

Nanoengineered Drug Delivery Systems for the Treatment of Sensorineural Hearing Loss

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ABSTRACT

Innear ear disease is the leading cause of hearing impairment in developed countries.

An estimated 466 million people suffer from hearing loss worldwide and this number is on the rise. Sensorineural hearing loss (SNHL) is the most common form of hearing impairment and is characterised by the degeneration of key structures of the sensory pathway in the cochlea of the inner ear (the cochlea) such as the sensory hair cells, the primary auditory neurons and their synaptic connection to the hair cells. Current research focuses on developing techniques to administer growth proteins such as neurotrophins to repair or regenerate damaged auditory neurons, as well as preventing loss of primary auditory neurons. Drug delivery systems are being developed to treat SNHL, such as cell-based drug delivery systems and gene vectors, however nanoengineered systems show promise to address the specific needs of neurotrophin-based therapies such as safety, high dosing and long-term delivery to the cochlea. Research carried out in this thesis has developed this technology further by the scale-up production of nanoengineered silica-based supraparticles (SPs) (particles comprised of primary silica nanoparticles that form together to produce large SPs (~550 μm) with high porosity) and the development of several strategies towards their application as viable drug delivery platforms for achieving sustained drug release in the inner ear, as detailed in Chapters 3–6.

In Chapter 3, a gel-mediated electrospray technique was developed to synthesise silica supraparticles (Si-SPs) in high yields. The Si-SPs were assembled from different primary silica particles i.e., particles with no pores, small pores (2–3 nm) and bimodal

large pores (2–3 nm and 15–64 nm). A high loading of fluorescently labelled model protein (fluorescein isothiocyanate (FITC)-lysozyme) and neurotrophic factor (a drug for the treatment of inner ear disease) in the Si-SPs was possible and the resulting particle system could achieve sustained drug release for over 150 days. The findings demonstrate that gel-mediated electrospray is a robust and automatable technology to produce Si-SPs, which is a promising platform for clinical translation and commercialisation.

In Chapter 4, the pharmacokinetics of the neurotrophin brain-derived neurotrophic factor (BDNF) from Si-SPs was examined as engineering drug delivery systems with well-defined pharmacokinetics is important for clinical translation. BDNF-loaded Si-SPs were surgically implanted either directly into the cochlea, or onto a semi-permeable membrane (the round window membrane; RWM) that is a boundary between the middle and inner ear. Treatment duration was for either 3 or 7 days whereby the fluids from the cochleae were sampled and tested for BDNF levels. The results showed that the BDNF released from the Si-SPs was detected in the cochlear fluids indicating that the approach has potential as a clinically relevant neurotrophin delivery strategy to treat people with hearing impairment.

In Chapter 5, a bioengineering coating strategy was developed for retarding the initial burst release of neurotrophins from the Si-SPs. Applying a fibrin coating on the surface of the Si-SPs and embedding the fibrin-coated Si-SPs within an alginate CaCO_3 hydrogel both slowed the initial burst release to improve the drug release kinetics. The results demonstrate the suitability of alginate CaCO_3 hydrogel systems for surgical handling of the Si-SP system.

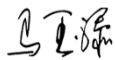
In Chapter 6, a chitosan and an alginate layer-by-layer coating on the Si-SPs was developed as an alternative strategy for delaying the initial burst release of uncoated Si-SPs. Chitosan and alginate are two biocompatible polysaccharides that interact electrostatically via the carboxyl groups from alginate (negatively charged) and the amine groups on chitosan (positively charged). By varying the layer number and hence, the thickness of the coating, different release profiles were attained. *In vitro* neurotrophins release profiles showed that chitosan-alginate-coated silica supraparticles (^(Chi/Alg)Si-SPs) experienced a delayed initial burst release. Spiral ganglion neurons culture and neurite length analysis indicated that the neurotrophins released from ^(Chi/Alg)Si-SPs had maintained biological activity. Functional hearing was tested using auditory brainstem responses (ABRs) to determine the safety profile of surgical delivery of coated SPs to the inner ear. Hearing thresholds were maintained within the normal range following RWM, however an increase in thresholds for high frequency sounds were observed following implantation of ^(Chi/Alg)Si-SPs into the cochlea. Scanning electron microscopy images of ^(Chi/Alg)Si-SPs collected following *in vivo* implantation along with a commercial viable fibrin sealant indicated the biodegradability of ^(Chi/Alg)Si-SPs post-implantation. These results indicate that ^(Chi/Alg)Si-SPs can potentially be used as a clinically applicable platform for sustained inner ear neurotrophin delivery.

In summary, this thesis expands knowledge in the development and engineering of Si-SPs in addressing key neurotrophin delivery issues for the treatment of hearing loss, including high yield, sustained drug release, well-defined pharmacokinetics and biodegradability.

DECLARATION

This is to certify that:

- (i) The thesis comprises only my original work towards the PhD except where indicated in the Preface;
- (ii) Due acknowledgement has been made in the text to all other material used;
- (iii) The thesis is less than 100,000 words in length, exclusive of tables, bibliographies, appendices and footnotes.



Yutian Ma

PREFACE

Part of this chapter has been included in the following publication. All text included in this thesis was independently written by the author of this thesis, with suggestions and feedback from co-authors of the publications.

Chapter 1

- **Yutian Ma**, Andrew K. Wise, Robert K. Shepherd, Rachael T. Richardson. “New Molecular Therapies for the Treatment of Hearing Loss.” *Pharmacology & Therapeutics* **2019**, *200*, 190-209. Copyright 2019 Elsevier.

Chapter 3

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Robert K. Shepherd, Prof. Christopher J. H. Porter and Prof. Frank Caruso provided helpful discussion for the development of ideas and experimental planning.

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Dr. Christina Cortez-Jugo helped with the cell culture. Dr. Jianhua Li contributed to the data collection. Zhixing Lin and Yiyuan Han assisted with SEM images and ICP measurements, respectively. A. Prof. Rachael T. Richardson and Dr. Orlagh M. Feeney assisted with the ELISA studies. Dr. Jiajing Zhou, Dr. Mattias Björnmalm, Qizhi Zhong and Prof. Christopher J.H. Porter provided helpful discussions on experimental planning. A. Prof. Andrew K. Wise and Prof. Frank Caruso contributed to the development of ideas and experimental planning.

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PUBLICATIONS

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6. Zhong, Q. Z.; Pan, S.; Rahim, M. A.; Yun, G.; Li, J.; Ju, Y.; Lin, Z.; Han, Y.; **Ma, Y.**; Richardson, J. J.; Caruso, F. “Spray Assembly of Metal–Phenolic Networks: Formation, Growth, and Applications.” *ACS Appl Mater Interfaces* **2018**, *10*, 33721-33729.

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7. **Ma, Y.** “Supaparticles with Sustained Drug Delivery for Treating Hearing Loss and Clinical Translation” Department of Materials, July 2019, Imperial College London, London, United Kingdom.
8. **Ma, Y.** “Fibrin Coated Silica Supraparticles with Alginate-Calcium Carbonate Particle Hydrogels for Long-term Controlled Drug Delivery” Nanostructured Interfaces

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Chapter 1

Introduction

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New molecular therapies for the treatment of hearing loss

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ABSTRACT

An estimated 466 million people suffer from hearing loss worldwide. Sensorineural hearing loss is characterized by degeneration of key structures of the sensory pathway in the cochlea such as the sensory hair cells, the primary auditory neurons and their synaptic connection to the hair cells – the ribbon synapse. Various strategies to protect or regenerate these sensory cells and structures are the subject of intensive research. Yet despite recent advances in our understandings of the capacity of the cochlea for repair and regeneration there are currently no pharmacological or biological interventions for hearing loss. Current research focusses on localized cochlear drug, gene and cell-based therapies. One of the more promising drug-based therapies is based on neurotrophic factors for the repair of the ribbon synapse after noise exposure, as well as preventing loss of primary auditory neurons and regrowth of the auditory neuron fibers after severe hearing loss. Drug therapy delivery technologies are being employed to address the specific needs of neurotrophin and other therapies for hearing loss that include the need for high doses, long-term delivery, localised or cell-specific targeting and techniques for their safe and efficacious delivery to the cochlea. Novel biomaterials are enabling high payloads of drugs to be administered to the cochlea with subsequent slow-release properties that are proving to be beneficial for treating hearing loss. In parallel, new gene therapy technologies are addressing the need for cell specificity and high efficacy for the treatment of both genetic and acquired hearing loss with promising reports of hearing recovery. Some biomaterials and cell therapies are being used in conjunction with the cochlear implant ensuring therapeutic benefit to the primary neurons during electrical stimulation. This review will introduce the auditory system, hearing loss and the potential for repair and regeneration in the cochlea. Drug delivery to the cochlea will then be reviewed, with a focus on new biomaterials, gene therapy technologies, cell therapy and the use of the cochlear implant as a vehicle for drug delivery. With the current pre-clinical research effort into therapies for hearing loss, including clinical trials for gene therapy, the future for the treatment for hearing loss is looking bright.

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Abbreviations: AAV, adeno-associated virus; AN, auditory nerve; BM, basilar membrane; BDNF, brain derived neurotrophic factor; CRISPR, clustered regularly interspaced short palindromic repeats; CSF, cerebrospinal fluid; iPSCs, induced pluripotent stem cells; NT-3, neurotrophin-3; OW, oval window; PLGA, poly (d,l-lactide-co-glycolide acid); RNA, RNA interference; RW, round window; SC, semi-circular canal; SM, scala media; ST, scala tympani; SV, scala vestibuli; SEM, scanning electron microscope; SGNs, spiral ganglion neurons; SNHL, sensorineural hearing loss; TMC1, transmembrane channel-like protein 1; VGLUT1/3, vesicular glutamate transporter type 1/3.

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1.1 SUMMARY

This chapter mainly focuses on introducing the main themes and relevant literature related to this thesis. This includes description of the auditory system, hearing loss and the potential for repair and regeneration in the cochlea, and drug delivery strategies to the cochlea with a focus on new biomaterials, gene therapy technologies, cell therapy and the use of the cochlear implant as a vehicle for drug delivery. The aim is to highlight the challenges and opportunities for pre-clinical research, which can provide guidance for the future treatment of hearing loss based on nanoengineered approaches.

LIST OF ABBREVIATIONS

AAV – adeno-associated virus

AN – auditory nerve

BM – basilar membrane

BDNF – brain derived neurotrophic factor

CRISPR – clustered regularly interspaced short palindromic repeats

CSF – cerebrospinal fluid

IPSCs – induced pluripotent stem cells

NT-3 – neurotrophin-3

OW – oval window

PLGA - poly (D,L-lactide-*co*-glycolide acid)

RW – round window

SC – semi-circular canal

SM – scala media

ST – scala tympani

SV – scala vestibuli

SEM – scanning electron microscope

SGNs – spiral ganglion neurons

SNHL – sensorineural hearing loss

TMC1 – transmembrane channel-like protein 1

VGLUT1/3 - vesicular glutamate transporter type 1/3

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1.2 BACKGROUND

Over 5% of the world's population is living with a disabling hearing loss impacting on the individual's ability to communicate with others.¹ Despite being a very important part of our lives, hearing is often taken for granted until it begins to deteriorate. The severity of the pathology ranges from loss of the synapse between the hair cells that convert sound input into electrical signals and the SGNs that transmit the electrical signals to the brain, to more severe cases with loss of hair cells and loss of SGNs. There are currently very few treatment options for people with hearing loss. Hearing aids may be used to amplify sounds for people with a mild or moderate hearing loss, whereas cochlear implants are suitable for people experiencing a more significant hearing impairment. Although hearing aids and cochlear implants partially reverse the symptoms of hearing loss, they do not restore the underlying pathology: cellular degeneration in the cochlea and loss of connection between the cochlea and the central auditory processing areas of the brain. At the pre-clinical level, there is mounting evidence of therapeutic protection and/or regeneration of hair cells, SGNs and other affected cell types with numerous examples of hearing protection and hearing recovery. Therapeutic strategies focus on overcoming some of the obstacles of providing safe and efficacious localised drug delivery to the cochlea. This chapter will provide an overview of the auditory system, hearing loss, capacity for repair and regeneration and the current status of targeted therapies for drug delivery to the cochlea.

1.3 NORMAL COCHLEAR ANATOMY AND FUNCTION

The peripheral auditory system consists of the external, middle and inner ear (or cochlea) (**Figure 1**).² The external auditory canal provides efficient transmission of the

acoustic stimulus to the tympanic membrane or ear drum. The middle ear cavity, bound by the tympanic membrane laterally and the cochlea medially, efficiently couples sound energy from the air-filled external auditory canal to the fluid-filled cochlea via the ossicular chain consisting of the malleus, incus and stapes.³ Fluid in the cochlea is displaced by movement of the stapes onto the OW located in the base of the cochlea (**Figure 2a**). The RW membrane, also located near the base of the cochlea, vibrates in opposite phase to the OW as the fluid in the cochlea is displaced in response to acoustic stimulation. The RW provides a site for surgical access to the cochlea for both cochlear implants and drug delivery, as discussed later in the chapter, but the potential risks of additional hearing loss from targeting this membrane must be carefully considered when developing safe and effective drug delivery therapies (**Figure 2a**).

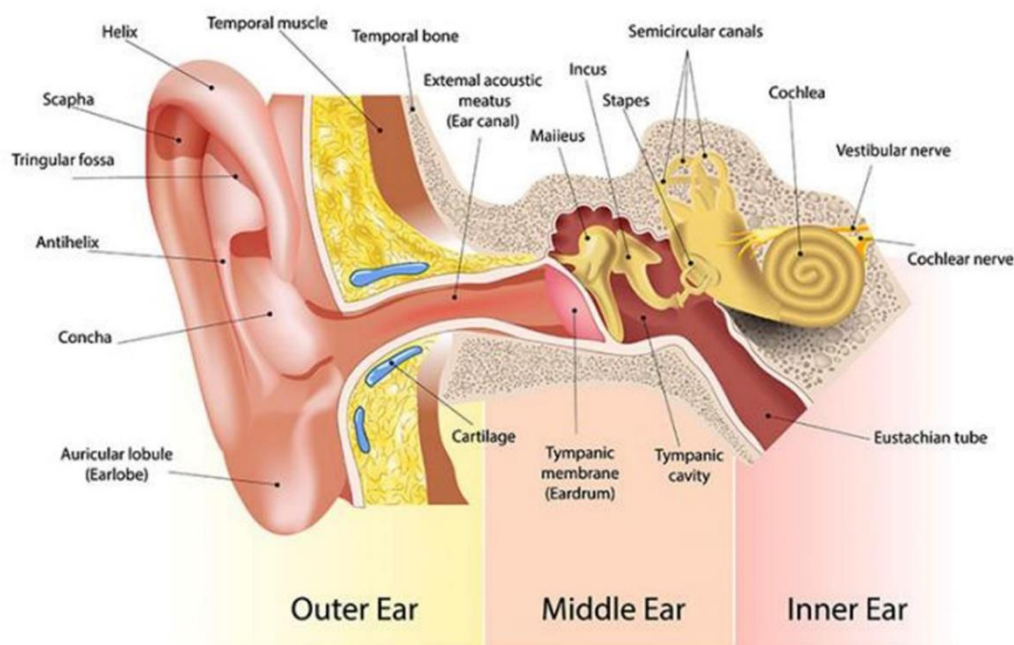


Figure 1. Coronal view of the ear showing the outer, middle, inner ear components.²

The cochlea is a spiral-shaped labyrinth embedded within the temporal bone. Three fluid-filled scalae form the membranous labyrinth (**Figure 2b**). The BM separates the ST from the SM while Reissner's membrane separates the SM from the SV (**Figure 2c**).

Both the ST and SV contain perilymph, an extracellular fluid similar to CSF.⁴ In contrast, the SM contains endolymph, a unique extracellular fluid that has a high concentration of potassium and a low concentration of sodium, a composition that is important for the normal process of transduction in the hair cells.⁵ The cells lining the perimeter of the SM are joined by intercellular tight junctions that maintain the unique electrolyte composition of endolymph.⁶ In the majority of cases, cochlear therapy techniques focus on delivery of a drug to the perilymph. In humans, the total perilymph volume is approximately 158 mm³ which includes the cochlea and SCs as there is free passage of fluids between these organs. The endolymph is sometimes targeted for therapies, with its volume being just 34 mm.^{3,7,8}

The sensory cells of the cochlea are located within the organ of Corti, a complex structure attached to the BM (**Figure 2d**). In the human, the BM is approximately 32 mm in length from the cochlea base to the apex. The mechanical characteristics of the BM allow the cochlea to resolve different frequencies at different sites along its length; higher frequencies in the base of the cochlea close to the middle ear and lower frequencies at progressively more distal sites within more apical regions of the cochlea.⁹

The sensory hair cells consist of a single row of inner hair cells and three rows of outer hair cells (**Figure 2d**). The human cochlea contains approximately 3200 inner hair cells and 10,000 outer hair cells.¹⁰ Both inner and outer hair cells contain up to 150 stereocilia protruding from their apical surface. On displacement of the basilar membrane, mechanically sensitive ion channels located in the stereocilia initiate depolarisation of the hair cell. This in turn results in the release of the excitatory neurotransmitter glutamate, depolarising primary afferent neurons innervating the hair cell, the SGNs.^{11,12} Both the hair cells and the SGNs are the most common target cell types for therapies to prevent or reverse hearing loss.

