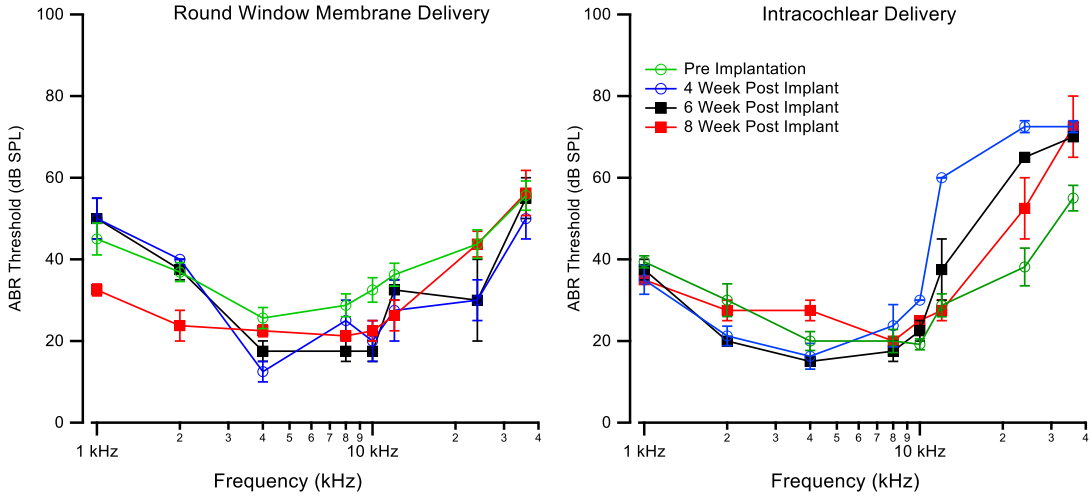


Figure 5. Cat auditory brainstem response (ABR) thresholds for tone pip acoustic stimuli for Round Window delivery and Intracochlear delivery. Average (±SEM) thresholds are shown prior to implantation and after 4-, 6- and 8-weeks post bilateral implantation of the ^{125}I NT3 loaded SPs. Round window, 4 (n=2), 6 (n=2) and 8 weeks (n=4 cochleae). Intracochlear, 4 (n=4), 6 (n=2) and 8 weeks (n=2 cochleae).

Impact of hyaluronic acid coating of the RWM in NT3 levels inside the guinea pig cochlea

Hyaluronic acid (HA) has previously shown to improve drug delivery across the RWM [31, 36]. We hypothesised that HA would increase RWM permeability and promote SP-released NT3 transfer into the cochlea. Therefore, we initially tested this approach in guinea pigs using standard SPs. To implant SPs onto the RWM, on one ear, HA was applied on the RWM for 30 minutes using gelfoam prior to the SPs (n= 4-5) being placed on the membrane. The contralateral ear was not treated with HA (SPs

only; control). The animals were terminated at 4 hours and 3-days post-implantation. Whole cochlear gamma counts were obtained to measure the total drug availability (inside the cochlea and external to the cochlea) at each time point. There was a decline in the amount of available NT3 after 3 days of treatment, averaging 72% (~21 µg) after 4 hours and 28% (~10 µg) at day 3 (Figure 6a and b). However, there was no statistically significant difference in the amount of available NT3 after treatment of the RWM with HA, compared to cochleae that were not pre-treated with HA (p=0.98, unpaired T test).

Autoradiography analysis of cochlear sections revealed a strong ^{125}I signal from the SPs on the basilar membrane (Fig 6d) indicating intracochlear delivery of NT3 following RWM placement of the NT3-SPs. To determine the extent of drug entry into the cochlea, we compared the radiolabel signal on the basilar membrane of the cochleae treated with the RW SPs and HA+RW SPs on day 3. There was no statistically significant difference in the amount of NT3 signal in the basilar membrane after HA treatment compared to no HA treatment (p=0.45, unpaired T test) indicating that HA was not effective in promoting NT3 transfer across the RWM.