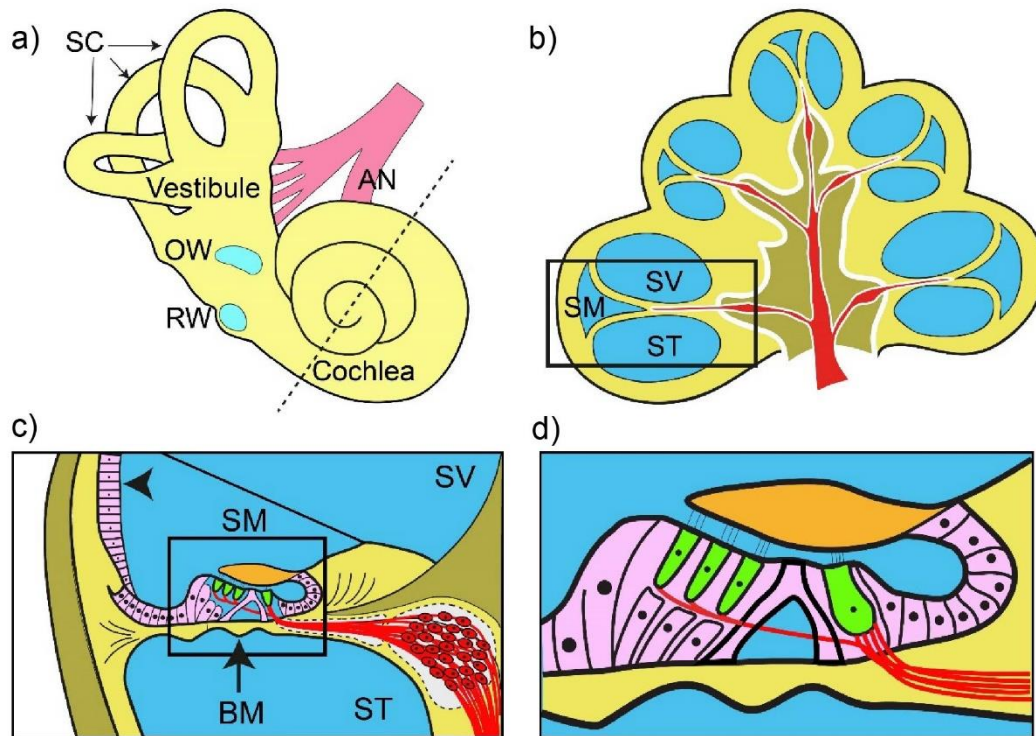


Figure 2. (a) Schematic image of the inner ear depicting the three SC, the OW, the RW and the AN. Drugs can be delivered to the inner ear via the semi-permeable round and OWs or directly into the SC or cochlea via a small hole drilled through the bony wall or by piercing the RW. The dotted line bisects the cochlea in half which is depicted schematically in (b). (b) Transverse section of the human cochlea illustrating the three fluid-filled cochlear ducts shown in blue i.e., SV, SM and ST - spiralling around the modiolus containing the AN (red). The boxed region is shown in (c) in greater detail. (c) The organ of Corti (boxed) is located on the BM (arrow) that separates the ST from the SM. The lateral wall of the SM contains the stria vascularis (arrow head), which is responsible for the generation of the endocochlear potential. The SGN soma (red) are located in a small bony canal (Rosenthal's canal) that also spirals from the cochlear base to its apex. (d) The organ of Corti contains the two types of sensory hair cells—a single row of inner hair cells and three rows of outer hair cells (green)—together with

supporting cells (pink). Peripheral fibres from SGNs (red) form synapses with the inner and outer hair cells.¹³

Inner and outer hair cells are innervated by specific SGN populations; the difference in their afferent innervation reflects the different roles these two hair cell types play in the transduction process. Type I SGNs, which represent ~90% of the total SGN population, exclusively innervate inner hair cells, whereas Type II SGNs exclusively innervate outer hair cells.¹⁴⁻¹⁶ The two hair cell populations play different and unique roles in auditory perception; inner hair cells convey auditory stimuli to the central auditory pathway via their innervation with the primary SGN population, whereas outer hair cells provide biological amplification within the cochlea that increases hearing sensitivity to low intensity stimuli.¹¹ The central fibres of the SGNs form the AN and projects to the cochlear nucleus—the first relay centre within the central auditory pathway.¹⁶ There are approximately 30,000 SGNs in a normal human cochlea.¹⁷

The peripheral fibres of both Type I and II SGNs project from their soma in Rosenthal's canal through a small opening in the bony wall called the habenula perforata to the organ of Corti. Each Type I peripheral fibre makes a single synaptic connection with the closest inner hair cell. This synapse, known as a ribbon synapse, promotes the rapid release of neurotransmitter and can maintain high firing rates important for the temporal fidelity of sound processing.¹⁸ In contrast, Type II peripheral fibres cross the floor of the organ of Corti and synapse with multiple outer hair cells.¹⁷ The ribbon synapses in the inner hair cells have recently been identified as sensitive to even moderate noise exposure, preferentially affecting high-threshold neurons and are therefore a specific target for drug therapies in addition to hair cells and SGNs more generally,¹⁹⁻²² as outlined later in the chapter.

1.4 DEAFNESS

1.4.1 IMPACT ON PEOPLE

Over 432 million adults and 34 million children have a disabling hearing loss, making it a major health and economic burden on society.¹ Hearing impairment can result in significant communication disorders including poor development of spoken and written language in children, leading to significant educational, social and vocational ramifications that can adversely affect quality of life. In adults, hearing loss affects employment, income, social interactions and self-esteem.^{23,24} Moreover, hearing loss is a potential risk factor for accelerated cognitive decline and cognitive impairment in the elderly associated with increased social isolation.²⁵⁻²⁹ There are also links to the development of depression and its impact on the individual.²⁹⁻³²

1.4.2 REHABILITATION

There are two forms of deafness: conductive and sensorineural (**Figure 3**). Conductive deafness is a mechanically based hearing loss associated with the external or middle ear. It can occur as a result of middle ear infections or ossification of middle ear bones that affect the capacity of the auditory structures to transmit sound information to the inner ear. It is typically a partial hearing loss, and surgery and/or amplification via a hearing aid are the standard treatments. SNHL is the most common form of deafness and typically occurs following damage to, or dysfunction of, the sensory hair cells within the inner ear,³³ their synapse with primary auditory neurons (termed synaptopathy)³⁴ or the stria vascularis.³⁵ Extensive hair cell damage can result in a hearing loss that encompasses the important frequency range for human speech (~300 Hz–8 kHz). Although a mild SNHL can be clinically managed using hearing aids,

people with severe-to-profound SNHL may benefit from a cochlear implant which uses electrical stimulation to activate the AN. Cochlear implants enable recipients to understand speech in good listening conditions; however, results can vary considerably and performance in challenging or noisy environments is typically poor.^{36,37}

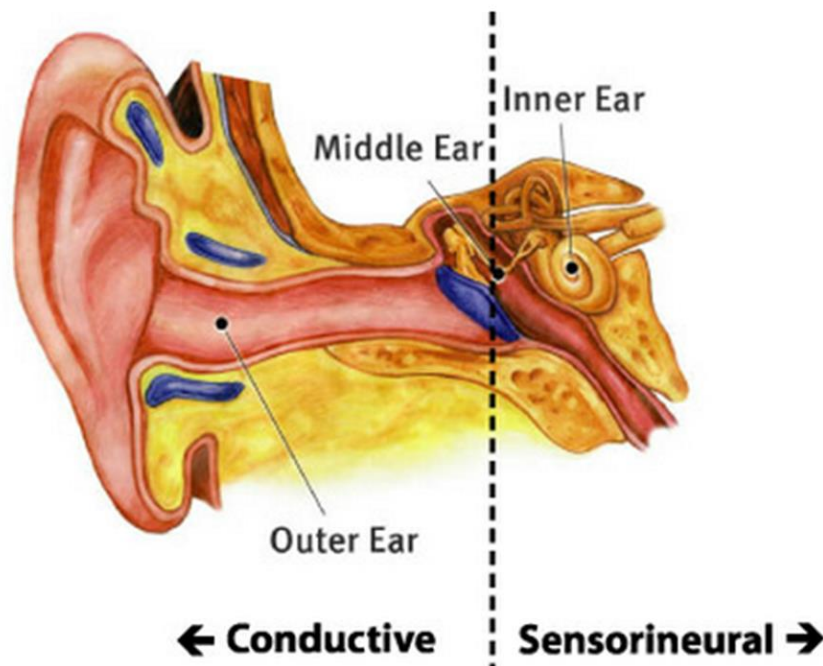


Figure 3. Schematic illustration of conductive hearing loss and SNHL.³⁸

Auditory rehabilitation, including the use of hearing aids and cochlear implants, results in positive improvements in terms of educational outcomes in children³⁹⁻⁴¹ as well as a decline in social isolation, depression and cognitive deterioration in the elderly.⁴² However, a perceived stigma of hearing loss in challenging environments often prevents people from taking up these technologies.²³ The implementation of drug therapies for hearing loss has the potential to not only reach a greater portion of affected individuals, but also improve the outcomes when used in conjunction with devices.

1.4.3 CAUSES OF SNHL

SNHL can be hereditary or may be acquired as a result of infection or injury, long-term exposure to loud noise or drugs that are toxic to hair cells. There is also a strong correlation between ageing and hearing loss suggesting that many factors increase the risk or susceptibility to SNHL later in life.⁴³ As discussed in this chapter, the aetiology of hearing loss has a major impact on the potential for repair and regeneration and shapes the development of technologies for the treatment of hearing loss.

1.4.3.1 GENETIC FACTORS

Over 60% of cases of SNHL can be attributed to a genetic cause⁴⁴ classified as non-syndromic (most common) or syndromic conditions affecting auditory function at different sites along the auditory transduction pathway (for review see⁴⁵). For instance, mutations to cochlear gap junctions is a common cause of genetic hearing loss, in particular connexin 26 that enables the passage of potassium ions during normal cochlear function.⁴⁶ Indeed, connexin 26 mutation is responsible for ~50% of cases of non-syndromic hearing loss.⁴⁷ Other causes of genetic hearing loss include mutations to genes important for hair cell function, for example the structural protein Myo7A,⁴⁸ or to genes that encode for transmembrane channels essential for mechanotransduction in the hair cell stereocilia such as TMC1.⁴⁹

1.4.3.2 INFECTIONS AND INJURY

Hearing loss can result from infections contracted or injuries sustained at any stage of life. Viral infections such as cytomegalovirus are a major cause of congenital hearing loss⁵⁰ and traumatic injuries to the temporal bone can cause significant hearing loss.

Diseases such as AN neuromas may require surgical intervention that can have significant impact on hearing.

1.4.3.3 AGEING

Hearing loss associated with ageing, termed presbycusis, can have significant implications to healthy ageing. Disentangling the specific effects of ageing on cochlear health can be problematic with life-long environmental factors such as exposure to noise in combination with genetic predisposition to hearing loss making the task difficult.⁵¹ Nevertheless, there is a clear link between progression of hearing loss and ageing with nearly two thirds of people over 70 having significant hearing impairment as assessed by standard audiometry testing.⁵² Typically hearing loss is symmetrical with a progressive loss of hearing sensitivity over time. The characteristics of hearing loss are variable between people. Similar to the effects of excitotoxic injury from noise, it is likely that presbycusis manifests as a combination of loss of hearing sensitivity brought about by loss of ribbon synapses, hair cells and SGNs. Animal models of presbycusis can exhibit significant SGN loss without appreciable hair cell loss^{53,54} suggesting that auditory neuropathy is a likely contributor to the loss of hearing function in humans.⁵⁵ Dysfunction to cochlear homeostasis, brought about by metabolic changes that affect the stria vascularis lining the outer wall of the SM, is also likely to contribute to age-related hearing loss.⁵⁶

1.4.3.4 NOISE EXPOSURE

Noise exposure can result in a temporary loss of hearing sensitivity, which can resolve after a few days or weeks. This temporary threshold shift was previously thought to reflect temporary changes to the structure of the outer hair cell stereocilia, the tectorial

membrane and other changes in the organ of Corti.⁵⁷ More recently, evidence has begun to emerge that more permanent changes occur in the cochlea despite a return to normal hearing thresholds.⁵⁸ As noted above, this phenomenon has been called synaptopathy or hidden hearing loss⁵⁹ as the deficit in hearing cannot be detected in common audiometry testing where hearing thresholds are monitored at specific frequencies using a pure tone audiogram. The mechanism for hidden hearing loss is thought to be the loss of synaptic connections between the SGN peripheral fibres and the inner hair cells.^{19,21,34,60} In particular, SGNs with higher activation thresholds are more susceptible to damage, whereas the low threshold neurons are more resilient to noise-induced damage.⁶¹ The resulting outcome is that hearing sensitivity is maintained but the capacity to process auditory information in more challenging environments is impacted due to the loss of information provided by the higher threshold neurons. There is degradation in the capacity to process temporal information of suprathreshold input. This type of deficit can manifest itself as difficulty in understanding speech in challenging sound environments such as a noisy restaurant. In terms of the impact on the SGNs, significant loss of synaptic contact can result in ongoing and progressive degeneration of the nerve fibres and ultimately death of the SGN,⁵¹ irrespective of the survival of the inner hair cells.⁶²

Longer noise exposure or exposure to louder noise can lead to excitotoxic hair cell damage and loss due to excessive glutamate release at ribbon synapses.⁶³ There is a release of reactive oxygen species, increases in calcium level in cochlear hair cells and a cascade of intracellular events within the hair cell leading to functional damage and death via apoptotic or necrotic mechanisms in the days to weeks following damaging noise exposure.⁶⁴ Local cochlear inflammation and the upregulation of pro-inflammatory cytokines also occur following noise exposure.⁶⁵ Excessive noise

exposure can also result in mechanical damage to the organ of Corti such as rupture of the reticular lamina that can allow the endolymph and perilymph to mix leading to further damage of the hair cells, supporting cells and nerve fibres.⁶⁶ The outer hair cells are typically damaged and lost prior to the loss of the inner hair cells.⁶⁷

1.4.3.5 OTOTOXIC DRUGS

Cochlear hair cells are sensitive to exposure to ototoxic drugs such as platinum-based chemotherapeutics (e.g. cisplatin) and aminoglycoside antibiotics (e.g. neomycin and kanamycin). Cisplatin is a chemotherapeutic agent widely used in the treatment of several forms of cancer. Ototoxicity is a common adverse side effect associated with the administration of this drug, particularly affecting the higher frequencies.⁶⁸ Cisplatin enters the hair cell cytoplasm mainly via mammalian copper transport 1 and organic cationic transporter, where it increases the oxidative stress of the cell promoting apoptotic and necrotic cell death.⁶⁹ It is anticipated that targeting these transporters and/or the application of antioxidants locally within the cochlea would reduce the incidence of hearing loss, while maintaining the efficacy of cisplatin as a chemotherapy agent.⁶⁹

The broad-spectrum antibacterial activity of aminoglycoside drugs is used to treat Gram-negative bacterial infections and those drugs are widely used worldwide, often as a last resort option. The ototoxic compounds enter the cochlea via the stria vascularis and then enter the apical surface of hair cells via the mechanotransduction channels located in the stereocilia. The compounds affect metabolic function of the hair cells by damaging mitochondrial ribosomes leading to the production of oxygen free radicals and initiating cell death mechanisms.⁷⁰ In animal models ototoxic drugs can result in profound deafness and elimination of all cochlear hair cells. The ototoxic drugs can

directly affect the cochlear synapses, causing rapid loss of the ribbon synapse and subsequent retraction of the peripheral nerve fibres.⁷¹ The peripheral nerve fibres degenerate and retract within their myelin sheath followed by the breakdown and loss of myelin sheath.⁷²

1.4.3.6 STRIAL/METABOLIC HEARING LOSS

The stria vascularis, located in the SM (**Figure 2c**), maintains a high concentration of potassium (150 nM K^+) in the endolymph via the functioning of ion pumps, producing the highly positive endocochlear potential (+80 to +100 mV). There are three main layers to the stria vascularis composed of marginal, intermediate and basal cells. Any dysfunction of the endocochlear potential could impact on hair cell depolarisation in response to acoustic input with the overall effect of reduced hearing sensitivity. For example, mutations in the potassium channel subunit KCNQ1 in marginal cells disrupt the endocochlear potential and cause severe congenital deafness—Jervell and Lange-Nielsen syndrome.⁷³ Strial dysfunction is also thought to contribute to age related hearing loss⁷⁴ with the cochlear apex (low frequency) less susceptible to changes in the endocochlear potential compared to the basal (high frequency) region. Consequently, the loss in hearing sensitivity due to strial dysfunction is likely to result in a gradual reduction in hearing sensitivity in the hearing frequency range.^{75,76}

1.4.3.7 LOSS OF RESIDUAL HEARING WITH COCHLEAR IMPLANTATION

Typically, patients that combine the residual low frequency acoustic hearing with the high frequency electric hearing from the cochlear implant have superior performance compared to profoundly deaf patients.⁷⁷ A significant concern and risk for these patients is the loss of residual hearing that can occur following cochlear implant surgery. The

loss can occur immediately post-surgery,⁷⁸ presumably due to factors such as altered cochlear mechanics, acute trauma or immediate inflammatory effects of implantation.⁷⁹⁻

⁸³ However, some patients can lose residual hearing months following implantation via mechanisms that are not well understood. Additional research is required to elucidate the causes of delayed loss of residual hearing. Other research has shown correlations between the extent of fibrous tissue in the cochlea (especially new bone growth), the population of remaining auditory neurons and cochlear implant performance.⁸⁴

1.4.4 POTENTIAL FOR REPAIR OR REGENERATION

Given the diverse causes of hearing loss, the pathology in the cochlea will also be extremely varied in terms of cells affected and the severity of damage. Damaged cells and debris are eliminated by resident and migrating macrophages.⁸⁵ If damage is severe and widespread, a complete loss of the sensory epithelium can occur resulting in a so-called flattened sensory epithelium. Such profoundly damaged cochleae represent a potentially insurmountable obstacle for regenerative therapies, essentially trying to recapitulate the highly structured and complex sensory epithelium from scratch.⁸⁶ Consequently, research efforts have been focused towards the development of protective therapies and therapies designed to restore hearing function in cochleae that have not sustained profound sensory cell loss. The main targets for these therapies are the hair cells, SGNs and the ribbon synapses.

1.4.4.1 HAIR CELLS

It has long been known that hair cells in birds and non-mammalian vertebrates have the capacity to regenerate following damage.⁸⁷⁻⁸⁹ Regeneration occurs via either direct transdifferentiation, whereby neighbouring supporting cells are converted into hair cells,

or by mitotic regeneration in which supporting cells divide and are subsequently converted into a hair cell. Conversely, in the mature mammalian auditory system, spontaneous regeneration of auditory hair cells following damage to the sensory epithelium is significantly limited and short lived.⁹⁰ Attempts to induce hair cell regeneration in the mature mammalian cochlea by cellular reprogramming have had varied success. Some studies have reported the conversion of supporting cells into hair cells by increasing the expression of the *Atoh1* gene, a basic helix-loop-helix transcription factor, in supporting cells via gene therapy or molecular techniques with associated recovery of hearing thresholds.^{91,92} Manipulation of the Notch signalling pathway is another method of increasing *Atoh1* gene expression and this has also successfully regenerated hair cells in the cochlea with recovery of hearing after noise injury.⁹³⁻⁹⁵ Others have reported hair cell conversion that was either too limited, short-lived or incomplete to result in recovery of hearing.⁹⁶⁻¹⁰⁴ Factors that may affect the efficacy of hair cell regeneration and hearing recovery include the degree of pathology in the cochlea, maturity of the organ of Corti or age-related epigenetic modifications, timing of the therapy and the expression dynamics of the transfected gene.¹⁰⁵ The sub-type of supporting cell being converted may also impact on outcomes with pillar and Deiters cells being more resistant to conversion.¹⁰⁶ Newly regenerated hair cells have been noted in some cases to attract neuronal fibres and express synaptic markers suggesting that the hair cells can be reconnected.^{97,99,106,107} It is also noteworthy that recovery of vestibular function has also been observed for *Atoh1* gene therapy following vestibular hair cell ablation as a result of vestibular hair cell regeneration.^{108,109}

A clinical trial is underway to investigate the potential for *Atoh1* gene therapy to restore hearing in patients with profound hearing loss (NCT02132130). An adenovirus carrying

the human Atonal transcription factor gene is being assessed for safety, tolerability and efficacy in patients with severe-to-profound hearing loss. An experimental dose of 20–60 μ L is delivered via the intra-labyrinthine route to access the cochlear-supporting cells in the sensory epithelium.