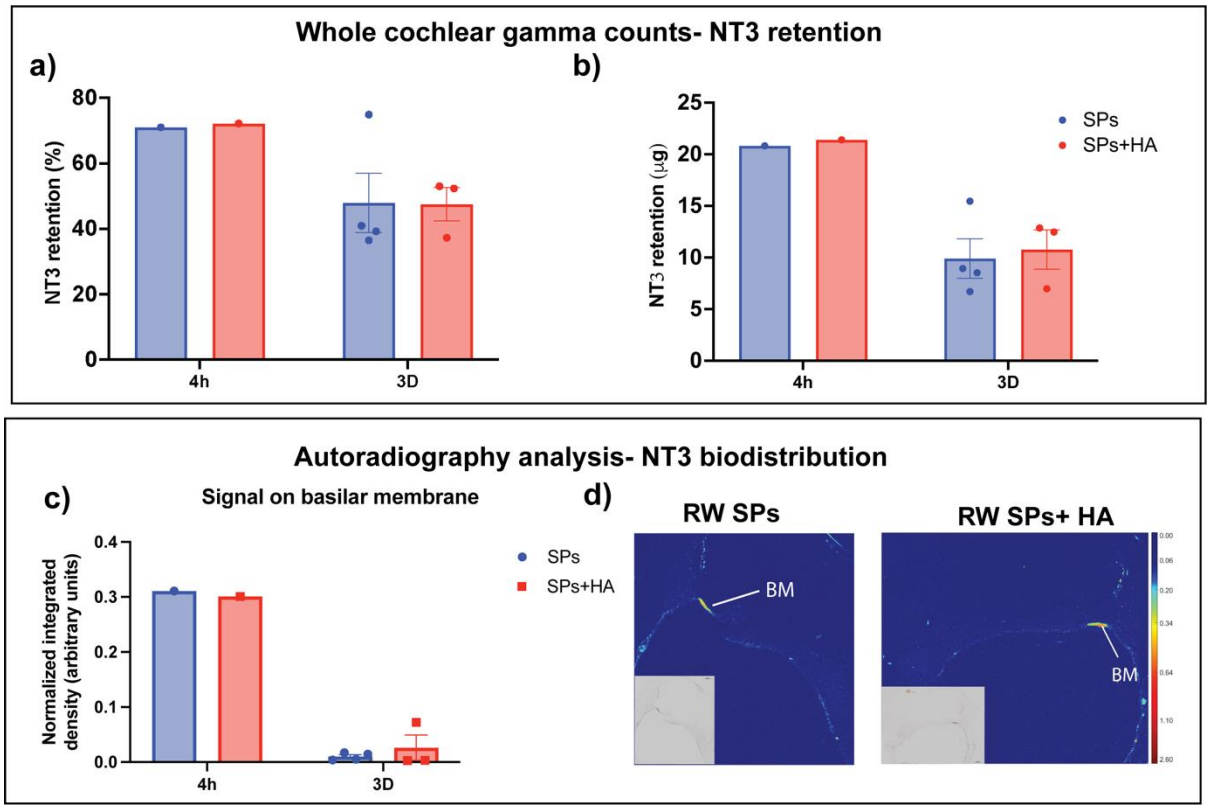


Figure 6: Impact of hyaluronic acid coating of the RWM in NT3 levels inside the guinea pig cochlea. (a) The percentage and (b) amount of NT3 retained was calculated from the gamma counts of the administered SPs and gamma counts of the whole cochleae post-surgery with and without hyaluronic acid (HA) treatment. The animals were bilaterally implanted- one ear with the RW SPs + HA and the other ear only the RW SPs (control) (c) Autoradiography analyses of ^{125}I signal density in the basilar membrane 4 hours post-treatment with and without HA. (d) High magnification images of H&E-stained cochlear lower basal turns showing representative images of the organ of Corti region (OC) containing the basilar membrane (BM) and Rosenthal's canal (RC) in the left (RW SP+HA-treated) and right ear (RW SP) of the same animal. Scale bars= 50 µm. Data presented as mean ± standard errors of mean (SEM). Day 1 (n=1) and 3 (n=4).

Table 2: Data summary of guinea pig and cat supraparticle RWM implantations. Round window membrane (RWM), basilar membrane (BM).

	Treatment	Timepoint	No. of cochleae	SP detected	Signal on RWM	Signal on BM	% of cochleae with signal on BM
Guinea pigs	SPs	4 hr	N=1	1/1	1/1	1/1	100%
		3D	N=4	4/4	4/4	2/4	50%
	SPs + HA	4 hr	N=1	1/1	1/1	1/1	100%
		3D	N=4	4/4	3/4	1/4	25%
	(Chi/Alg)SP	2 weeks	N=8	8/8	4/8	2/8	25%
		4 weeks	N=12	12/12	1/12	1/12	8.3%
Cats	SPs	2 weeks	N=8	8/8	7/8	5/8	62.5%
	(Chi/Alg)SP	4 weeks	N=2	2/2	1/2	1/2	50%
		6 weeks	N=2	0/2	0/2	0/2	0%
		8 weeks	N=4	2/4	1/4	1/4	25%

5. DISCUSSION

There is a need to improve intratympanic administration of neurotrophins to the cochlea as a treatment for hearing loss. The application of SPs holds promise as a vehicle for transferring high drug load capacities via the RWM, with maintained bioactivity over an extended period [24]. We previously showed that SP delivery of ^{125}I NT3 using the RWM approach led to some accumulation of NT3 within the RWM niche and the basal region of the guinea pig cochlea [24]. The signal was detected in the cochlea for up to one month. While these results were promising, further optimisation of the SP delivery approach could improve the levels of drug transfer across the RWM for clinical translation of this therapy. To improve the capacity to deliver NT3 over longer periods of time, in this study, we developed a coating technique that reduces NT3 elution over the first few weeks to extend the elution profile. We show using two animal species that the uncoated SPs and coated (Chi/Alg)SPs can be used to effective release NT3 for transfer across the RWM, but with low efficiency and variability between animals. Encouragingly, with the intracochlear approach, a weak ^{125}I NT3 signal was detected inside the cochlea even after 2 months post-treatment with (Chi/Alg)SPs, thus supporting the long term drug deliverability of the system.

The coated (Chi/Alg)SPs combined with fibrin sealant improved drug elution in vitro but not in vivo.

The coated (Chi/Alg)SPs reduced the burst-release profile of the SPs at the early elution timepoints. When combined with the fibrin sealant the (Chi/Alg)SPs had a sustained drug elution profile *in vitro*. To evaluate *in vitro* elution, the (Chi/Alg)SPs were immersed in PBS buffer with the buffer collected at various time points. Immersing the loaded SPs in PBS leads to disruption of the electrostatic interactions established between the positively charged neurotrophins and negatively charged silica SPs, prompting drug release *in vitro*. In both guinea pigs and cats, RWM implantation of (Chi/Alg)SPs led to a progressive decrease in ^{125}I NT3 levels, but still detectable at the latest timepoints analysed (0.38 µg for guinea pig cochlea after 4 weeks; ~2.1% and 0.46 µg for cat cochlea after 8 weeks; ~0.27%). Although this was promising, the uncoated SPs performed significantly better with the guinea pig cochlea retaining an average of ~2.3 µg (corresponding to ~5%) of drug after 4 weeks, while for the cats, an average of ~54 µg (corresponding to ~37%) of drug was remaining in the

cochlea after 2 weeks. These findings contrasted with our hypothesis whereby coated SPs were expected to exhibit greater NT3 retention over time therefore enabling improved drug delivery at later timepoints (weeks to months) than that achievable with uncoated SPs. While there are several obvious differences between *in vitro* and *in vivo* environments, another potential factor could be the chemistry of the coated SPs reducing capacity for the (Chi/Alg)SPs to interact with the RWM and/or increasing the propensity for immunoclearance of the (Chi/Alg)SPs from the middle ear [37]. Of note, previous studies that applied chitosan coated nanoparticles for cochlear viral transfection and neomycin delivery reported inconsistent transfection *in vitro* and *in vivo* [38, 39]. They attributed this variability to chitosan causing a transient increase in thickness of the RWM, impacting substance transfer [39]. We also have some preliminary data suggesting that the elution profile of NT3 *in vivo* is faster compared to elution *in vitro* (unpublished). Collectively, although chitosan is accepted as safe and effective for drug delivery in other systems, investigation of the mechanism underlying the discrepant results in the cochlea is required.

Given that there are many factors that contribute to the efficiency of NT3 delivery across the RWM (e.g. residence time, middle ear clearance, local tissue response, RWM permeability), we used intracochlear delivery to examine the ability of the (Chi/Alg)SPs to deliver drug over time without the influence of the above factors. In terms of clinical applicability, the intracochlear delivery route is most suited when the cochlea is already being accessed during cochlear implant surgery and NT3 can be applied to improve auditory neuron survival and patient outcomes [12-21]. We previously showed that intracochlear delivery of SPs carrying neurotrophin significantly improved auditory neuron survival in deafened guinea pigs [26], providing strong evidence that sufficient bioactive neurotrophin is released from the particles. We also showed that after intracochlear SPs implantation to guinea pigs, there was widespread drug distribution inside the cochlea, with an average of ~0.18 μg (corresponding to ~3.6%) of ^{125}I NT3 remaining after two weeks [24]. SPs implantation into cat cochleae led to accumulation of the ^{125}I NT3 signal at the basilar membrane of the lower basal turn, with up to ~0.9 μg (corresponding to ~2.5%) of drug remaining in the cochlea after four weeks. However, with the (Chi/Alg)SPs , there was much less NT3 within the cochlea at the same timepoint, nearly a 4.5 fold lower level of drug retained, with only ~0.2 μg (corresponding to ~0.66%) of ^{125}I NT3 detected. Nevertheless, in one of the cat cochleae treated with (Chi-Alg)SPs, accumulation of ^{125}I NT3 was detected on the basilar membrane even after 8 weeks indicating that the delivery system is capable of long-term delivery of a therapeutic molecule into the inner ear environment. Although only a small cohort of cats were used in this experiment this data indicates that there was no benefit of coating SPs with Chi/Alg to improve NT3 delivery into the cochlea over time. There are several potential reasons for the lower NT3 retention in (Chi/Alg)SPs *in vivo*. Firstly, the cochlear perilymph fluid is comprised of a complex chemical composition [40], that may interact with the (Chi/Alg)SPs and prompt its degradation. For instance, the presence of Ca^{2+} may react with the alginate and prompt erosion of the chitosan/alginate network. Secondly, flow of the perilymph fluid (albeit very slow) inside the cochlea may also replenish the ions the SPs are exposed to, thus further accelerating the eroding process of the (Chi/Alg) coating. Finally, the degradation of the (Chi/Alg)SPs is also influenced by properties of the polymers including molecular weight, viscosity, stiffness [41, 42].