A single-factor approach to reprogramming of supporting cells may be too simplistic,¹¹⁰ with some reports of increased conversion and improved survival and function of converted hair cells with a combinatorial approach. Co-factors to the Atoh1 gene have included beta-catenin (a component of the Wnt signalling pathway),⁹⁹ transcription factors Gfi1 and Pou4f3,^{106,111} deletion of p27KIP1 (a factor associated with quiescence of hair cells and supporting cells)¹⁰⁶ and the GATA3 transcription factor.¹⁰⁶

Direct transdifferentiation of supporting cells to hair cells depletes the supporting cell population that normally plays a critical mechanical role in auditory transduction and homeostasis. To overcome this, the supporting cell population may first require proliferation prior to transdifferentiation to hair cells (mitotic regeneration, **Figure 4**). Recent research has shown that the inner ear contains a distinct population of progenitor cells that can form new hair cells and neurons in culture.¹¹² These resident cells serve as hair cell precursors during development.^{113,114} By altering intracellular signalling pathways, such as Wnt and Notch activation, the progenitor cells can be ‘switched on’ and stimulated to divide and differentiate into cochlear hair cells.¹¹⁵ In explanted cochlear tissue depleted of hair cells by aminoglycosides, the addition of small molecules that activate Wnt and Notch signalling pathways resulted in an increase in the number of hair cells by stimulating the division and differentiation of Lgr5-positive cells without the loss of supporting cells that occurs with Atoh1 gene therapy.¹¹⁵

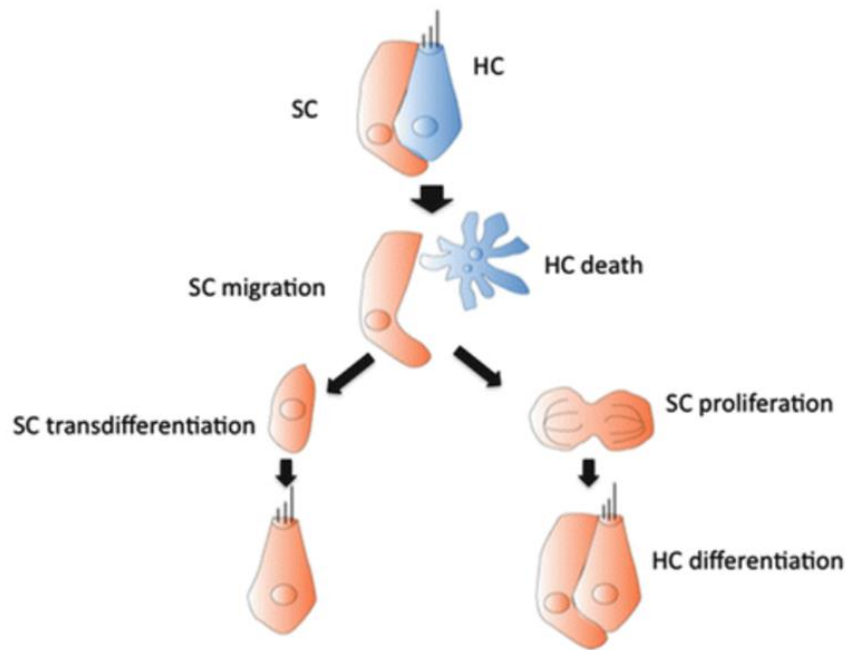


Figure 4. Proposed mechanisms of regeneration of hair cells from proliferation or transdifferentiation of supporting cells (SC).¹¹⁶

1.4.4.2 SGNs

Hair cell loss leads to retraction of the SGN peripheral fibres. The SGN cell bodies within Rosenthal's canal shrink before loss of the neuronal cell body via apoptotic or necrotic processes.⁷² Interestingly, once the neuronal cell body has degenerated the cochlear, the Schwann cell that provides myelin to the SGN appears to alter its phenotype towards a non-myelinating form and interacts with remaining neurons.⁷² The dynamic behaviour of the remaining Schwann cells is likely to facilitate regrowth of the peripheral fibres of the remaining neurons, which often occurs spontaneously after a cochlear insult such as aminoglycoside damage but can be enhanced by the delivery of neurotrophic growth factors. In terms of functional changes in the deafened cochlea as a consequence of neural and myelin degeneration, changes in AN functioning in response to electrical stimulation are more subtle. Auditory neurons maintain the ability

to respond to electrical stimuli, however, reductions in refractory periods and increases in electrical thresholds have been reported in long-term deafened animals¹¹⁷ and myelin-deficient mice.¹¹⁸

The relative slow decline of SGNs (compared to hair cell loss) makes them an amenable target for drug therapies in the cochlea. Given that the number of SGNs is an important factor influencing post-implant hearing performance clinically,^{84,119} developing effective strategies aimed at their rescue is likewise important. There is a large body of work on the use of neurotrophins in the cochlea for the protection of SGNs, regrowth of the peripheral fibres and repair of the hair cell ribbon synapses (see Section 1.4.4.3. and review¹²⁰).

1.4.4.3 NEUROTROPHINS AND THEIR ROLE IN THE TREATMENT OF HEARING LOSS

Neurotrophins are a family of proteins that play a major role in both the development and maintenance of neurons in the central and peripheral nervous systems.¹²¹ During cochlear development, SGNs receive neurotrophic support, including BDNF and NT-3, from hair cells, supporting cells of the organ of Corti and second order cochlear nucleus neurons.¹²²⁻¹²⁵ The high-affinity receptors for these neurotrophins (TrkB and TrkC, respectively) are expressed on SGNs.^{123,124} Importantly, the recent identification of BDNF and its high affinity receptor in developing human cochleae suggests a similar role in human SGNs.¹²⁶

Both BDNF and NT-3 also play a role in the maintenance of SGNs in the adult inner ear.^{124,127} Removal of this support leads to the gradual degeneration of SGNs via apoptotic cell death.^{122,128} Significantly, the neuroprotective effects of exogenous neurotrophins on SGNs *in vivo* have been demonstrated (**Figure 5**).¹²⁹⁻¹³⁴ Associated

with this rescue effect is regrowth of the SGN peripheral fibres compared with deafened controls,¹³⁴⁻¹³⁷ with implications in reducing excitation thresholds when electrically stimulated with a cochlear implant.¹³⁸ Finally, exogenous neurotrophins have been shown to promote synaptic regeneration of the SGN peripheral fibres to the hair cell (i.e. the ribbon synapse) and rescue of hearing function in adult animals following acoustic trauma.¹³⁹⁻¹⁴¹ While protective effects of neurotrophin administration have been observed for at least 2 weeks post-therapy,^{141,142} it appears that long-term exogenous neurotrophin delivery to the cochlea may be required for ongoing SGN protection.¹⁴³ In contrast, promoting SGN peripheral fibres to re-synapse with sensory hair cells via exogenous neurotrophin delivery would unlikely require long treatments as the connection would presumably be maintained by the endogenous supply via the hair cell and supporting cells of the organ of Corti.^{140,141}

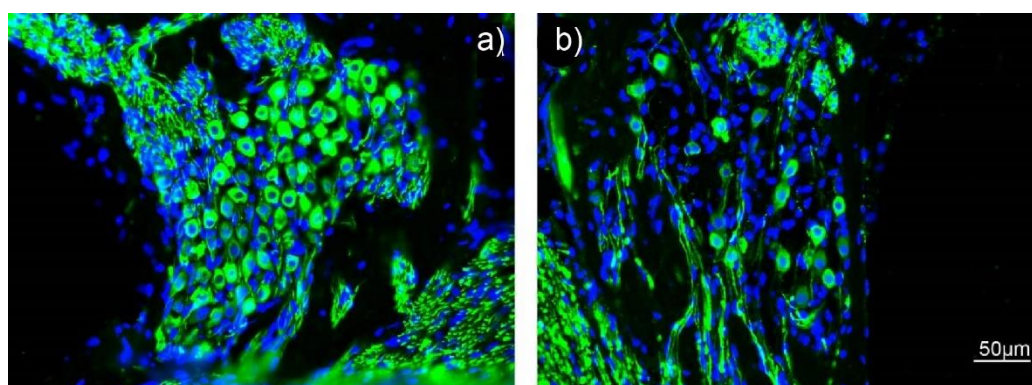


Figure 5. Neurotrophin therapy results in SGN survival after hearing loss in guinea pigs. (a) An intra-cochlear BDNF therapy applied 1 week after ototoxic hearing loss maintains the survival of SGN cell bodies (green) in Rosenthal's canal as well as the peripheral fibres over a 4-week period. (b) Decline of SGN population over 5 weeks in deafened guinea pigs that receive a control therapy.¹⁴⁴

These pre-clinical studies have shown that there are a number of opportunities for drug therapies for hearing loss that each presents a set of unique requirements, such as

specific cellular targeting or slow-release delivery, as well as universal requirements such as the need to protect residual cochlear function and for reliable dosing. Section 1.5 and 1.6 will focus on current and new technologies being developed to meet the demand for a drug therapy that can be applied to the cochlea for preservation and regeneration of hair cells, SGNs, ribbon synapses or other affected cell types.

1.5 DELIVERY OF DRUGS TO THE INNER EAR

Drug-based therapies targeting inner ear disease have been used clinically for over 60 years, initially using systemic administration to deliver aminoglycosides for the treatment of severe bilateral Meniere's disease and more recently the application of steroids for sudden SNHL. Although still in clinical practice, these therapies exhibit significant limitations including highly variable pharmacokinetics due to the blood – cochlear barrier and clinical variability (e.g. patient age, renal function, aetiology, previous inner ear pathology, genetic disposition) and potential undesirable side effects associated with systemic drug administration.¹⁴⁵

In an attempt to improve clinical outcomes, investigators developed drug delivery techniques specifically targeting the inner ear by delivering drugs directly to the RW membrane at the base of the cochlea. Intra-tympanic clinical delivery techniques include direct trans-tympanic injection into the middle ear or the use of Gelfoam™ as a means to maintain drug contact with the RW membrane. Similarly, the Silverstein Microwick™ (Summit Medical Inc.) is a device that is placed onto the RW via a small incision in the tympanic membrane, allowing drugs to be transported from the external auditory meatus to the RW. These techniques have been used to provide a more targeted approach in the treatment of Meniere's disease and sudden SNHL after systemic treatment has failed.^{146,147} This route offers a short-term delivery strategy that is suitable

for some clinical applications but also has a number of limitations including loss of drug through the eustachian tube, variability in pharmacokinetics due to variability and thickening of the RW membrane,¹⁴⁸ and the very slow diffusion of the drug through the cochlea.^{147,149}

The most efficient route is delivery of the drug directly into the inner ear. The anatomy of the cochlea makes it relatively isolated and local therapy can be applied with few off-site effects. The ST provides small, relatively contained fluid-filled compartments into which drugs can be delivered at high doses to reach all target cells including hair cells, supporting cells, SGNs and the stria vascularis;¹⁵⁰⁻¹⁵² only the SM is not accessible via this route due to the presence of tight junctions.¹⁴⁷ In addition to the distinct advantage of delivering predictable amounts of a drug into the inner ear, this technique offers the potential for safe delivery of drugs that have a history of adverse side effects when administered systemically. While efficient, direct administration of drugs to the cochlea brings with it increased risks of both short- and long-term damage to inner ear structures and therefore requires careful preclinical investigation to develop safe surgical access and delivery techniques prior to clinical application. This is particularly important in mild forms of SNHL where further loss of sensory hair cells or their peripheral fibres could result in a profound hearing loss. In cases involving cochlear implantation, where surgical access to the inner ear is part of the procedure and there is little or no residual hearing, the risks of ‘doing harm’ are significantly reduced. It is for this reason that a major clinical application of therapeutic drugs into the cochlea, at least in the short term, will be performed in association with cochlear implantation with a focus on preserving residual hearing and/or promoting SGN survival and peripheral fibre regeneration.^{153,154}

1.6 NOVEL COCHLEAR THERAPY TECHNOLOGIES

The key features of a clinically relevant drug delivery strategy are that the system must be capable of delivering a therapeutic dose of the drug safely to the inner ear over durations that are required for a clinically meaningful outcome. The active agent is typically delivered to the RW membrane or to the intracochlear environment depending upon the clinical indication that is being targeted. Although there are sophisticated microfluidic-based drug delivery pumps under development with application to the cochlea,¹⁵⁵ the present review concentrates on non-pump-based delivery techniques. Some of the considerations for these systems are described in the following section.

1.6.1 BIOMATERIALS

The observations in preclinical studies that neurotrophin delivery (often using mini-pump systems) can protect and promote re-sprouting of SGNs in deafened models have been the motivation for the development of new strategies to deliver neurotrophins that are suitable for clinical application. As neurotrophins have a short biological half-life, poor pharmacokinetics and poor permeability across biological barriers,^{156,157} it is difficult and expensive to systemically administer them for therapy in the cochlea. Thus, localised biocompatible nanotechnology-based neurotrophin delivery systems are being developed such as nanogels, hydrogel, micelles, microspheres, electrospun nanofibres, nanoparticles and supraparticles. Neurotrophins can be immobilised in nanotechnology-based systems via physical entrapment, adsorption or electrostatic interaction. The sustained release of neurotrophins from the drug carriers can then be achieved via the diffusion of neurotrophins and/or the degradation of the carriers. Controlled and sustained release systems can increase the residence time of

neurotrophins in the middle ear/cochlea. In this section, most commonly used delivery strategies will be discussed under investigation for neurotrophin therapy (or other therapeutic molecules) for hearing impairment: hydrogels, nanoparticles and supraparticles.

1.6.1.1 HYDROGELS

Hydrogels are constituted of a porous network of lightly hydrophilic cross-linked polymers, which enables them to easily swell or dehydrate in aqueous environments based on triggering mechanisms such as pressure, temperature, electrical potential or pH, thus achieving drug loading and release.¹⁵⁸ Hydrogels can be synthesised by various polymerisation approaches^{158,159} and have been widely used as drug delivery reservoirs in tissue engineering and neuroscience applications. For the treatment of hearing loss, hydrogels loaded with neurotrophins are typically applied onto the RW membrane.^{160,161} Because of their high viscosity and viscoelastic properties, they prevent the neurotrophins from rapidly flowing through the eustachian tube in the middle ear, thus increasing the residence time to achieve more uniform delivery to the inner ear. Release of the drug is often rapid and occurs by diffusion.^{160,162} The injection of hydrogels onto the RW membrane is less invasive compared to the intracochlear administration route and is therefore a potential platform to translate this approach into clinical application.

Collagen, gelatin and agarose are natural biomaterials that can easily form a hydrogel or be simply modified into a synthetic injectable hydrogel.¹⁶³ Pre-clinical studies using hydrogels infiltrated with neurotrophins have been performed towards evaluating the technique for the protection or rescue of SGNs post-hearing loss. For example, encapsulation of BDNF in a biodegradable hydrogel and its implantation onto the RW

membrane of deafened guinea pigs resulted in BDNF delivery to the cochlea over a 7-day period, protecting SGNs and significantly lowering electrically evoked auditory brain-stem response thresholds compared to controls.¹⁶⁴ Similarly, Gelfoam™, a biodegradable gelatin matrix, loaded with BDNF and positioned onto RW membrane of a deafened guinea pig, promoted survival of SGNs in the basal region of the cochlea after treatment for 2 weeks¹⁶¹ and after treatment with NT-3 for 4 weeks via alginate.¹⁶⁰

For more sustained drug release, research has focused on layering technologies. Sustained release of lysozyme, a similar protein in size, molecular weight and isoelectric point to BDNF, for up to a month was reported from an agarose hydrogel where the protein was encapsulated in poly(ethylene glycol)/poly (acrylic acid) that was assembled using a layer-by-layer approach.¹⁶⁵

The ease of application of hydrogels delivered transtympanically for treatment of hearing loss is offset by some potential challenges such as the relatively quick release profile of most hydrogels which might not be applicable for long-term drug delivery and the variations in the thickness of the RW membrane observed in the clinical setting.

1.6.1.2 NANOPARTICLES

Because neurotrophins are rapidly released from most hydrogels, it is essential to develop other drug delivery carriers that form strong bonds with neurotrophins such as electrostatic interaction for longer term release. Nanoparticle/nanocarrier systems are promising drug delivery technologies that are able to encapsulate and transport neurotrophins to the inner ear (**Figure 6**). Encapsulation of neurotrophins results in stabilisation of the protein and controlled and sustained drug release, avoiding neurotoxicity of highly concentrated neurotrophins.^{140,147,166}

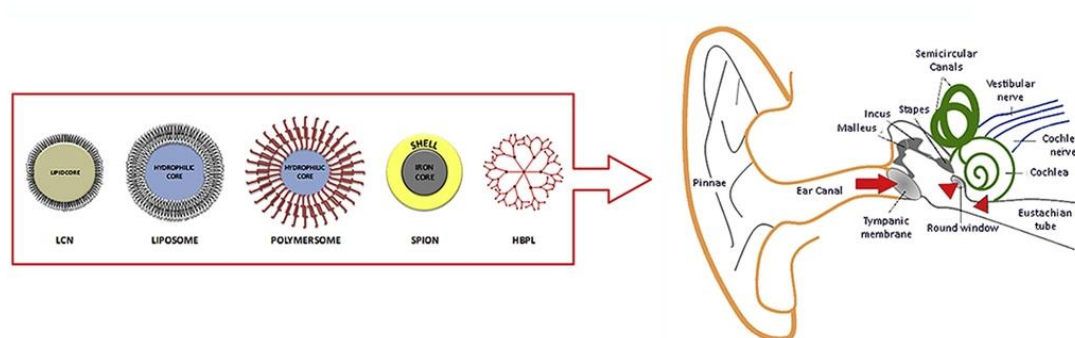


Figure 6. Local drug delivery based on nanoparticles systems to inner ear including lipid core nanoparticle (LCN), liposome, polymersome, supermagnetic iron oxide nanoparticle (SPION), and hyperbranched poly-L-lysine nanoparticle (HBPL).¹⁶⁷

Specific targeting of nanoparticles to auditory hair cells and SGNs has been achieved via surface modifications. For instance, PLGA nanoparticles functionalised with hydrophilic molecules such as poloxamer 407 showed greater cellular uptake in outer hair cells compared to unmodified PLGA nanoparticles and PLGA nanoparticles with chitosan and methoxy poly(ethylene glycol) modifications.¹⁶⁸ Moreover, poly(ethylene glycol)-*b*-poly (ϵ -caprolactone) nanoparticles with surface modifications of nerve growth factor derived peptides demonstrated specificity for SGNs.¹⁶⁹ Recently, a peptide (termed A666) with specific binding affinity for prestin, an abundant outer hair cell protein, was conjugated to poly(lactide) nanoparticles to specifically target outer hair cells. The peptide was coupled via the SH-terminal to maleimide groups of poly(ethylene glycol) on the surface of the nanoparticles. The aim was to use the nanoparticles to deliver a protective payload of dexamethasone to outer hair cells for protection of hearing during cisplatin-based cancer treatment, where ototoxicity is a

common side effect.⁶⁸ As a result, dexamethasone-loaded A666 nanoparticles enhanced outer hair cell viability and afforded some hearing protection following cisplatin therapy in guinea pigs, with a slight advantage over dexamethasone or non-coated dexamethasone-loaded nanoparticles. The nanoparticles were confirmed to cross the intact RW membrane and target outer hair cells, with internalisation of the nanoparticles shown in an inner ear cell line HEI-OC1.¹⁷⁰

The stabilisation and sustained drug release properties of nanoparticles have made them an attractive means for the delivery of neurotrophins to the cochlea as a hearing loss therapy. For example, Tan et al. demonstrated poly(L-glutamic acid) (PGA) nanoparticles loaded with BDNF could achieve sustained delivery for 70 days although nearly 90% of the BDNF was released in the first 30 days. The biological activity of BDNF was not affected by interaction with the nanoparticles and when applied to the cochlea of deafened guinea pigs prevented the degeneration of SGNs (**Figure 7**). However, the long-term stability of the BDNF in the nanoparticles was not measured.¹⁷¹ Similarly, phytantriol lipid-based crystalline nanoparticles were shown to load a large amount of nerve growth factor and improve the availability of nerve growth factor in the cochlea following placement on the RW membrane of the cochlea as compared to application of nerve growth factor in the absence of nanoparticles.¹⁷² Recently, Schmidt et al. has used silica nanoparticles with an amino group surface modification to encapsulate BDNF and demonstrated sustained release for a period of 80 days. The BDNF released from the silica nanoparticle-based drug delivery system was shown to retain bioactivity for at least 39 days that was equivalent to or greater than the addition of 50 ng/mL BDNF to cultured SGNs.¹⁷³ Technologies that maintain the activity of neurotrophic factors are an important consideration in the development of successful slow release delivery systems.