To evaluate the functional changes of (Chi/Alg)SPs -mediated neurotrophin delivery to normal hearing cats, hearing function was tested. The delivery of SPs to the RWM had minimal effect on hearing function across the frequencies analysed. The intracochlear approach showed some hearing loss at the higher frequencies after surgery. The hearing loss was mostly likely caused by penetration of the RWM, surgical trauma from drilling the bulla and/or the presence of the SPs in the scala tympani. These findings reiterate the notion that the RWM approach is the safest route for drug

delivery and warrants further study into developing approaches to improve drug transfer across the RWM.

In our previous study, we showed that delivery of SPs using both the intracochlear and round window route promoted drug retention in the guinea pig cochlea even after one-month post-implantation [24]. In addition, we showed that the intracochlear delivery approach led to widespread drug distribution throughout the cochlea. While there was some variability in drug distribution with the RWM approach, across nearly all the animals tested, ^{125}I NT3 was detected in the basal region of the cochlea and within the RWM niche. Although the use of biocompatible coatings was effective in modifying drug release characteristics in *in vitro* elution studies, this was not observed when tested *in vivo*. These results highlight the importance of *in vivo* validation studies to test drug pharmacokinetics, whereby a more complex biological environment can have an impact. Nevertheless, we conclude based on our previous and current findings that the SPs are a viable approach to effectively deliver neurotrophins to the cochlea for extended durations.

No statistically significant difference in ^{125}I NT3 distribution after acute treatment of the RWM with HA and SPs.

HA is a viscous, high-molecular-weight polysaccharide that is broadly expressed in epithelial, connective, and neural tissue. It is approved by the United States Food and Drug Administration for use in cosmetic and ophthalmic surgeries or as a substrate for drug delivery. In the cochlea, both pre-clinical and clinical data indicate that intratympanic administration of steroids such as dexamethasone with HA produces a higher concentration of drug in the perilymph and restores auditory function (compared to dexamethasone alone; [43, 44]). The mechanism of HA activity in the cochlea is unknown, but it has been hypothesised that it increases the osmotic pressure above the RWM, improves membrane permeability, and/or promotes long-term contact of a drug/substance to the RWM due to its high viscosity promoting drug crossing across the RWM [31].

Given the clinical relevance of HA, we tested the potential for HA to improve the permeability of SP released NT3 across the guinea pig RWM. Here, the membrane was acutely treated with HA using gelfoam (~30 min) prior to SP implantation and analysed 4 hours and 3 days post-treatment. However, we found that there was no statistically significant difference in the retention of ^{125}I NT3 inside the cochlea after 3 days based on the whole cochlear gamma counts and autoradiography analyses. Other approaches to partially digest and permeabilize the RWM include chemicals such as sodium carprate [31, 45, 46], ultrasound microbubble disruption [47], creation of a micro-perforations [48], and/or incorporation of the Si-SPs in hydrogels to improve retention on the RWM [49]. Testing these approaches to improve diffusion of SP-released NT3 across the RWM warrants further investigation.

Understanding the mechanisms of drug transfer across the RWM is needed to advance clinical translation of hearing therapeutics

Several factors may have contributed to the low permeability of the RMW to ^{125}I NT3. In this study, the outer layer of the RWM is directly exposed to the SPs and the released ^{125}I NT3. The first factor that may have impacted drug transfer is the residence time of the SPs on the RWM [50]. Our data suggests that residence time may be a factor related to the level of drug inside the cochlea. In cases where there was an absence of SPs located on the RWM at the time of termination there was a higher propensity for the intracochlear signal to be reduced or absent (Table 2). Secondly, considerable evidence has revealed that the modality of transfer across the RWM varies based on the drug or substance placed, which remains unknown for the SPs and NT3. For example, the molecule may diffuse through the cytoplasm of the cells of the RWM (e.g., exotoxin), transverse via pinocytotic vesicles (e.g. cationic ferritin) or in some cases enter through different channels in between cells in

the outer epithelium [7, 8]. As the drug enters the second layer of the RWM, there is a possibility that the residing immune cells may phagocytize the substance and/or cause the drug to penetrate blood or lymph vessels in this layer before entering the perilymph contributing to the variable outcomes between animals. Thirdly, permeability of the RWM to a molecule may be impacted by the size, configuration, concentration, liposolubility and electrical charge of the molecule. Of note, NT3 is a large molecule with a size of 27.3 kDa and a positive charge. Finally, the thickness and condition of the RWM also plays a key role in drug transfer. Of note, the thickness of the RWM varies between species, whereby for guinea pigs, the thickness 10-14 μm, for cats its 20-40 μm and for humans its ~70 μm [7-9]. There is also the potential that with age or progression of hearing loss, there are structural changes to the RWM, which may further impact the mechanics of drug transfer across the membrane [51]. Although relevant to the clinical population, this may not have been a contributing factor in this study as age-matched normal hearing animals were used. Therefore, the passage of substances through the RWM is not a mere mechanical passage through an inert three-layered wall but represents a more dynamic biological mechanism whereby every substance that interacts with the RWM is dealt with in a different manner. As such, delivery strategies that overcome the RWM barrier to achieve effective long term drug treatment are likely to progress the development of clinically effective therapeutic treatments for hearing impairment.