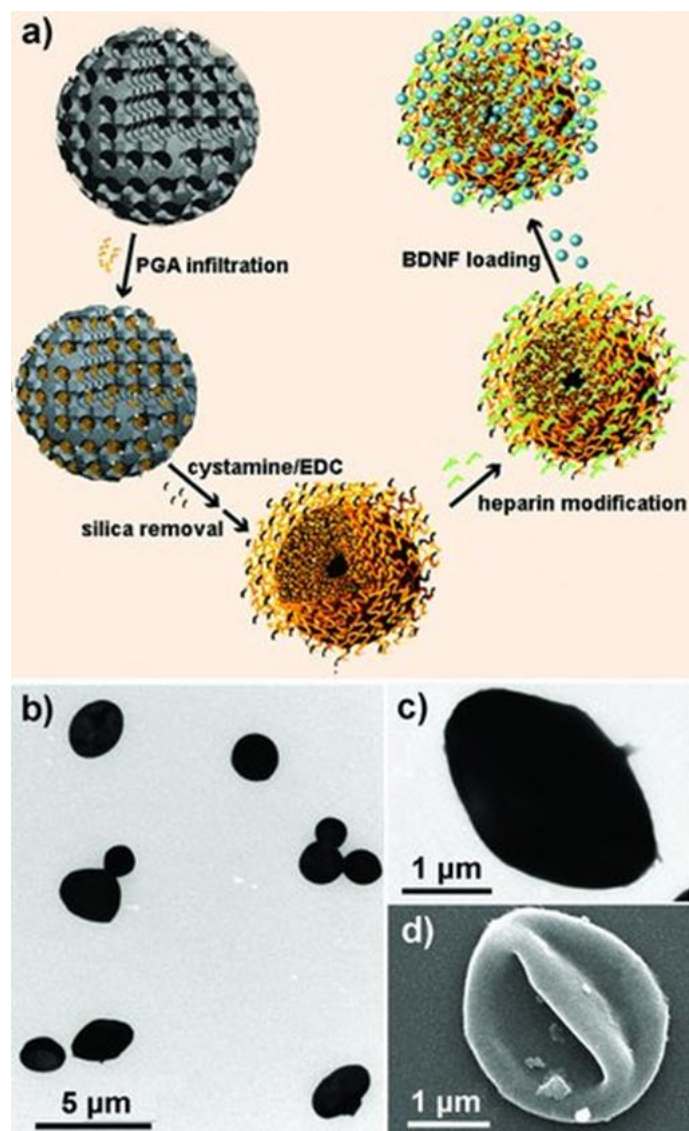


Figure 7. (a) Schematic illustration of neurotrophins encapsulation in PGA particles. Transmission electron microscopy (TEM) images of PGA particles at (b) low and (c) high magnifications. (d) SEM image of a PGA particle.¹⁷¹

Exosomes are cell-derived membrane lipid nanoparticles secreted by many cells to mediate cell-to-cell communication by delivering proteins, nucleic acids or metabolites. They are now being considered as a drug delivery tool due to their intrinsic biocompatibility, stability, targeting properties and ability to overcome natural barriers.¹⁷⁴ Exosomes can be used to package and deliver genetic material such as RNA, DNA, gene editing tools and viral gene therapy as well as small molecules and peptides.

In the auditory system, exosomes have been shown to enable delivery of AAV to otherwise hard-to-transfect outer hair cells of the cochlea, outperforming conventional viral vectors.¹⁷⁵

Small nanoparticles have the potential to migrate from the cochlea to the brain via the cochlear aqueduct—a small canal connecting the basal turn of the ST to the subarachnoid space of the cranium,⁴ presenting a safety concern that is not unique to nanoparticles.^{176,177} Moreover, some nanoparticle technologies exhibit a rapid release profile over the first few days followed by prolonged release of only very small amounts of neurotrophin.^{171,178} Many therapeutic applications in the auditory system have a need for a more regulated release of neurotrophins over a longer period of time.

1.6.1.3 SUPRAPARTICLES

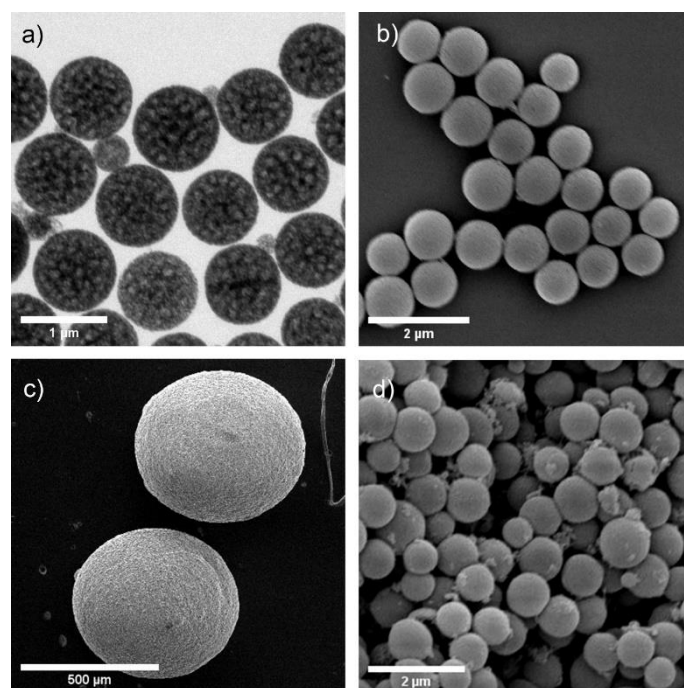


Figure 8. Characterisation of silica nanoparticles and silica supraparticles. (a) TEM image of silica nanoparticles. SEM images of the (b) silica nanoparticles and (c) silica

supraparticles. (d) SEM image of the surface structure of a silica supraparticle where individual nanoparticles can be seen clustered together.¹⁷⁹

Supraparticles consist of thousands of colloidal nanoparticles with their size ranging from 10 to 1000 μm . The overall larger size of supraparticles together with the large surface area provided by the individual porous nanoparticles and the pores between the nanoparticles provide an opportunity for a large drug loading capacity compared to individual nanoparticles (**Figure 8a, b**).¹⁸⁰

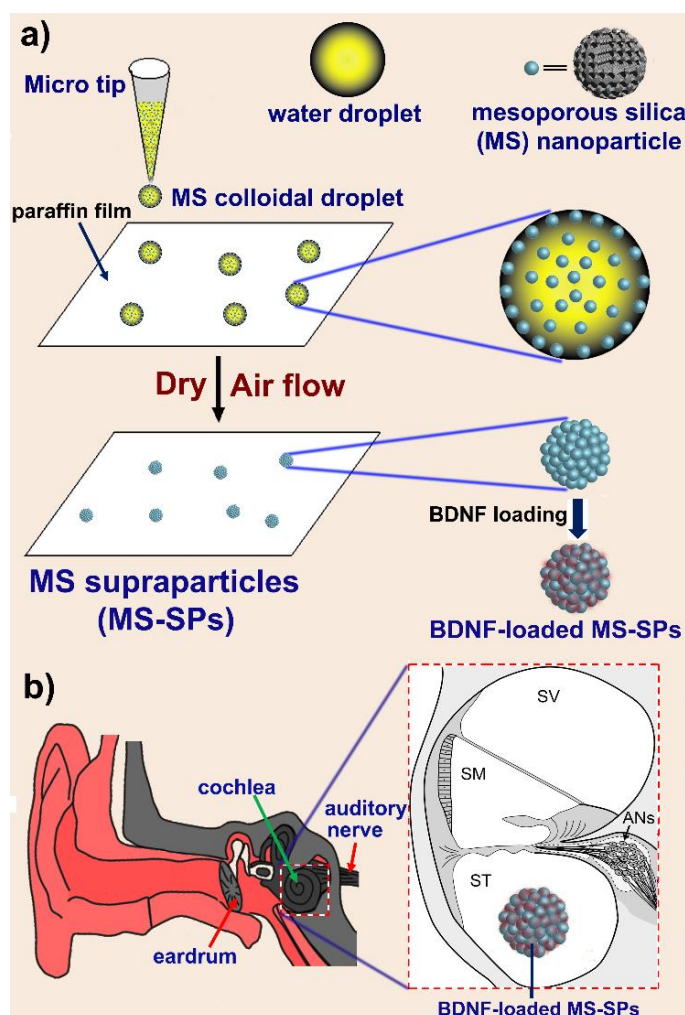


Figure 9. (a) Schematic illustration of the preparation of supraparticles. (b) Surgical implantation of supraparticles to the inner ear.¹⁸¹

The larger size of supraparticles also addresses the safety concerns of nanoparticle delivery systems where nanoparticles may unintentionally flow into cerebral spinal fluid through the cochlear aqueduct (**Figure 8c, d**).¹⁷⁶ Furthermore, BDNF-loaded supraparticles synthesised by an evaporation-induced self-assembly method (silica nanoparticles water droplet was dripped on superhydrophobic surface followed by air flow and condensed with furnace (**Figure 9**)) showed a reduction in the initial burst of release commonly seen with other biomaterials and demonstrated sustained BDNF release over at least 70 days *in vitro*, presumably due to the highly convoluted path that the neurotrophin must take to elute from the particle.¹⁸¹ After 70 days, there was still about 89% of the initial amount remaining in the particle and with the release rate still steady. When applied to deafened guinea pig cochleae, the BDNF-loaded particles maintained SGN numbers at near-normal levels after 28 days treatment.^{144,181} Co-delivery of two drugs important for hearing loss therapies, BDNF and dexamethasone, was demonstrated over 30 days using gelatin hybrid mesoporous silica supraparticles synthesised via a polydimethylsiloxane mould approach (nanoparticles was cross-linked by gelatin to form big aggregation in polydimethylsiloxane (PDMS) moulds (**Figure 10**)).¹⁸² In an important step towards clinical translation and commercialisation, large porous silica supraparticles shown in could also be prepared by an automatable electrospray technique prior to lysozyme or BDNF encapsulation as an approach for long-term drug delivery to treat inner ear disease.¹⁷⁹

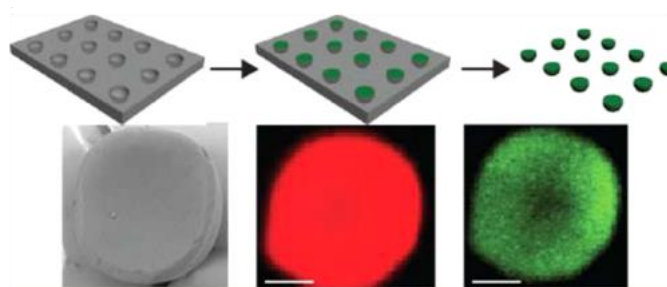


Figure 10. Schematic showing the preparation of supraparticles using a mould-templating approach.¹⁸²

Overall, the studies on silica supraparticles have played an important role in the development of controlled and sustained neurotrophins delivery. However, the pharmacokinetics of neurotrophins in the cochlea released from supraparticles has not been well studied. In addition, the therapeutic dose of neurotrophins and the distribution of the degradation components from supraparticles in different organs have not been investigated. These studies are required to translate this drug delivery system into clinical applications.

1.6.2 GENE THERAPY

Recent developments in the field of cochlear molecular therapies are showing exciting trends towards clinical therapies for hearing loss, particularly for the repair of genetic defects that result in loss of hair cells. Molecular therapies include gene replacement, gene augmentation, gene editing, post-translational modifications and alternative splicing. The transfer of genetic material into cells can be achieved via viral or non-viral vectors, including adenovirus, AAV, herpes simplex virus I, vaccinia virus and non-viral technologies such as lipid gene transfer systems. The challenges for the field of gene therapy have been to prove that the technique applied to the cochlea is safe, highly efficacious and targeted. Recent advances in the field are stepping up to meet

these goals through improvements in the mode of delivery to the cochlea and advances in viral vector technology.

1.6.2.1 VIRAL VECTORS

AAVs are generally considered to have the best profile of safety and efficacy for clinical translation.¹⁸³ AAVs are non-pathogenic, have low immunogenicity and provide long-term gene expression in non-dividing cells. The safety profile of AAVs has improved with all viral genes removed from the current generation of viruses. The multitude of serotypes gives diverse targeting capability, thus specific AAV serotypes can be matched to different indications. The main limitations of AAVs are a restriction on the size of the gene of interest and pre-existing immunity to AAV, which may render the treatment ineffective.

The limited cargo capacity of AAVs was previously an obstacle for genes such as otoferlin, also known as DFNB9. Mutations in the large otoferlin gene cause profound hearing loss due to defects in vesicle exocytosis.¹⁸⁴ Researchers have overcome this limitation through a dual AAV approach where large genes have been split between two AAV vectors. The gene is then reassembled in the transfected cells. Partial restoration of hearing thresholds was observed in otoferlin knockout mice following delivery of dual AAV vectors into the cochlea.¹⁸⁵ Full length otoferlin was observed in an average of 19 or 30% inner hair cells (based on two different dual AAV strategies) with restoration of fast vesicle exocytosis. Weaker expression was observed in outer hair cells. With further improvements to the dual AAV vectors to achieve higher full-length protein expression, this study demonstrates the potential for this technique for transferring large genes into cells within the auditory system.

Research is also underway to address the problem of pre-existing immunity to AAVs. The synthetic vector, AAV2/Anc80L65, first described by Zinn et al., is comprised of ancestral sequences of AAV capsids.¹⁸⁶ Cross reactivity to AAV2/Anc80L65 was partially reduced in AAV8 pre-immunised animals, whereas AAV8 was completely neutralised.¹⁸⁶ The AAV2/Anc80L65 vector also heralded an important breakthrough in terms of cellular targeting in the cochlea. AAV2/Anc80L65 targeted both inner and outer hair cells of the cochlea with high efficiency, whereas other viral vectors had previously shown poor uptake in outer hair cells. Furthermore, AAV2/Anc80L65 transfected SGNs more efficiently than had been previously achieved following gene therapy into the fluids of the cochlea.¹⁸⁷ The vector also had faster transfection kinetics compared to other AAVs with expression visible as early as 1 day post-therapy.¹⁸⁸

AAVs are the subject of numerous clinical safety and efficacy studies for various diseases including inherited blindness, Parkinson's disease and cystic fibrosis to name a few. Many studies are showing promising results such as improvements in visual acuity in blind patients with choroideremia.¹⁸⁹ However, an adenovirus is the subject of the only current clinical trial (NCT02132130) for the treatment of hearing loss, as discussed previously.

1.6.2.2 APPLICATION AND DISTRIBUTION

Typically, gene therapy viral vectors are delivered directly to the cochlear fluid compartments: the perilymph or the endolymph. The perilymphatic compartment can be accessed via the RW or OW membranes of the cochlea or by drilling a cochleostomy through the otic capsule of the cochlea to directly access the ST (**Figure 2a**). The RW approach is reported to be the least damaging to residual hearing,¹⁹⁰ sometimes with no reported loss of hearing thresholds following surgical access and injection of viral

vectors.¹⁹¹ While early studies on gene therapy to the cochlea were often hampered by low transfection efficiency, largely a result of the method of delivery to the cochlea or the cell specificity of the viral serotype, recent developments in mode of delivery and viral vector technology in cochlear gene therapy research are eliminating this problem.¹⁹²

Enormous inter-subject and inter-study variation in transfection levels following direct injection into the cochlear perilymph fluid highlight the difficulty of this approach experimentally. Reported transfection rates to inner hair cells following RW membrane or cochleostomy injection of viral vectors varied from 15% to 100% at the base of the cochlea, with declining transfection rates towards the apex.^{190,193-195} Up to 50% inter-subject variation was also reported.¹⁹⁴ A major cause of variation is the rapid efflux of the perilymphatic fluid that occurs once the otic capsule or the RW membrane has been opened while gaining access to the cochlea for application of gene therapy. During efflux of perilymph from the cochlea, CSF flows into the cochlea via the cochlear aqueduct to maintain fluid homeostasis.⁴ Injection of a viral vector into the cochlea is therefore against the perilymph/CSF flow with inevitable loss of the injected material, unknown dosing and difficulty in transfecting the apical turns of the cochlea. Techniques designed to carefully seal potential fluid leaks from the cochlea during the injection such as with gels or glues have shown increased efficacy.¹⁹⁶ Another group addressed low transfection rates by creating a flow-system during injection in which the cochlea was inoculated with AAV2/9 via the RW membrane while perilymph was drawn out from a distal location in the SCs.¹⁹⁷ The resulting near-complete transfection in inner hair cells was consistent throughout the cochlea in both young (P15-16) and adult (P56-60) mice, while the effect on hearing was minimal. In direct comparison, injection through the RW membrane without distal venting resulted in 68% inner hair

cell transfection in the base, only 21% in the middle turn and as little as 2% in the apex with high variability between animals.¹⁹⁷ Finally, other causes of variation in transfection may come from the use of different AAV serotypes, different doses, animal age/strain differences and variations in surgical experience.