Given the complexity and multitude of factors impacting transfer of small molecules, macromolecules, and viruses across the RWM, the development of *in vitro* or *ex vivo* RWM models will help to improve our understanding of drug transfer across the RWM [52-55]. Of note, a recent study developed and characterised an easy-to-build ex vivo porcine RWM model [56]. The porcine RWM is a similar thickness and size to human. They demonstrated the mechanics of the passage of the non-salt form of dexamethasone (Dex), a drug known to be highly permeable across the RWM, and the low-permeability non-salt form of Dex conjugated with fluorescein (DexF). Furthermore, the impact of permeabilization of the RWM on DexF passage using saponin and collagenase was presented. We believe the integration of models such as the above combined with computational modelling and ultimately *in vivo* testing, will be paramount for studying and optimising the passage of SP-released NT3 through the RWM to the cochlea.

6. CONCLUSIONS

Intratympanic administration of a therapeutic drug is the most favoured route for drug delivery across the RWM and into the cochlea as there is minimal risk to residual hearing. We previously reported that silica supraparticles (SPs) are an efficient modality for the delivery of bioactive NT3 into the cochlea. However, the effectiveness of SPs in releasing drug reduces over time both *in vitro* and *in vivo*. In this study, we developed a novel coating strategy to extend the drug release capacity of SPs by reducing the amount of drug eluted within the first few weeks. While the coated particles significantly improved the longevity of drug elution *in vitro*, no benefit of the coated particles was observed inside the cochlea, with elution potentially occurring much faster *in vivo*. We also noted considerable variability between animals in the amount of drug entering the cochlea. This may be attributed to the permeability of the RWM being highly variable between animals with up to 10-fold differences in drug adsorption across the round window [57]. We believe there is a need to understand the interaction and mechanism of the SP released NT3 across the RWM. Current studies are underway to develop novel approaches to improve drug entry across the RWM using SPs and evaluate the functional outcomes of the treatment in deaf animal models.

7. CREDIT AUTHOR STATEMENT

Niliksha Gunewardene: conceptualisation, methodology, formal analysis, investigation, data curation, writing-original draft, visualisation. Yutian Ma: conceptualisation, methodology, formal analysis, investigation, data curation, writing-original draft, visualisation. Patrick Lam: conceptualisation, methodology, formal analysis, visualization. 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.

8. ACKNOWLEDGEMENTS

This work was funded with support from the Department of Defence USA, Hearing Restoration Research Program, Translational Research Award W81XWH1810276, The Passe and Williams Foundation, the Australian National Health and Medical Research Council (GNT 1142910). F.C. acknowledges the award of a National Health and Medical Research Council Leadership Fellowship (GNT2016732). The Bionics Institute acknowledges the support it receives from the Victorian Government, Australia, through its Operational Infrastructure Support Program. The authors would like to thank Trung Nguyen, Donna Tuari, James Firth, Sayward Barone, Brianna Flynn, Alex Thompson, Ella Trang and Caitlin Singleton from the Bionics Institute, Melbourne, Australia, for technical assistance, Patricia WM Ho from St Vincent's Institute, Melbourne, Australia for the use of the gamma counter and David Casley from ProSearch International, Melbourne, Australia, for technical assistance with protein radiolabelling. Zhixing Lin from University of Melbourne, Australia, for technical assistance with SEM-EDX.

9. DATA AVAILABILITY

Source data are available upon request from the corresponding author.

10. 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.

REFERENCES

[1] N. Inamura, A.N. Salt, Permeability changes of the blood-labyrinth barrier measured in vivo during experimental treatments, Hear Res, 61 (1992) 12-18.
[2] K. Mizutari, M. Fujioka, M. Hosoya, N. Bramhall, H.J. Okano, H. Okano, A.S. Edge, Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma, Neuron, 77 (2013) 58-69.
[3] L.P. Rybak, A. Dhukhwa, D. Mukherjea, V. Ramkumar, Local Drug Delivery for Prevention of Hearing Loss, Front Cell Neurosci, 13 (2019) 300.
[4] C.R. Anderson, C. Xie, M.P. Su, M. Garcia, H. Blackshaw, A.G.M. Schilder, Local Delivery of Therapeutics to the Inner Ear: The State of the Science, Front Cell Neurosci, 13 (2019) 418.
[5] A.A. Mikulec, S.K. Plontke, J.J. Hartsock, A.N. Salt, Entry of substances into perilymph through the bone of the otic capsule after intratympanic applications in guinea pigs: implications for local drug delivery in humans, Otol Neurotol, 30 (2009) 131-138.
[6] S.K. Plontke, R. Mynatt, R.M. Gill, S. Borgmann, A.N. Salt, Concentration gradient along the scala tympani after local application of gentamicin to the round window membrane, Laryngoscope, 117 (2007) 1191-1198.