Outer hair cells of the cochlea are typically more difficult to transfect with viral vectors compared to inner hair cells. As mentioned previously, the AAV2/Anc80L65 synthetic vector drastically improved targeting to outer hair cells compared to other AAV serotypes.^{187,198} Another group employed an exosome delivery technique to improve targeting to both inner and outer hair cells with AAV1 or AAV9. The *in vitro* data demonstrated that exosome-AAV1 vectors could improve inner hair cell transfection (65% transfected) and outer hair cells (50% transfected) compared to non-exosome based AAV vectors in cochlear explant cultures (as low as 20% for AAV1 for both inner and outer hair cells). Up to 95% outer hair cells were targeted with exosome-AAV9, demonstrating significantly enhanced efficiency of gene delivery with the exosome packaging.¹⁷⁵ Similar enhanced transfection of hair cells was demonstrated by injecting exosome-AAV1 vectors into the cochlear perilymph of neonatal mice with 88% inner hair cell transfection (compared to 65% for conventional AAV1) and 28% outer hair cell transfection (compared to 14% for conventional AAV1). The gene delivery technique was tested in *Lhfpl5*^{-/-} mice, a strain of mice with profound hearing loss and vestibular dysfunction due to deficiency in a protein integral to mechanotransduction in inner and outer cells.¹⁹⁹ Presence of the correctly localised *Lhfpl5* protein was demonstrated in inner and outer hair cells, with overall improved hair cell survival, functional mechanotransduction, partial improved hearing thresholds in 9 out of 12 animals (thresholds at ~70 dB SPL), a detectable startle response and rescue of balance in 5 animals.¹⁷⁵

A few studies have investigated delivery to the endolymph within the SM (**Figure 2b**), which is much smaller and more difficult to access surgically. Access is achieved by injection through the BM but causes more hearing damage compared to perilymphatic injection^{200,201} or via a cochleostomy through the stria vascularis, also with the potential to damage the stria cells responsible for cochlear fluid dynamics and homeostasis.³⁵ In the guinea pig, injection of an adenovirus carrying neurotrophin genes directly into the endolymph resulted in greater transfection in the hair cells and supporting cells of the organ of Corti compared to perilymphatic injection, attributable to the more contained nature of the endolymphatic space compared to the perilymph.^{200,201} Endolymphatic injection was also successfully applied to mice for the correction of a genetic defect resulting in hearing loss. KCNQ1 is a potassium channel subunit essential for the generation of the endocochlear potential and the high potassium content of endolymph which are crucial for the transduction of sound by hair cells.^{202,203} Mutations in KCNQ1 are responsible for Jervell and Lange-Nielsen syndrome resulting in severe congenital deafness.⁷³ Neonatal mice lacking the KCNQ1 gene were injected with an AAV carrying the KCNQ1 gene via the SM with resulting expression in 61–75% of cochlear marginal cells of the stria vascularis in all cochlear turns. KCNQ1 gene therapy to the endolymph, but not the perilymph, corrected the severe morphological defects caused by the mutation, restored the endocochlear potential and improved hearing thresholds.²⁰⁴

The risk of loss of residual cochlear function is obviously a significant issue when it comes to therapies that aim to protect or restore hearing function. One method employed to minimise the impact on hearing function was gene therapy via the posterior SC, first trialled with adenovirus carrying a reporter gene with a significant advantage in hearing preservation.²⁰⁵ AAV2/Anc80L65 containing a reporter gene was injected

into the posterior SC of 6 week old mice, with resulting transfection of nearly 100% of cochlear inner hair cells and high transfection of outer hair cells throughout the cochlea without affecting hearing thresholds.¹⁹⁸ Furthermore, up to 10% of SGNs in the cochlea were also transfected, with greater efficiency in the apical turn. Vestibular hair cells, supporting cells and ganglion cells were also transfected without any obvious vestibular dysfunction or changes in vestibular evoked thresholds. This technique therefore has merits for drug therapies targeting both cochlear and balance disorders.¹⁹⁸ A similar finding was reported for neonatal mice injected with AAV8 via the posterior SC that showed moderately high cochlear inner hair cell and vestibular hair cell transfection without balance or hearing dysfunction.^{206,207}

1.6.2.3 GENE REPLACEMENT

In animal models of genetic loss-of-function hearing loss, gene therapy has demonstrated successful repair of genetic defects in hair cells and partial or near-complete restoration of hearing, although often the therapy is required at early stages of hearing development with obvious translational challenges.

The Whirlin gene is required for actin polymerisation in hair cell stereocilia. As the name suggests Whirler mice exhibit circling and head tossing behaviour indicative of vestibular dysfunction as well as a profound hearing loss due to a frameshift mutation in the Whirlin gene.²⁰⁸ Delivery of a viral vector containing the Whirlin gene to the perilymph of the cochlea via the RW of neonatal Whirler mice restored the morphology of the stereocilia in transfected cells (**Figure 11**).¹⁹³ However, there was no improvement in overall hearing sensitivity as a result of the low transfection efficiency in this study (15% hair cells at the base of the cochlea).¹⁹³

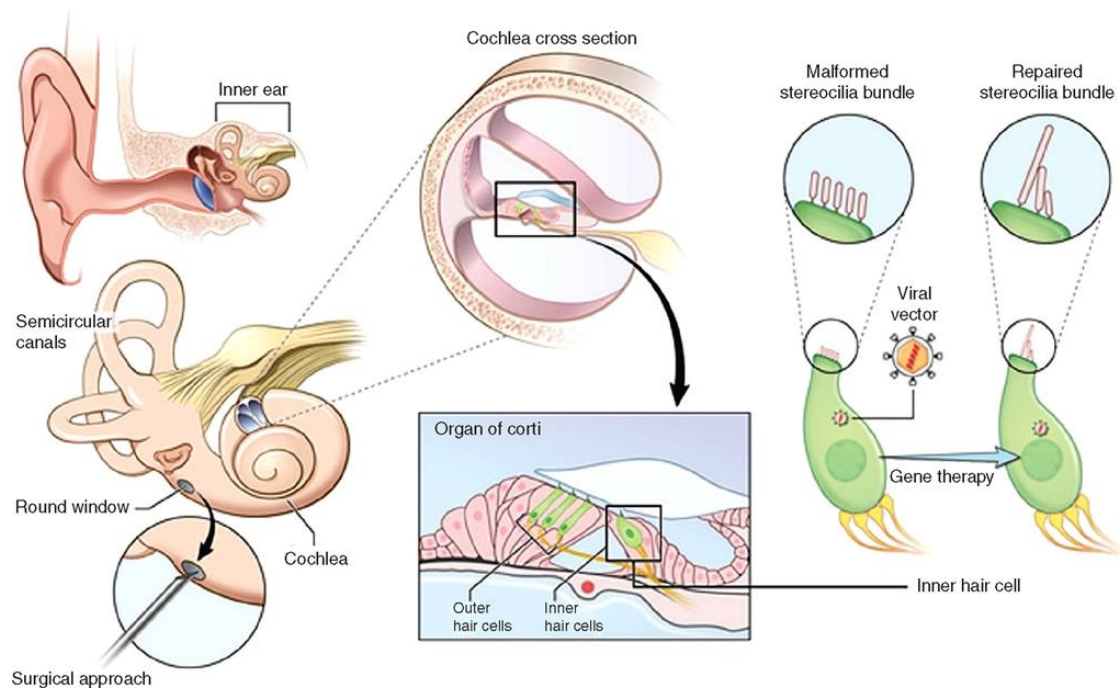


Figure 11. The inner ear structure and schematic illustration of whirlin gene therapy to restore hair cell stereocilia morphology and function in whirler mutant mice.¹⁹³

TMC1 is another gene required for hair cell transduction channel function (**Figure 12b**).²⁰⁹ Delivery of an AAV2/1 through the RW membrane of neonatal mice that were bred for recessive TMC1 deafness resulted in nearly 60% inner hair cell transfection and the return of some hearing function.¹⁹⁴ To target outer hair cells more efficiently, the group then used the synthetic AAV2/Anc80L65 serotype in the TMC1 model of hearing loss and found dramatically improved transfection in both inner (91%) and outer hair cells (50%). Restoration of hearing thresholds of up to 50 dB was observed in TMC1 mutant mice.²¹⁰ As the cochlear injection approach also resulted in expression in vestibular hair cells, the researchers tested whether gene therapy could restore balance and vestibular function in TMC1/TMC2 mutant mice, as vestibular hair cells express both TMC1 and TMC2.²¹¹ Following neonatal injection of AAV2/Anc80L65 containing the TMC1 or TMC2 gene into the cochlea via the RW the mice showed vestibular function similar to wild type mice.²¹⁰

Vesicular glutamate transporter type 3 (VGLUT3) is required for synaptic transmission in hair cells (**Figure 12a, c**). Mice lacking the VGLUT3 gene are profoundly deaf due to lack of glutamate release from hair cells.²¹² Restoration of auditory brainstem responses to within 10 dB of wild-type mice as well as a partial restoration of behavioural responses to auditory stimuli were demonstrated following injection of an AAV1 containing VGLUT3 into VGLUT3 knockout neonatal mice via the RW membrane. In this case, mostly all inner hair cells were transfected, demonstrating the enormous potential of gene therapy when efficient and timely targeting of cells is achieved.¹⁹⁵ However, variable loss of hearing was then observed approximately 7 weeks after gene therapy, suggesting transgene inactivation or a slow progressive hearing loss resulting from the surgical delivery of the transgene.¹⁹⁵

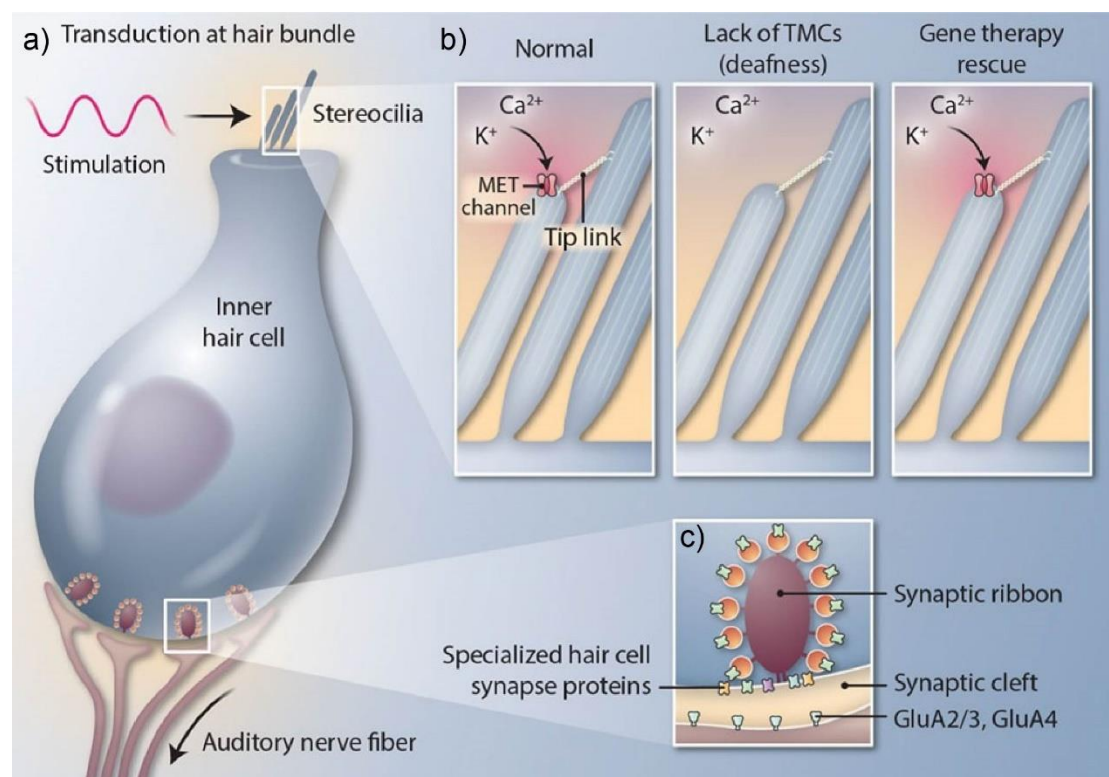


Figure 12. (a) Schematic showing an inner ear hair cell structure. (b) Transduction was reinstated via AAV-mediated gene therapy for some type of hearing impairment owing to the lack of TMC1/TMC2 in which the transduction fails. (c) AAV-mediated gene therapy rescue hearing by adjusting the expression of VGLUT3 in inner hair cells.²¹³

Ushers syndrome is responsible for 3–6% of childhood deafness.²¹⁴ Patients with the most severe form experience profound hearing loss, balance issues and progressive blindness. A disrupted harmonin protein is responsible for this phenotype, causing disorganised hair cell stereocilia bundles and loss of mechanotransduction. Using the synthetic AAV serotype AAV2/Anc80L65, harmonin-a1 or harmonin-b1 was introduced into the cochlear and vestibular hair cells in a mouse model of Usher syndrome. Following neonatal cochlear injection, when there are still surviving hair cells, inner hair cell transfection rates were 80–90% with correctly localised harmonin protein at the tips of the stereocilia. Partial recovery of inner and outer hair cell function, auditory thresholds and behaviourally relevant auditory function were observed in the majority of mice injected with harmonin-b1 6 weeks after injection, particularly in the low frequencies.²¹⁵ Out of the five mice examined 6 months after injection, three mice showed continued low frequency hearing attributable to the survival of outer hair cells.²¹⁵

To summarise, gene therapy to the cochlea via AAV viral vectors has resulted in partial or sometimes near-complete correction of genetic defects in the auditory pathway. Several challenges remain such as the timing of treatment with respect to the degenerative changes of the disease. In many cases experimentally, gene therapy is administered to neonatal mice in order to repair the gene in the hair cell prior to hair cell degeneration. Given that the development of human hearing occurs prior to birth,²¹⁶

the translation of these approaches would potentially require gene therapy *in utero*. Characterisation of long-term expression and outcomes will also be required for each type of gene therapy as long-term outcomes are variable.

1.6.2.4 MODULATING GENE TRANSLATION

Gene replacement is not the only strategy for the repair of the hearing defect. Some point mutations result in aberrant gene splicing such that the translated protein is truncated or frame-shifted and inactive. In such cases, manipulating splicing could potentially correct the defect by blocking the offending splice site and directing splice site selection to normal. As mentioned above, harmonin is a protein encoded by the *USH1C* gene and is located in stereocilia and ribbon synapses of hair cells in the cochlea and vestibular system where it plays a role in stereocilia structure and mechanotransduction.²¹⁷ About 6–8% of mutations in Usher syndrome are in the *USH1C* gene, attributed to a *USH1C* c.216G>A mutation that causes mis-splicing and ultimately a truncated non-functional harmonin protein.^{217,218} Antisense oligonucleotide therapy was developed to base-pair at the position of the mutation, blocking access of splicing factors to the aberrant splice site and redirecting the splicing to the correct location in the gene. Deviating from many of the other gene therapy strategies described above where the gene therapy is localised to the cochlea, antisense oligonucleotide therapy in a mouse model of this particular Usher mutation (Lentz 2007) was administered via systemic administration in neonates²¹⁹⁻²²¹ or embryonically via the amniotic fluids²²² as there is a critical time period during the development of hearing in which therapy must be administered. Antisense oligonucleotide therapy afforded long-term significant rescue of hearing and correction of vestibular function associated with restoration of hair cell stereocilia morphology.²¹⁹⁻²²¹ Earlier treatment had a greater

impact on hearing restoration consistent with the developmental pattern of expression of harmonin that occurs between P4 and P16.²²³

The ease of delivery, specificity and efficacy of antisense oligonucleotides make this form of therapy an exciting option for altering gene expression in diseases with a known genetic component. Splice-switching oligonucleotide therapy is currently in clinical trials for the treatment of Duchenne muscular dystrophy and spinal muscular atrophy²²⁴ showing relatively low toxicity and good tolerability.

RNA interference is a technique that can be used to silence specific genes linked to a disease, particularly for gain-of-function mutations. Gain-of-function mutations occur when a mutation results in an altered gene product which then interferes with the normal functioning of a biological pathway. An example in the auditory system is connexin-26, encoded by the Gap Junction Protein Beta 2 gene (*GJB*) which is a part of a larger complex of proteins that form a connexon, the pore-forming unit of gap junctions.²²⁵ A point mutation in connexin-26 results in a protein that still complexes with the other five connexins but interferes with the function of gap junctions, preventing hearing transduction in the cochlea through deficient potassium recycling.²²⁶⁻²²⁹ To suppress the mutant protein, a short-interfering RNA was complexed to a liposome delivery system and applied to the intact RW membrane of a mouse model of the connexin point mutation. The RNA interference treatment significantly and specifically suppressed the mutant *GJB* mRNA, preventing hearing loss in the mouse model.²³⁰ Similarly, in a mouse model of a gain-of-function *TMC1* mutation known as Beethoven,²³¹ a single intracochlear injection of a microRNA delivered by an AAV slowed the progression of hearing loss through suppression of the mutated gene. At the 35-week time point, mice in the RNA interference group had a 40 dB improvement in hearing thresholds over non-treated controls.²³² Other gain-of-function mutations in the auditory system that

may benefit from RNA interference include missense mutations of the KCNQ4 gene that affect potassium channels^{233,234} and the DFNA5 gene which produces a protein of currently unknown function.^{235,236}

As discussed in Section 1.4.4.1, new hair cells can be generated in mammals by increasing expression of the Atoh1 transcription factor, decreasing Notch signalling or regulating other pathways following hearing loss.²³⁷ Silencing of hairy and enhancer of split-1 (Hes1), a down-stream effector of Notch signalling and inhibitor of Atoh1,^{238,239} is also sufficient to promote transdifferentiation of supporting cells to hair cells in the mouse cochlear tissue.²⁴⁰⁻²⁴² To test these results *in vivo*, a Hes1 small interfering RNA was delivered to adult guinea pig cochleae via PLGA nanoparticles 72 h after noise-induced hearing loss by mini-osmotic pump infusion into the perilymph over 24 h. As a result of the treatment, there were fewer missing inner and outer hair cells across the entire length of the cochlear spiral, some correctly positioned and some ectopic. There was also progressive recovery of hearing of more than 10 dB, particularly in the epicentre of the noise-induced damage and near the infusion site.⁹⁴

In an exciting development for gene modulation therapies, the US Food and Drug Administration has approved the first gene-silencing technology based on RNA interference: the drug, patisiran, which has improved outcomes in patients with a rare heart and nerve function condition.²⁴³

1.6.2.5 CELL TARGETING

Most experimental cochlear gene therapies to date aim to target cells in the sensory epithelium such as the hair cells or supporting cells. SGNs have proven to be relatively resistant to transfection. There is recent interest in transducing SGNs, particularly for optogenetics research that requires expression of a light-sensitive ion channel in SGNs.

The aim is to develop an optical cochlear implant for improved spectral resolution during SGN stimulation in the cochlea.^{244,245} Initially, embryonic gene therapy was performed in order to achieve efficient SGN transfection.²⁴⁶ When E11.5-12.5 embryos were transfected, there was 40% SGN transfection which was sufficient to evoke activity within the central auditory pathway to optical stimulation of the transfected SGN. Clinical translation of this technique requires a solution in which post-natal, preferably adult SGNs are transfected. A solution was the use of a novel AAV serotype that improved SGN transfection in post-natal mice. In 5–7 day old early post-natal mice, strong SGN expression of the opsin was achieved across all turns (about 55% in base and 75% in apex) when using a novel serotype with improved neural transfection, AAV-PHP.B²⁴⁷ via a RW membrane injection²⁴⁸ or 74% transfection when using the AAV2/Anc80L65 viral vector in P4 mice.²⁴⁹ Interestingly, another solution came in the form of a novel mode of delivery to the cochlea. In adult gerbils, AAV2/6 was injected directly into the modiolus of the cochlea. This technique resulted in up to 30% transfection in SGNs in all turns of the cochlea in about half of the injected animals. However, the technique was damaging to SGNs, resulting in approximately 25% loss of SGNs.²⁵⁰ Subsequent optical stimulation of SGNs expressing the light-sensitive ion channels resulted in auditory percepts and behavioural responses in transfected gerbils.²⁵⁰

1.6.2.6 GENE EDITING

Usher syndrome is an inherited disorder causing deafness and blindness with up to 13 associated genes. More than 50% of Usher syndrome cases harbour a mutation in the USH2A gene. The large size of the USH2A gene makes it difficult to develop a conventional gene therapy for these patients, but the CRISPR/Cas9 gene editing

technology now offers an alternative strategy. The Cas9 nuclease is guided with precision by small RNAs to the target sequence for editing. Successful targeting and repair of the USH2A mutation was demonstrated in fibroblasts *in vitro*.²⁵¹ More recently CRISPR/Cas9 technology has been used to restore hearing in animals with a genetic form of deafness that normally results in loss of hair cells. The faulty TMC1 gene present in cochlear hair cells was targeted by the gene-editing tool to repair a single base-pair mutation. In a novel take on the CRISPR/Cas9 technology, the authors used a special DNA-free, virus-free delivery technique in which the Cas9 complex was encapsulated in lipid packaging to directly deliver the Cas9 complex inside the hair cell rather than having the cell make the Cas9 from DNA.²⁵² This reduced the off-target editing thereby improving the precision of the editing. The outcome in these mice was significantly greater hair cell survival and prevention of loss of hearing.²⁵³

Genome editing with nucleases such as Cas9 can be subject to extraneous insertions and deletions (indels) and is largely restricted to mitotic cells.²⁵⁴ To overcome these limitations, researchers have used a nuclease-free base editing technique which increased the ratio of editing compared to indels.²⁵⁵ Lipid-mediated delivery of the beta-catenin base editor to the mouse inner ear resulted in the replacement of Ser 33 to Tyr in the beta-catenin gene. This resulted in altered post-translational protein modification (prevention of phosphorylation) and therefore, up-regulation of Wnt signalling. This in turn promoted mitosis in supporting cells of the cochlea and reprogramming of the cells towards the hair cell lineage.⁹⁵

1.6.2.7 GENE AUGMENTATION

Viral gene therapy is not just limited to the correction of hereditary disorders. Gene therapy can be used to augment gene expression, for example to restore neurotrophin

levels in the cochlea following the loss of hair cells and supporting cells that occurs with SNHL.^{137,200,256-258} When neurotrophin gene therapy was administered to the SM of guinea pigs that were deafened by aminoglycosides there was a preservation of the SGN population along with outgrowth of the peripheral fibres towards neurotrophin-expressing cells in the sensory epithelium (**Figure 13**).^{200,258} It is yet to be determined whether neurotrophin gene therapy results in improvements in cochlear implant function as has been shown with long-term recombinant BDNF delivery to the cochlea.²⁵⁹ Guinea pig cochleae that were inoculated with AAV containing the NT-3 gene and then implanted with an electrode array showed measurable and long-lasting SGN survival, but variability in transfection made electrophysiological assessment difficult.²⁶⁰

Finally, the potential to use the cochlear implant to drive transfection of DNA into resident cells lining the ST via brief but intense pulses from the electrode array provides a unique transfection mechanism for gene therapy. Using this electroporation technique Housley and colleagues were able to transfect mesenchymal cells with a complementary DNA (cDNA) gene construct resulting in the expression of BDNF.²⁶¹ Using a deafened guinea pig model, they were able to show reduced electrical thresholds and improved SGN survival with significant regrowth of the nerve fibres within the ST. The resultant improvement in the electrode-neural interface may lead to clinical improvements for cochlear implant recipients following completion of an upcoming Phase 1 clinical trial.

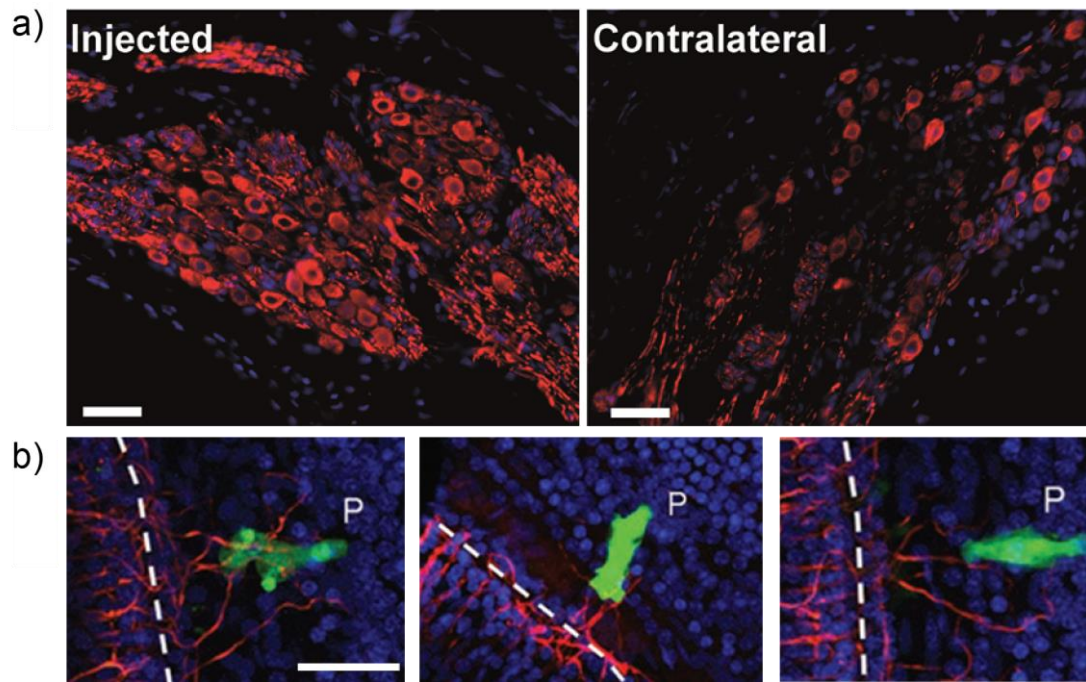


Figure 13. Neurotrophin gene therapy provides cellular sources of neurotrophins for SGN survival after hearing loss. (a) Comparison of SGN cell bodies (red) in a cochlea subjected to neurotrophin gene therapy and the contralateral non-injected cochlea. Guinea pigs were deafened with aminoglycosides 1 week prior to gene therapy with adenoviral vectors containing BDNF and NT3 genes into the ST. After three weeks, there was a dramatic difference in the number of surviving SGNs between the treated and non-treated cochleae. Scale bars are 50 μ m. (b) Surface view of the organ of Corti with peripheral fibres of SGNs (red) attracted towards pillar cells (P) that have been transfected with neurotrophin genes (as labelled by a green-fluorescent protein reporter gene). DAPI counterstain is in blue. Scale bars are 50 μ m. The dashed line indicates the position of the habenula perforata.¹³

1.6.3 COCHLEAR IMPLANT COMBINATION THERAPIES

Cochlear implant electrode arrays are implanted into the ST of severe-to-profoundly deaf patients. There is therefore the potential to use the device to deliver therapeutics

to the cochlea. The validity of this approach was first investigated by manufacturing a cannula internally to the electrodes that was connected to a pump for the continuous delivery of neurotrophins to the guinea pig cochlea following hearing loss.²⁶² SGN survival was enhanced compared to deaf controls and further improvement in the SGN density was observed in animals that were simultaneously given electrical stimulation.^{259,263} However, the presence of a cannula or any cavity in the electrode array is problematic as it increases the infection risk and significantly alters the mechanical characteristics of the electrode array. For this reason, some studies examined drug release from a cochlear implant coating that has been modified to be loaded with drugs for subsequent release into the cochlea. These have included a coating of cells that have been modified to express and release neurotrophins and degradable coatings.

In one study cochlear implant electrodes were coated with the electro-active polymer polypyrrole. The polypyrrole could be doped with NT-3 or BDNF that was only released during electrical stimulation.^{136,264-266} Arrays that were coated with polypyrrole containing NT-3 were implanted in guinea pigs that had been ototoxically deafened and electrical stimulation was provided for 4 weeks. It was found that there was greater nerve survival in guinea pigs implanted with coated compared to control arrays²⁶⁷ although the technique is limited to a relatively small and finite payload of neurotrophins.

Inflammation and fibrosis following cochlear implantation is believed to be the cause of some post implantation-related hearing loss that can occur in recipients with residual hearing.^{268,269} Pharmacological interventions to reduce the immune response are being investigated with a particular emphasis on the anti-inflammatory drug dexamethasone. Local delivery of dexamethasone to the cochlea can prevent some of the loss of residual

hearing and reduces the tissue response around the implanted array.²⁷⁰ Some researchers are investigating the cochlear implant itself as a means to elute drugs such as dexamethasone over the time frame of inflammation and fibrosis, which occurs in stages during the days and weeks following cochlear implantation. The dexamethasone was added during the polymerisation of silicone elastomer such that the drug is an integral part of the implant.²⁷¹ When tested in guinea pigs the dexamethasone-eluting cochlear implant attenuated some inflammatory cell infiltration in the cochlea between 3 and 13 days post-implantation.²⁷² This approach has also demonstrated a trend towards hearing protection, however the results to date have not been statistically significant and may depend on the level of damage during insertion.^{273,274}

1.6.4 CELL-BASED THERAPIES

Another potential solution to the damage or loss of hair cells or SGNs is cell-based therapies for the replacement or restoration of these cells. Stem cells are one of the most commonly used in the field of cell-based therapies due to their potential to differentiate into different cell types including hair cells and SGNs. To date, there are quite a few studies examining the differentiation of murine embryonic stem cells²⁷⁵⁻²⁷⁷ or human embryonic stem cells²⁷⁸⁻²⁸⁰ into functional hair cells or auditory neurons.

When transplanted into the cochlear environment, differentiated stem cells have been shown to survive and integrate into the tissue.^{281,282} Strikingly, transplanted neural progenitor cells appeared to respond to natural differentiation and guidance cues in the tissue environment with engrafted embryonic stem cells in the cochlear nerve trunk projecting fibres towards the sensory epithelium.²⁸³ Human otic progenitor cells that were differentiated towards cells with morphological and electrophysiological features of hair cells and SGNs *in vitro* promoted recovery of auditory brainstem response

thresholds when transplanted into SGN-depleted gerbils.²⁸⁴ iPSCs, differentiated cells that have been reprogrammed to an embryonic state, also received much attention as they can be transplanted without immune response as they are generated from the patients' own cells.²⁸⁵ In one study, mouse IPSC-derived neural progenitors could be differentiated into a neural lineage. After transplantation into the mouse cochlea *in vivo*, a small percentage of the transplanted IPSC-derived neural progenitors expressed VGLUT1, a marker for glutamatergic neurons.²⁸⁶

In addition to the transplantation of differentiated neural stem cells to replace degenerated sensory cells or neurons in deafened cochlea, a second cell-based therapy approach involves the delivery of cells designed to rescue sensory cells or neurons from ongoing degeneration. For example, *in vitro* experiments indicated that genetically modified Schwann cells could maintain the survival of SGNs.²⁸⁷ To avoid recognition by the immune system and cellular migration from the implantation site, BDNF-over-expressing Schwann cells were encapsulated by a semi-permeable alginate membrane where oxygen and nutrients could diffuse into the cells and the BDNF and waste could diffuse away from the cells (**Figure 14**).²⁸⁸ The results showed that alginate encapsulated BDNF-over-expressed Schwann cells could promote the survival of SGNs in deafened guinea pigs. Further, porcine choroid plexus cells, which naturally secrete a cocktail of neurotrophins,²⁸⁹ encapsulated in an alginate membrane promoted survival of SGNs and their peripheral fibres for several months in deafened guinea pigs with a combination of chronic electrical stimulation via cochlear implantation with minimal tissue response to the grafted cells.¹⁵³

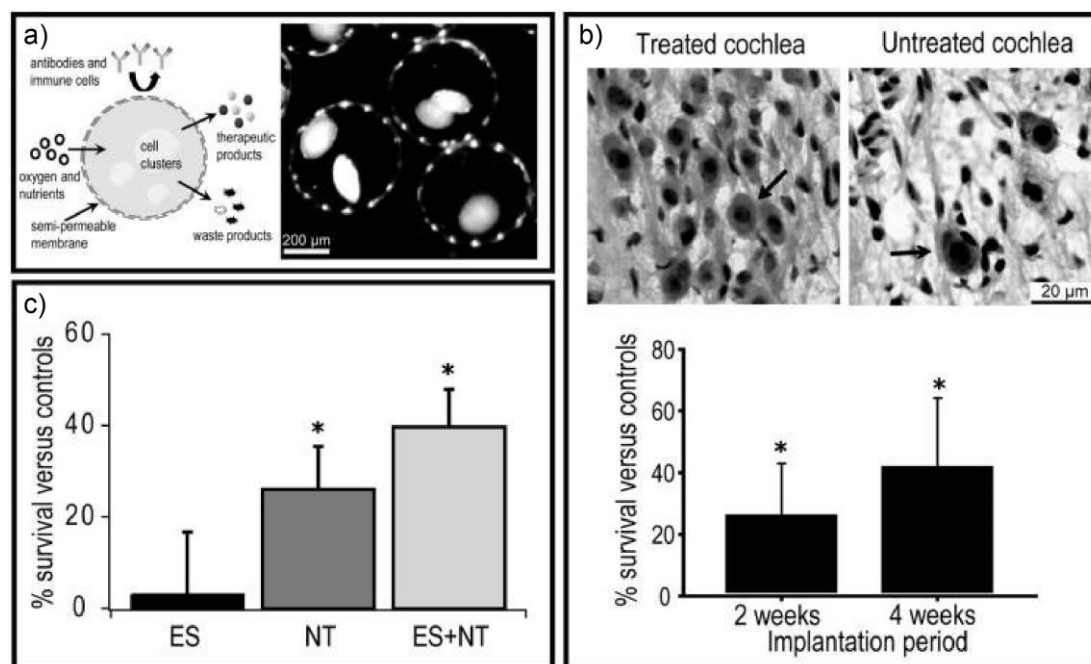


Figure 14. (a) Schematic showing the basic concept of cell-based therapy with encapsulation of therapeutic products and an example of cells encapsulated in alginate membrane. (b) Histological images and percentage of SGN survival of deaf guinea pig cochlea after the implantation of BDNF-encapsulated Schwann cells and control capsules. (c) Percentage of SGN survival based on electrical stimulation, choroid plexus cells neurotrophin delivery and the combination of those two strategies over 6 months in a deafened cat.¹⁷⁸

There are still a number of challenges for clinical application of cell-based therapies. It is essential that the transplanted cells survive for long periods of time and not interfere with normal cochlear function or affect residual hearing.^{290,291} Potential immunological responses to embryonic stem cells must be considered²⁹² alongside the risk of tumorigenesis if they are not fully differentiated.^{293,294} An additional uncertainty is whether the generated cells have the same functional response as normal SGNs or hair cells.^{295,296} It is also challenging to precisely inject the cells into the target without

damage to existing structures and for the cell to integrate into the existing tissue and to form functional connections.^{291,296-298}

1.7 HOW CLOSE AND WHICH DELIVERY TECHNIQUE IS BEST?

This chapter has covered a number of novel drug therapy technologies for the potential treatment of hearing loss. The therapies are varied by necessity to mirror the diverse aetiologies of hearing loss. A number of the therapies discussed have demonstrated partial reversal or slowed progression of hearing loss in relevant animal models. These have included hair cell regeneration following acquired damage to hair cells via the manipulation of transcription factor pathways by gene or drug therapies.⁹¹⁻⁹⁵ The most promising hair cell regeneration therapies are those that use a combination of factors^{99,106,111} and those that promote mitotic regeneration such that the supporting cell population is maintained during hair cell regeneration.¹¹⁵ For acquired synaptopathy or hidden hearing loss, delivery of neurotrophic factors such as NT-3 to the cochlea has been shown to rescue hearing function via the regeneration of the ribbon synapse following noise trauma.^{140,141} The simplicity of the trans-tympanic delivery technique will make this therapy readily translatable despite the short window of opportunity for effective treatment. Neurotrophins also protect SGNs that can otherwise degenerate following hearing loss and hence are a promising drug therapy that could be applied to a range of hearing loss conditions. In many cases, extended exposure to neurotrophins is beneficial to the therapy, hence research into drug delivery technologies such as biomaterials (incorporating hydrogels, nanoparticles, supraparticles) or cell-based therapies that can not only stabilise the drug over time, but also provide sustained release of the drug. With so many tools available, gene therapy has the potential to treat a diverse range of SNHL such as gene editing of point mutations, replacement of

defective genes, silencing of gain-of-function mutations, suppression of aberrant splice variants, alteration of post-translational modifications, manipulation of transcription factor pathways for regeneration and augmentation with therapeutic genes such as neurotrophins. Using mouse models of known human mutations or acquired damage to hair cells, there have been many reports of long-term correction of genetic defects ranging from partial to near-complete reversal/prevention of hearing loss with early intervention.^{185,191,194,195,204,210,215,219-221,230,232,253} Furthermore, with a direct read-out of transfection efficiency in the form of a fluorescent reporter gene, gene therapy research has led to significant improvements in cochlear delivery techniques that will benefit all forms of therapy to the cochlea. In contrast, there have been relatively few examples of hearing restoration by cell based therapies²⁸⁴ compared to gene therapy techniques highlighting the challenges of differentiation stem cells into functional auditory sensory cells, integrating the cells into the auditory pathway and maintaining long-term survival in the cochlea.

Although there are a relatively large number of clinical trials incorporating trans-tympanic drug delivery into the middle ear,^{155,299} there are few trials incorporating drug delivery directly into the cochlea due to concerns with potential damage to residual hair cells. As noted above, the only clinical trial registered with ClinicalTrials.gov incorporating direct intra-labyrinthine drug delivery is designed to evaluate the safety, tolerability and the potential ability of the drug CGF166, an adenovirus containing the human *Atoh1* gene, to improve hearing in patients with severe-profound SNHL (NCT02132130).

The various delivery systems have specific advantages and disadvantages that must be taken into consideration in developing a clinical-based delivery system. For example, delivery using biomaterials, particularly nano- and supraparticles, can deliver large

payloads including a cocktail of drugs delivered over different time-constants. These systems can deliver both native protein and appropriate synthetic drugs. Biomaterial-based delivery techniques typically have short or moderate release profiles and must be carefully tuned to ensure they do not produce high concentrations of the drug(s) at the target site. In contrast, cell and gene-based delivery techniques offer potentially longer-term solutions, whether for gene augmentation, genetic correction or cell regeneration, with a high degree of cell specificity. However, many of the promising therapies for hereditary hearing loss would require early intervention before complete loss of the hair cells or irreversible morphological changes.

In addition to these technical considerations, it is important when developing these systems to consider the manufacturing and clinical implications of the technology.³⁰⁰ For example, in a clinical setting the technology will need to conform to Good Manufacturing Practice guidelines and recognised standards of sterilisation for healthcare products and packaging systems and show a reasonable shelf-life at room temperature. If drug, gene or cell loading is to be performed during surgery it must be undertaken using sterile techniques under appropriate guidelines for handling biological material.

Finally, it is likely that the initial delivery techniques will target hearing preservation,³⁰¹ SGN peripheral fibre regeneration^{138,302} and a reduction in inflammation³⁰³ in cochlear implant patients. This work, together with novel techniques designed to safely target either the round or OWs to treat disorders including Meniere's disease³⁰⁴ and hidden hearing loss^{139,141} are likely to dominate inner ear drug delivery in the near-term. With the clinical experience gained from these approaches, more trials evaluating direct drug delivery to the cochlea in patients are expected. Disorders including tinnitus, sudden

hearing loss and hearing protection following exposure to loud noise or ototoxic drugs would be potential candidates for this route.

1.8 CONCLUSIONS AND OUTLOOK

Hearing loss is a significant problem worldwide. The sensory hair cells and SGNs of the cochlea are often affected and do not spontaneously regenerate in humans. A number of factors have been identified that can preserve, repair or regenerate these cell types with a growing number of studies showing recovery of hearing in animal models of acquired and genetic forms of hearing loss. The anatomy of the cochlea presents some unique challenges for the development of a therapy for hearing loss. The cochlear tissues and membranes are extremely sensitive and difficult to access, and consistent dosing is often difficult to achieve. To overcome these limitations, researchers are using novel drug delivery techniques and technologies. The cochlear implant itself is also the subject of a range of drug delivery strategies, from electroactive biomaterial coatings to using electrical stimulation to transfect cochlear cells with DNA encoding neurotrophic factors. Hydrogel biomaterials are being considered for the simplicity of application to the RW of the cochlea for non-invasive and safe drug delivery to the cochlea. Nano- or supraparticle biomaterial technologies offer slow-release and high payload drug delivery properties, which are attractive for neurotrophin delivery to the cochlea to protect SGNs from degeneration. There are currently numerous examples of hearing restoration in genetic models of hearing loss following gene therapies due to new viral vector and gene editing technologies and improvements in delivery techniques. Activating progenitor cells in the cochlea has also been explored towards hair cell regeneration alongside cell-based therapies for repopulating the SGN population or delivering neurotrophins for SGN protection after hearing loss. As the

potential for a drug treatment to restore hearing becomes evident in pre-clinical studies, advancements in drug therapy technologies such as those described in this thesis will help ensure that translation of these discoveries can be achieved.