[7] M.V. Goycoolea, Clinical aspects of round window membrane permeability under normal and pathological conditions, Acta Otolaryngol, 121 (2001) 437-447.
[8] M.V. Goycoolea, D. Muchow, G.C. Martinez, P.B. Aguila, H.G. Goycoolea, C.V. Goycoolea, P. Schachern, W. Knight, Permeability of the human round-window membrane to cationic ferritin, Arch Otolaryngol Head Neck Surg, 114 (1988) 1247-1251.
[9] M.V. Goycoolea, A.M. Carpenter, D. Muchow, Ultrastructural studies of the round-window membrane of the cat, Arch Otolaryngol Head Neck Surg, 113 (1987) 617-624.
[10] Y. Hamaguchi, T. Morizono, S.K. Juhn, Round window membrane permeability to human serum albumin in antigen-induced otitis media, Am J Otolaryngol, 9 (1988) 34-40.
[11] J. Suzuki, G. Corfas, M.C. Liberman, Round-window delivery of neurotrophin 3 regenerates cochlear synapses after acoustic overexposure, Sci Rep, 6 (2016) 24907.
[12] P. Ernfors, M.L. Duan, W.M. ElShamy, B. Canlon, Protection of auditory neurons from aminoglycoside toxicity by neurotrophin-3, Nat Med, 2 (1996) 463-467.
[13] S.L. McGuinness, R.K. Shepherd, Exogenous BDNF rescues rat spiral ganglion neurons in vivo, Otol Neurotol, 26 (2005) 1064-1072.
[14] A.K. Wise, R. Richardson, J. Hardman, G. Clark, S. O'Leary, Resprouting and survival of guinea pig cochlear neurons in response to the administration of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3, J Comp Neurol, 487 (2005) 147-165.
[15] A.J. Evans, B.C. Thompson, G.G. Wallace, R. Millard, S.J. O'Leary, G.M. Clark, R.K. Shepherd, R.T. Richardson, Promoting neurite outgrowth from spiral ganglion neuron explants using polypyrrole/BDNF-coated electrodes, J Biomed Mater Res A, 91 (2009) 241-250.
[16] L.N. Gillespie, G.M. Clark, P.L. Marzella, Delayed neurotrophin treatment supports auditory neuron survival in deaf guinea pigs, Neuroreport, 15 (2004) 1121-1125.
[17] T.G. Landry, A.K. Wise, J.B. Fallon, R.K. Shepherd, Spiral ganglion neuron survival and function in the deafened cochlea following chronic neurotrophic treatment, Hearing research, 282 (2011) 303-313.
[18] R.K. Shepherd, A. Coco, S.B. Epp, Neurotrophins and electrical stimulation for protection and repair of spiral ganglion neurons following sensorineural hearing loss, Hear Res, 242 (2008) 100-109.
[19] A.K. Wise, R. Richardson, J. Hardman, G. Clark, S. O'Leary, Resprouting and survival of guinea pig cochlear neurons in response to the administration of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3, The Journal of comparative neurology, 487 (2005) 147-165.
[20] R. Glueckert, M. Bitsche, J.M. Miller, Y. Zhu, D.M. Prieskorn, R.A. Altschuler, A. Schrott-Fischer, Deafferentation-associated changes in afferent and efferent processes in the guinea pig cochlea and afferent regeneration with chronic intrascalar brain-derived neurotrophic factor and acidic fibroblast growth factor, J Comp Neurol, 507 (2008) 1602-1621.
[21] P.A. Leake, G.T. Hradek, A.M. Hetherington, O. Stakhovskaya, Brain-derived neurotrophic factor promotes cochlear spiral ganglion cell survival and function in deafened, developing cats, The Journal of comparative neurology, 519 (2011) 1526-1545.
[22] R.T. Richardson, Q.Y. Hu, F. Shi, T. Nguyen, J.B. Fallon, B.O. Flynn, A.K. Wise, Pharmacokinetics and tissue distribution of neurotrophin 3 after intracochlear delivery, J Control Release, 299 (2019) 53-63.
[23] Y. Ma, A.K. Wise, R.K. Shepherd, R.T. Richardson, New molecular therapies for the treatment of hearing loss, Pharmacol Ther, 200 (2019) 190-209.
[24] N. Gunewardene, P. Lam, Y. Ma, F. Caruso, S. Wagstaff, R.T. Richardson, A.K. Wise, Pharmacokinetics and biodistribution of supraparticle-delivered neurotrophin 3 in the guinea pig cochlea, J Control Release, 342 (2022) 295-307.
[25] P. Lam, N. Gunewardene, Y. Ma, F. Caruso, T. Nguyen, B. Flynn, A.K. Wise, R.T. Richardson, A radiolabeled drug tracing method to study neurotrophin-3 retention and distribution in the cochlea after nano-based local delivery, MethodsX, 7 (2020) 101078.
[26] A.K. Wise, J. Tan, Y. Wang, F. Caruso, R.K. Shepherd, Improved Auditory Nerve Survival with Nanoengineered Supraparticles for Neurotrophin Delivery into the Deafened Cochlea, PLoS One, 11 (2016) e0164867.
[27] Y. Ma, M. Bjornmalm, A.K. Wise, C. Cortez-Jugo, E. Revalor, Y. Ju, O.M. Feeney, R.T. Richardson, E. Hanssen, R.K. Shepherd, C.J.H. Porter, F. Caruso, Gel-Mediated Electrospray