1.9 THESIS SCOPE

In this chapter, current challenges with delivering drug to inner ear via different strategies, especially nanoengineered drug delivery systems, were discussed and examples of how those drug delivery systems can be applied to solve some of the difficulties were presented. Although supraparticles have demonstrated potential for neurotrophin delivery to the inner ear, their current synthesis approach and drug delivery profiles need further improvement before translation of this system to clinical trials can be realised.

In this thesis, the motivation is to address key issues associated with using nanoengineered particles for delivering neurotrophins to treat hearing impairment. Specifically, this thesis describes the design and the development of a supraparticle drug delivery platform in three related aspects: (i) to develop a robust and automatable technique for nanoengineered particles assembly with high yields, (ii) to improve the pharmacokinetics of neurotrophins released from nanoengineered particles to the inner ear and (iii) to develop strategies to prolong neurotrophins release from nanoengineered particles.

In Chapter 2, the main instruments and methods associated with synthesising and characterising the nanoengineered particles are described.

In Chapter 3, the design and development of a robust and automatable alginate gel-mediated electrospray approach for nanoengineered drug delivery system is described.

Supraparticle assembly on a large scale provides an opportunity for translating this drug delivery system to clinical trials and commercialisation. Supraparticles assembled from primary silica particles with large pores and subjected subsequently to calcination to remove the alginate matrix are shown to be a promising platform for sustained and long-term neurotrophin delivery to the inner ear to treat hearing loss.

In Chapter 4, the pharmacokinetic profile of BDNF in guinea pig cochlea is investigated. BDNF-encapsulated supraparticles are implanted either in the inner ear or on the RW membrane. The results show that BDNF can be successfully released from supraparticles in an *in vivo* animal model for at least 7 days. This chapter provides a basic understanding of the mechanisms of BDNF dispersal in the inner ear.

In Chapter 5, the design and development of engineered biocoatings for supraparticles to reduce the initial rapid release of the loaded drug is described. Sustained release is desired due to potential drug toxicity caused by burst or rapid release of drugs. The results highlight the versatility of the supraparticle drug delivery platform, combining large loading capacity with sustained drug release that is tailorable using different modes of controlled delivery approaches.

In Chapter 6, an alternative bioengineered coating is developed to optimise the drug release behaviour of the supraparticles and their performance in the inner ear. The novel coating strategy shows that: (i) the released neurotrophins from the coated supraparticles remain active and (ii) “normal hearing” can be preserved via intratympanic administration route, while high frequency thresholds were increased following the implantation of coated supraparticles into the cochlea.

In summary, there are a number of nanoengineered drug delivery systems under investigation to treat inner ear disease, but only a few have progressed to the clinic. The

overall aim of this thesis is to develop a controlled and long-term drug delivery system that can be used to overcome the challenges of inner ear drug delivery both via assembly strategies and optimising the performance of particles in terms of drug release to facilitate their future clinical use.

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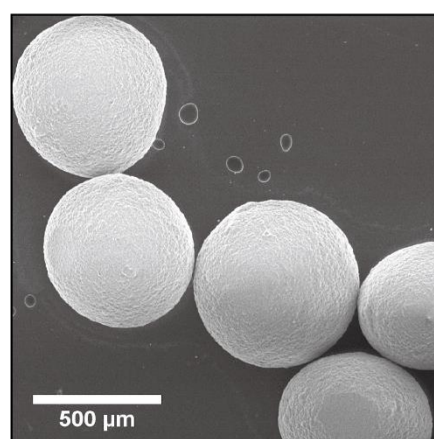
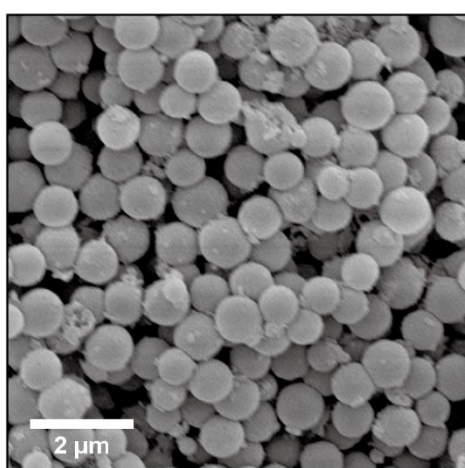
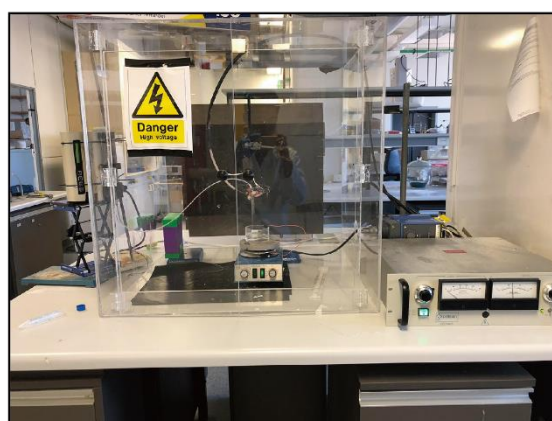
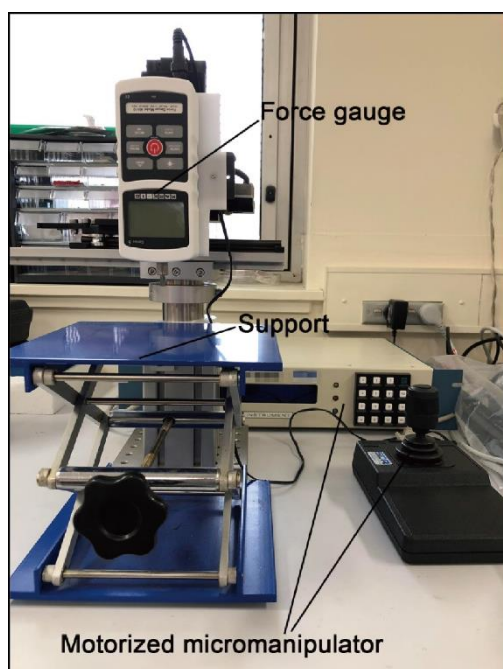
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Chapter 2

Instrumentation and Techniques



2.1 SUMMARY

This Chapter provides a brief description of the instruments and methods used in this thesis. The description mainly focuses on the theoretical principles, characterisation techniques and general operating procedures of the instruments. More detailed descriptions of specific instruments are provided in relevant chapters in this thesis.

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2.2 ELECTROSPRAY SET-UP

Electrospraying was first introduced by Cooley in 1903.¹ It was first applied to fabricate filaments and the paper describes the dispersion of charged fluids of various viscosities. Currently, electrospray technology can be applied in many engineering fields, including ink-jet printing, spray drying and atomisation, emulsification, dispersion and production of functional particles.² A typical electrospray set-up consists of a high voltage power supply, syringe pump, electrospray nozzle, flexible tube and a collector (such as petri dish) (**Figure 1**). The basic concept of electrospraying is to apply a high electric field to break a liquid jet into small droplets. Compared to traditional mechanical atomisers, the electrospray technique can reduce the size of the droplet to less than 10 μm .³ By adjusting the instrumental parameters, such as the distance between the nozzle and the collector, voltage (electric field), flow rate of syringe pump, the diameter of the nozzle, as well as the surface tension, electrical conductivity and viscosity of the liquid jet, different sizes and shape of electrospray beads/particles such as hollow microspheres, cup-shaped nanoparticles, porous microcarriers, cell-shaped microparticles, core-shell/multilayered microspheres and Janus particles can be formed.

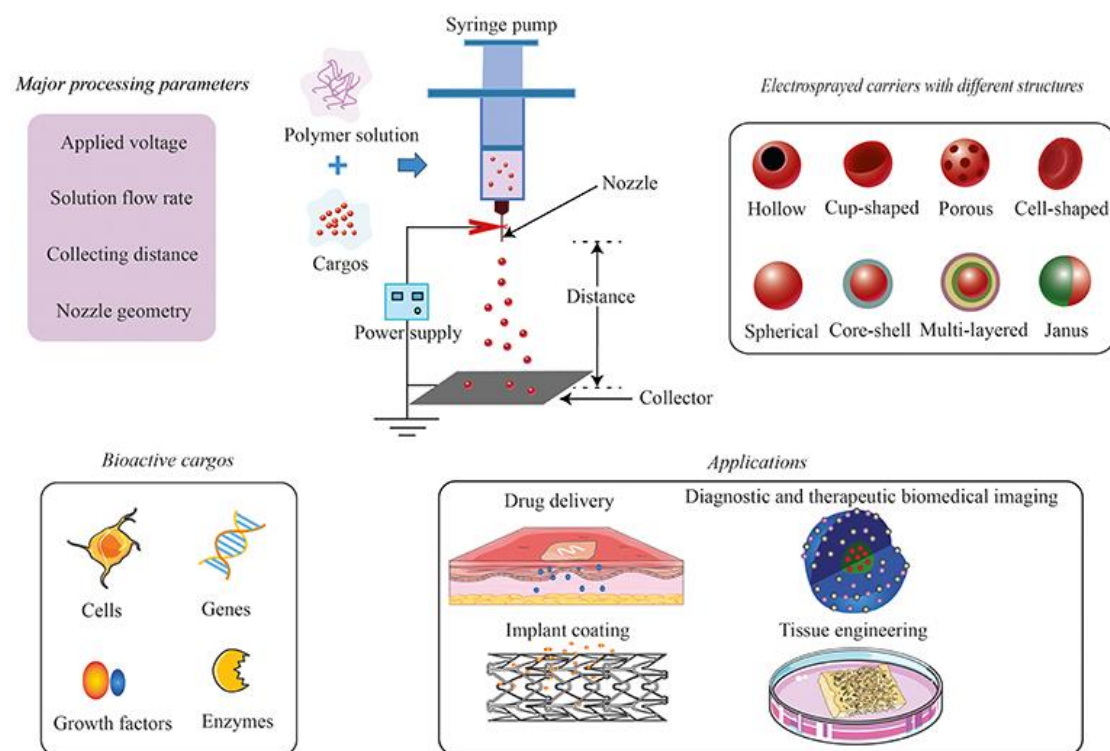


Figure 1. Illustration of basic electrospray set-up and fabrication and application of electrosprayed carriers.⁴

When the charged polymer solution is exposed to an electric field which is supplied by the high voltage power supply, it starts to accumulate charge on the surface (**Figure 2a**). As the voltage increases, the charged droplet will be elongated where a cone shape starts forming and the electrostatic force is still smaller than the surface tension (**Figure 2b**). As the voltage is higher than V_c (a certain threshold voltage), a cone-jet is formed due to the charge-charge repulsion (**Figure 2c**). Due to the viscosity of the charged polymer solution and the presence of entanglements, the jet still remains stable.⁵ The value of the electric field can be calculated using the equation as follows:

$$E = \frac{4U}{D_n \ln \left(\frac{8L}{D_n} \right)} \times 100\% \quad (1)$$

where E is the electric field, D_n is the diameter of the nozzle, U is the applied voltage, L is the distance between the nozzle and the collector.⁶

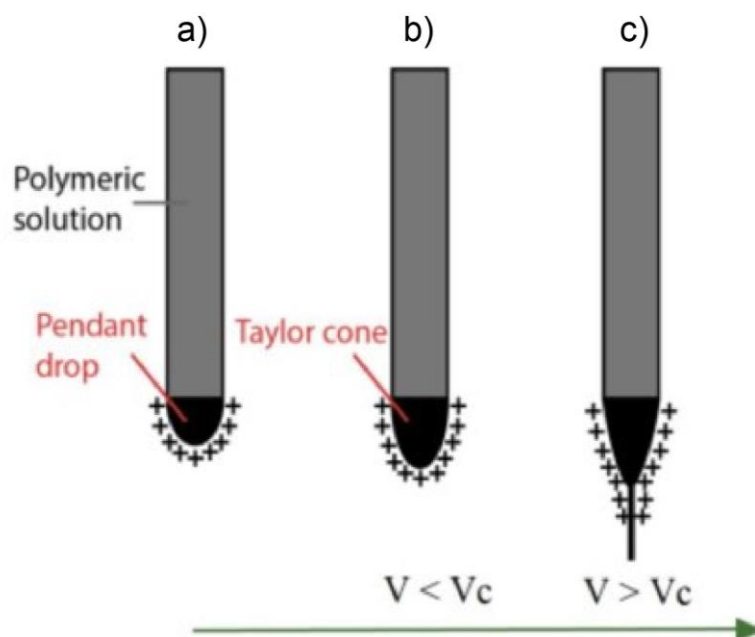


Figure 2. Illustration of the formation of Taylor cones from electrospraying. (a) Charge accumulation on the surface of the droplet when the electric field applied. (b) Electric field increases but still lower than V_c . (c) Electric field increases higher than V_c .⁷

There are quite a few advantages of using electrospray to produce particles or therapeutic carriers for biomedical applications. Firstly, the particle synthesis requirements are not harsh, which means they can be performed at ambient conditions. Secondly, the encapsulation efficiency of therapeutics or cargo into the carriers can be maximised.⁸ Thirdly, the synthesised nano/microparticles have uniform size distribution. Therefore, the electrospray technique can serve as a promising platform to synthesise drug delivery systems/carriers and scale up the manufacturing protocol towards clinical translation and commercialisation. In this thesis, electrospray was used to produce supraparticles in an alginate matrix at high throughput.

2.3 DIFFERENTIAL INTERFERENCE CONTRAST (DIC) MICROSCOPY

Differential interference contrast (DIC) microscopy is a qualitative technique that is typically used to increase the contrast of transparent or unstained specimens.⁹ Basically, in DIC microscopy, light is generated by an illuminator and polarised at 45° (**Figure 3**). Two beam-splitting prisms (Wollaston/Nomarski prisms) can generate two closely spaced partially coherent but orthogonal to sample specimens. The specimen optical path differences (unequal in phase) are mainly controlled by the differences of refractive index, thickness and surface properties of the products as the light beams traverse the sample. After the beams have interfered, the high contrast pseudo three-dimensional images with brighter and darker fields are generated. In this thesis, DIC microscopy was used to characterise the size distribution of silica supraparticles.



Figure 3. A DIC microscope. Polarised light enters a Nomarski prism and reorientates to produce a wavefront. Subsequently, the wavefront passes through a condensing lens to form parallel components and that probed onto the sample. After that, the

two beams are collected by objective lens and enter a second Nomarski prism. Finally, the analyser blocks the linear polarised light, generating a pseudo three-dimensional image through interference contrast.¹⁰

2.4 FLUORESCENCE MICROSCOPY

Fluorescence microscopy uses fluorescence and phosphorescence to study the structural organisation, spatial distribution of organic or inorganic samples that can be used in the field of biology, biomedicine and materials sciences.^{11,12} A fluorescence microscope works by the combination and magnification of the optical microscope properties. Typically, a fluorescence microscope consists of a light source (xenon or mercury arch lamp), excitation filter, the dichroic mirror (beam splitter), emission filter and objective lens (**Figure 4**). A high intensity light passes through an excitation filter to excite fluorophores in the fluorescence labelled samples. Then a dichroic mirror (placed 45° between excitation filter and emission filter) reflects and transmits the excitation signals to an emission filter. Finally, the emission filter will further filter out the incident excitation light and transmit the emission signals of the fluorophore to the objective lens to form images. In this thesis, fluorescence microscopy was used to characterise the FITC-lysozyme loading in silica nanoparticles.

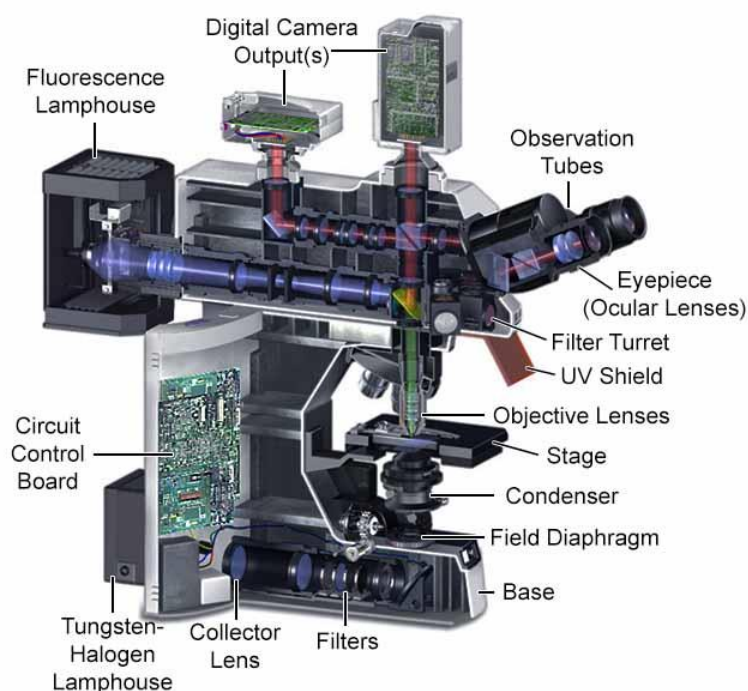


Figure 4. Schematic representation of a fluorescence microscope.¹¹

2.5 CONFOCAL LASER SCANNING MICROSCOPY

Confocal laser scanning microscopy (CLSM) is an advanced fluorescence microscopy technique that can detect fluorescent molecules within a sample in three dimensions.^{13,14} A typical confocal laser scanning microscope consists of the laser, beam splitter, scanner, objective lens, z-control, pinhole and photomultiplier tubes (PMTs) (**Figure 5**). Compared to a conventional fluorescence microscope, the laser light intensity of a confocal laser scanning microscope can be regulated by neutral density filters and transmitted to scanning mirrors that can move them precisely and rapidly. Then the light passes through a semi-transparent mirror in which the light can be reflected away from the laser and goes into pinhole aperture. This pinhole aperture will filter out a large percentage of out-of-focus light to the light detector by adjusting its size. Then the signal will enter the PMTs in which low intensity light can be amplified and finally passes through the detection system to form a real-time image of

the specimen. In this thesis, CLSM was used to characterise the FITC-lysozyme loading in silica supraparticles and confirm the successful FITC-chitosan coating on the surface of the silica supraparticles.