Assembly of Silica Supraparticles for Sustained Drug Delivery, ACS Appl Mater Interfaces, 10 (2018) 31019-31031.
[28] Y. Ma, C. Cortez-Jugo, J. Li, Z. Lin, R.T. Richardson, Y. Han, J. Zhou, M. Bjornmalm, O.M. Feeney, Q.Z. Zhong, C.J.H. Porter, A.K. Wise, F. Caruso, Engineering Biocoatings To Prolong Drug Release from Supraparticles, Biomacromolecules, 20 (2019) 3425-3434.
[29] R.T. Richardson, Q.-Y. Hu, F. Shi, T. Nguyen, J.B. Fallon, B.O. Flynn, A.K. Wise, Pharmacokinetics and tissue distribution of neurotrophin 3 after intracochlear delivery, Journal of Controlled Release, 299 (2019) 53-63.
[30] R.T. Richardson, A. Wise, S. O'Leary, J. Hardman, D. Casley, G. Clark, Tracing neurotrophin-3 diffusion and uptake in the guinea pig cochlea, Hear Res, 198 (2004) 25-35.
[31] S.B. Shibata, S.R. Cortez, J.A. Wiler, D.L. Swiderski, Y. Raphael, Hyaluronic acid enhances gene delivery into the cochlea, Hum Gene Ther, 23 (2012) 302-310.
[32] M. Faria, M. Björnmalm, K.J. Thurecht, S.J. Kent, R.G. Parton, M. Kavallaris, A.P. Johnston, J.J. Gooding, S.R. Corrie, B.J. Boyd, Minimum information reporting in bio–nano experimental literature, Nature nanotechnology, 13 (2018) 777-785.
[33] J. Hatami, S.G. Silva, M.B. Oliveira, R.R. Costa, R.L. Reis, J.F. Mano, Multilayered films produced by layer-by-layer assembly of chitosan and alginate as a potential platform for the formation of human adipose-derived stem cell aggregates, Polymers, 9 (2017) 440.
[34] N.-J. Cho, K.K. Kanazawa, J.S. Glenn, C.W. Frank, Employing two different quartz crystal microbalance models to study changes in viscoelastic behavior upon transformation of lipid vesicles to a bilayer on a gold surface, Analytical chemistry, 79 (2007) 7027-7035.
[35] S. Hatsushika, R.K. Shepherd, Y.C. Tong, G.M. Clark, S. Funasaka, Dimensions of the scala tympani in the human and cat with reference to cochlear implants, Ann Otol Rhinol Laryngol, 99 (1990) 871-876.
[36] Y.J. Hwang, M. Park, M.K. Park, J.H. Lee, S.H. Oh, M.W. Suh, High-Molecular-Weight Hyaluronic Acid Vehicle Can Deliver Gadolinium Into the Cochlea at a Higher Concentration for a Longer Duration: A 9.4-T Magnetic Resonance Imaging Study, Front Neurol, 12 (2021) 650884.
[37] K. Paunovska, C.D. Sago, C.M. Monaco, W.H. Hudson, M.G. Castro, T.G. Rudoltz, S. Kalathoor, D.A. Vanover, P.J. Santangelo, R. Ahmed, A.V. Bryksin, J.E. Dahlman, A Direct Comparison of in Vitro and in Vivo Nucleic Acid Delivery Mediated by Hundreds of Nanoparticles Reveals a Weak Correlation, Nano Lett, 18 (2018) 2148-2157.
[38] A. Saber, S.P. Strand, M. Ulfendahl, Use of the biodegradable polymer chitosan as a vehicle for applying drugs to the inner ear, Eur J Pharm Sci, 39 (2010) 110-115.
[39] A. Saber, C. Gont, S. Dash-Wagh, M. Kirkegaard, S. Strand, M. Ulfendahl, Assessment of in Vitro and in Vivo Transfection Efficiency of the Biodegradable
Polymer Chitosan in the Inner Ear, Journal of International Advanced Otology, 6 (2010) 307-315.
[40] J.C. Palmer, M.S. Lord, J.L. Pinyon, A.K. Wise, N.H. Lovell, P.M. Carter, Y.L. Enke, G.D. Housley, R.A. Green, Comparing perilymph proteomes across species, Laryngoscope, 128 (2018) E47-E52.
[41] E. Axpe, M.L. Oyen, Applications of alginate-based bioinks in 3D bioprinting, International journal of molecular sciences, 17 (2016) 1976.
[42] T. Boontheekul, H.-J. Kong, D.J. Mooney, Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution, Biomaterials, 26 (2005) 2455-2465.
[43] S.S. Chandrasekhar, R.Y. Rubinstein, J.A. Kwartler, M. Gatz, P.E. Connelly, E. Huang, S. Baredes, Dexamethasone pharmacokinetics in the inner ear: comparison of route of administration and use of facilitating agents, Otolaryngol Head Neck Surg, 122 (2000) 521-528.
[44] O.A. Selivanova, H. Gouveris, A. Victor, R.G. Amedee, W. Mann, Intratympanic dexamethasone and hyaluronic acid in patients with low-frequency and Meniere's-associated sudden sensorineural hearing loss, Otol Neurotol, 26 (2005) 890-895.
[45] H. Wang, R. Murphy, D. Taaffe, S. Yin, L. Xia, W.W. Hauswirth, M. Bance, G.S. Robertson, J. Wang, Efficient cochlear gene transfection in guinea-pigs with adeno-associated viral vectors by partial digestion of round window membrane, Gene Ther, 19 (2012) 255-263.
[46] W. Li, J.J. Hartsock, C. Dai, A.N. Salt, Permeation Enhancers for Intratympanically-applied Drugs Studied Using Fluorescent Dexamethasone as a Marker, Otol Neurotol, 39 (2018) 639-647.

[47] Y.C. Lin, C.P. Shih, H.C. Chen, Y.L. Chou, H.K. Sytwu, M.C. Fang, Y.Y. Lin, C.Y. Kuo, H.H. Su, C.L. Hung, H.K. Chen, C.H. Wang, Ultrasound Microbubble-Facilitated Inner Ear Delivery of Gold Nanoparticles Involves Transient Disruption of the Tight Junction Barrier in the Round Window Membrane, Front Pharmacol, 12 (2021) 689032.
[48] C.M. Kelso, H. Watanabe, J.M. Wazen, T. Bucher, Z.J. Qian, E.S. Olson, J.W. Kysar, A.K. Lalwani, Microperforations significantly enhance diffusion across round window membrane, Otol Neurotol, 36 (2015) 694-700.
[49] C. Honeder, C. Zhu, H. Schopper, J.C. Gausterer, M. Walter, L.D. Landegger, N. Saidov, D. Riss, R. Plasenzotti, F. Gabor, C. Arnoldner, Effects of sustained release dexamethasone hydrogels in hearing preservation cochlear implantation, Hear Res, 341 (2016) 43-49.
[50] A.N. Salt, D.B. Sirjani, J.J. Hartsock, R.M. Gill, S.K. Plontke, Marker retention in the cochlea following injections through the round window membrane, Hear Res, 232 (2007) 78-86.
[51] S. Jiang, T.W. Seale, R.Z. Gan, Morphological changes in the round window membrane associated with Haemophilus influenzae-induced acute otitis media in the chinchilla, Int J Pediatr Otorhinolaryngol, 88 (2016) 74-81.
[52] R. Singh, B. Birru, J.G.S. Veit, E.M. Arrigali, M.A. Serban, Development and Characterization of an In Vitro Round Window Membrane Model for Drug Permeability Evaluations, Pharmaceuticals (Basel), 15 (2022).
[53] J.J. Sun, Y. Liu, W.J. Kong, P. Jiang, W. Jiang, In vitro permeability of round window membrane to transforming dexamethasone with delivery vehicles--a dosage estimation, Chin Med J (Engl), 120 (2007) 2284-2289.
[54] M.C. Witte, J.L. Kasperbauer, Round window membrane permeability to transforming growth factor-alpha: an in vitro study, Otolaryngol Head Neck Surg, 123 (2000) 91-96.
[55] L.A. Lundman, L. Holmquist, D. Bagger-Sjoback, Round window membrane permeability. An in vitro model, Acta Otolaryngol, 104 (1987) 472-480.
[56] A. Moatti, D. Silkstone, T. Martin, K. Abbey, K.A. Hutson, D.C. Fitzpatrick, C.J. Zdanski, A.G. Cheng, F.S. Ligler, A. Greenbaum, Assessment of drug permeability through an ex vivo porcine round window membrane model, iScience, 26 (2023) 106789.
[57] H. Hahn, B. Kammerer, A. DiMauro, A.N. Salt, S.K. Plontke, Cochlear microdialysis for quantification of dexamethasone and fluorescein entry into scala tympani during round window administration, Hear Res, 212 (2006) 236-244.

Highlights

- Supraparticles are a viable approach to deliver neurotrophin to the cochlea
- Neurotrophin was detected in the cochlea 8 weeks post-supraparticle implantation
- Variable drug distribution after round membrane implantation of supraparticles
- Coated supraparticles improve the longevity of drug elution *in vitro*, but not *in vivo*