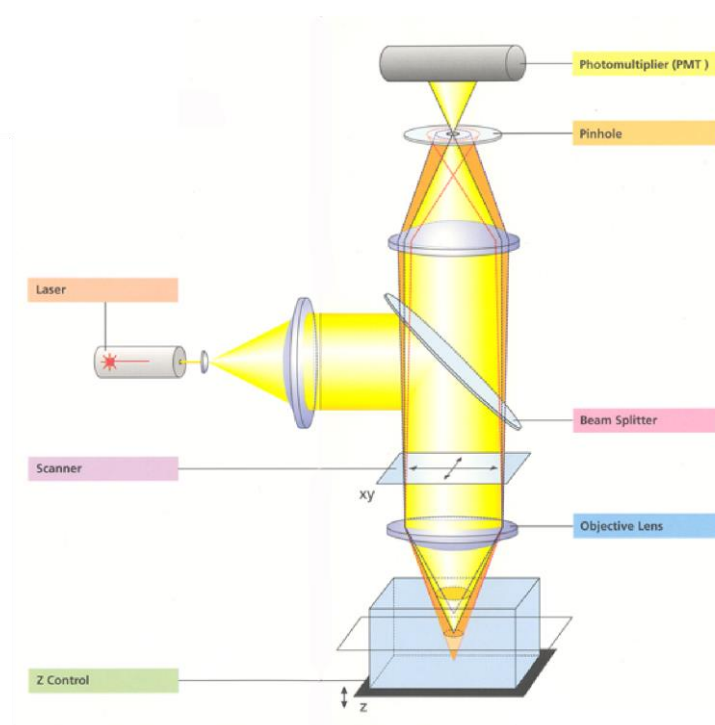


Figure 5. Basic set-up of a confocal laser scanning microscope.¹⁵

2.6 TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) is a microscopy technique that relies on the production of high-energy electron beams towards the specimen to generate high-resolution images down to the nano scale compared to light microscopy in which the resolution of the images depends on the wavelength of light (**Figure 6**).^{16,17} Therefore, TEM can be used as a powerful tool in the field of nanoengineering to determine the structure, morphology and elemental composition of samples in both two and three dimensions. The transmission electron microscope is typically enclosed in a thick shield

to avoid users from harmful x-rays that are emitted due to electron-matte interactions.¹⁸

The microscope consists of an electron source, electromagnetic condenser lens, objective lens, projection lens and an electron detector. Electrons are emitted by the electron source/gun and accelerated by the anode. The electrons are deflected and collected by condenser lens and objective lens, followed by focusing the electrons onto a fluorescent phosphor screen to visualise the images. In this thesis, TEM was used to characterise the size and morphology of the silica nanoparticles.

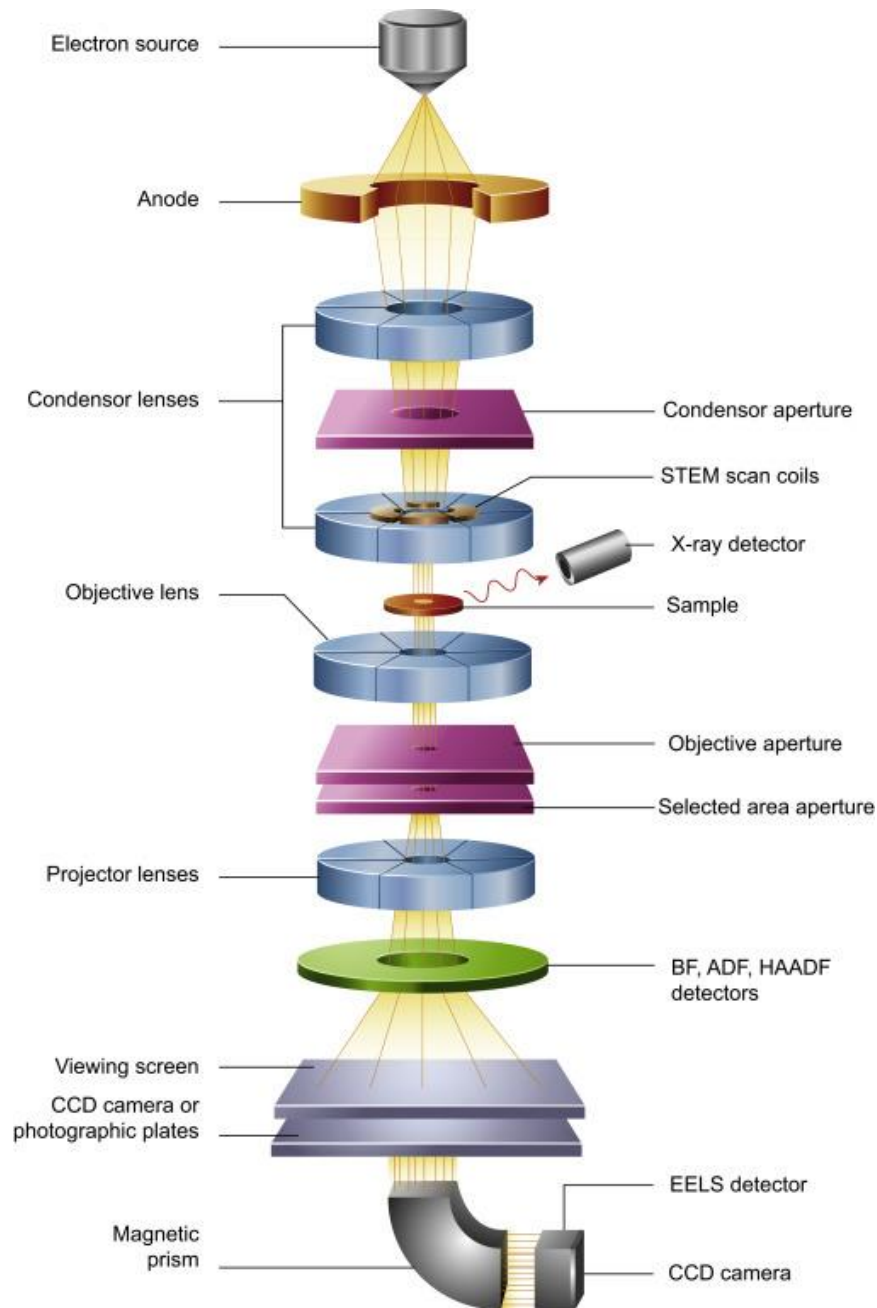


Figure 6. Schematic representation of a transmission electron microscope.¹⁹

2.7 SCANNING ELECTRON MICROSCOPY AND ENERGY DISPERSIVE X-RAY (EDX) SPECTROSCOPY

Scanning electron microscopy (SEM) is an imaging technique with microscope components similar to those found in a transmission electron microscope including an electron source/gun, condenser lens, focus lens and electron detectors.^{20,21} In SEM, the

electrons are generated by the electron gun (**Figure 7**). Then they are reflected and focused by a set of lenses followed by the measurement by the detector. SEM is typically used to investigate the surface morphology of samples. Different structures on the surface of the samples will scatter electrons differently which provides the contrast of the image. Normally, a focused electron beam (2–10 keV) scans the surface of samples and the space resolution can be as low as 1 nm. Poorly electrically conductive samples (such as silica particles) require a coating of a thin layer of gold or platinum. To further study the three-dimensional structures of particles, SEM samples can be tilted at different angles.

SEM coupled with energy-dispersive X-ray (EDX) spectroscopy can also provide analysis of the elemental composition of a specimen. A beam of electrons can ionise the atoms of the samples and generate X-ray emissions at different points of the specimen. The emitted X-ray corresponds to different types of elements as different elements have different atom structures, which gives the elemental composition of the specimen. In this thesis, SEM was used to characterise the size and morphology of silica supraparticles.

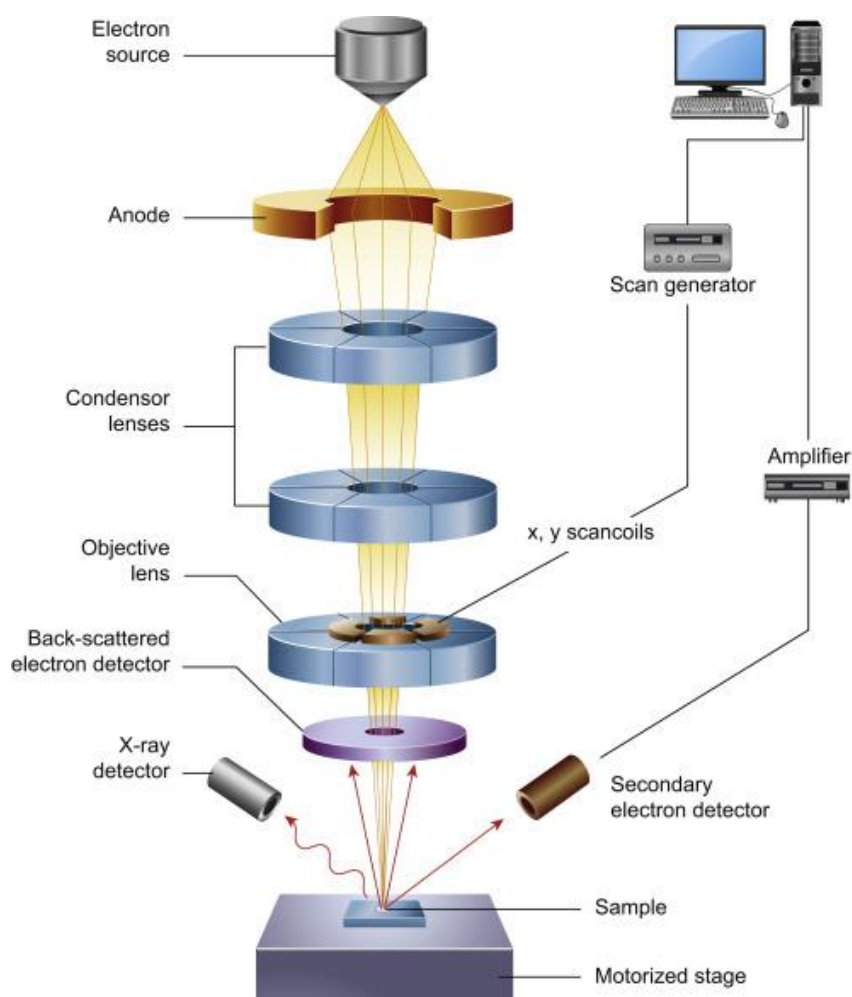


Figure 7. Schematic representation of a scanning electron microscope.¹⁹

2.8 FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy is an easy and powerful tool to measure and detect the fluorescence intensity of fluorescent species with high sensitivity, and thus, it is generally used to characterise the presence and concentration of the fluorophore-conjugated compounds.^{22,23} **Figure 8** illustrates how different electronic states of a molecule transition in the process of fluorescence. In general, fluorophores are excited with a specific wavelength of light where ground state electrons will absorb energy and jump to excitation state (S_2 in **Figure 8**). During this process, the electrons will experience the internal conversion process because of the heat loss from the

environment and vibration relaxation.²⁴ Finally, a photon will be emitted from the lowest lying singlet excited state, which is the fluorescence. Additionally, several factors will influence the fluorescence spectra such as quenching by other molecules/light, energy transfer from other molecules, binding, local polarity, solvent composition, temperature, pH value or aggregation.

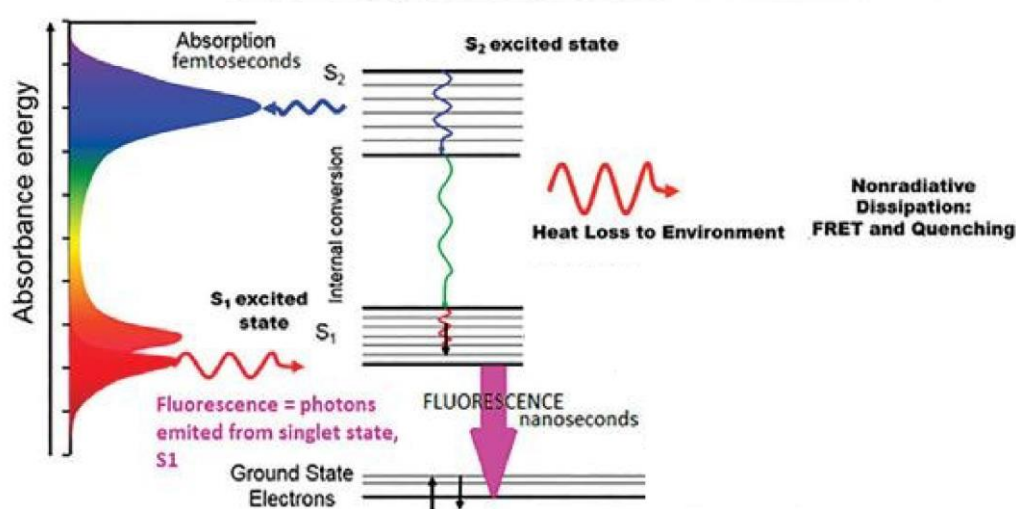


Figure 8. Jablonski diagram of molecular fluorescence and absorbance. Adapted from reference 24.²⁴

A general fluorescence spectrometer consists of a light source (a mercury-vapor or xenon lamp), an excitation monochromator, an emission monochromator, a sample holder and a photon detector (**Figure 9**). Excitation light is emitted by a light source with a specific wavelength and is then tuned by the excitation monochromator and further transmitted to the emission monochromator to reducing the background noise and for further analysis. Because of the lower energy of the emitted light than the exciting light, the emission light can be separated from the excitation light. In this thesis, fluorescence spectroscopy was used to measure the FITC-lysozyme loading and release kinetics.

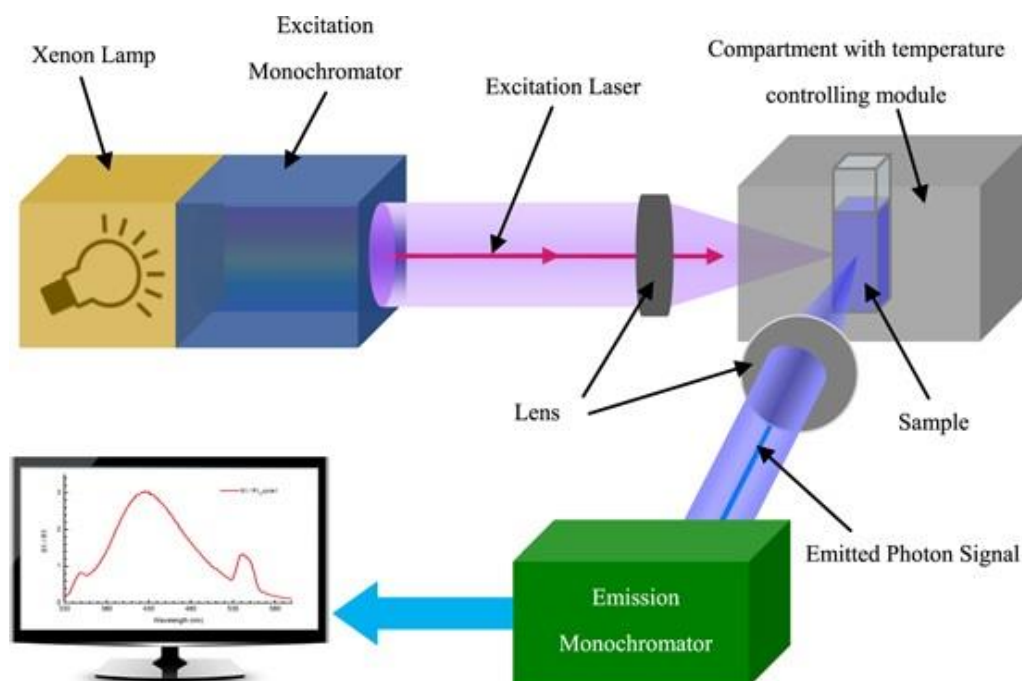


Figure 9. Schematic representation of a fluorescence spectrometer.²⁵

2.9 ULTRAVIOLET-VISIBLE SPECTROSCOPY

Ultraviolet and visible (UV–vis) spectroscopy is a powerful technique to determine the chemical information of samples and is used routinely in many fields, including biochemistry, environmental sciences, food quality and forensic science.²⁶ In chemical analysis, UV–vis spectroscopy is used to quantitatively characterise the concentration of the solution. The principle of UV–vis spectroscopy is to measure the attenuation of a beam of incident light after it passes through or reflects from a sample surface.²⁷ The UV–vis spectrometer consists of a light source, a set of monochromators (for narrowing the light bands) and a detector (**Figure 10**). A beam of light is generated by a light source and diffracted by a diffraction grating and further transmitted to the specimen. The intensity of the transmitted light can be related to the measured absorption value, which can be calculated by Beer-Lambert Law as follows²⁸

$$A = -\log_{10} \left(\frac{I}{I_0} \right) = \epsilon Lc \quad (2)$$

where, A is the light absorption value at a specific wavelength, I_0 and I are the intensity of the incident light and transmitted light, respectively, ϵ is the molar decadic extinction coefficient, L is the path length and c is the concentration of absorbing species. In this thesis, UV–vis spectroscopy was used to measure the concentration of released FITC-lysozyme and measure the cytotoxicity of supraparticles.

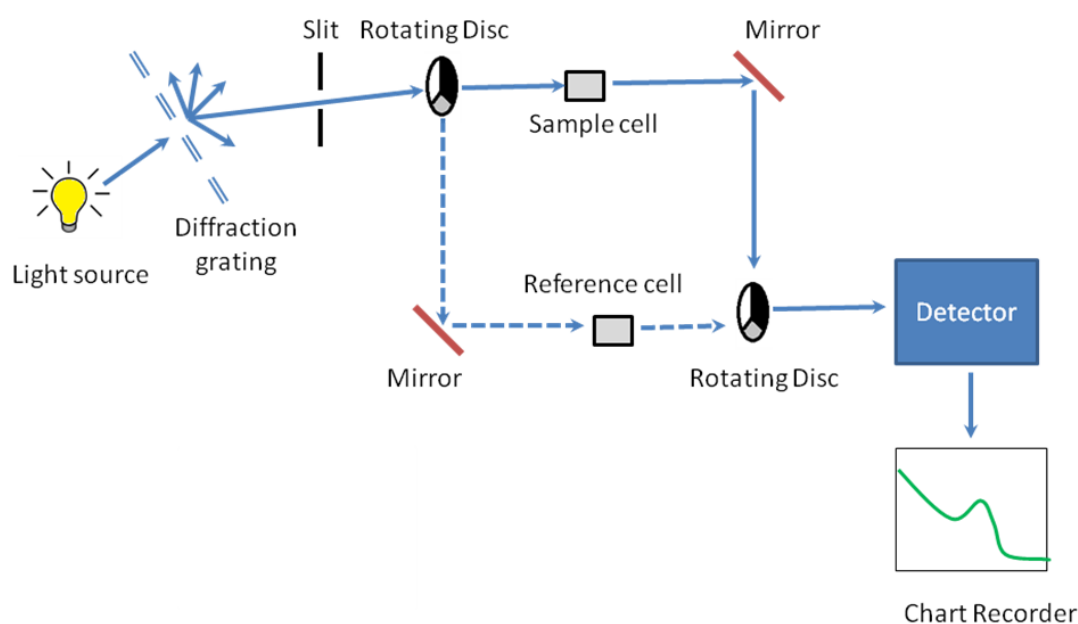


Figure 10. Schematic representation of a UV–vis spectrometer.²⁹

2.10 GEL ELECTROPHORESIS

Gel electrophoresis is a commonly used technique to separate biomolecules such as nucleic acids or protein based on their molecular weight.^{30,31} As proteins are normally folded into different structures and have functional groups with either positive or negative charges along the chain, to linearise a given protein in denaturing gel electrophoresis, the protein is first treated with an anionic detergent (sodium dodecyl sulfate (SDS)) to cover the protein and make it negatively charged. Then, the samples

are heated and treated with reducing agents to break off disulphide bridges.³² Subsequently, the protein samples are placed into a polyacrylamide-based gel followed by filling with a sufficient amount of buffer to stabilise the consistent charges of the proteins. After the application of an electric field, the negatively charged proteins will migrate from cathode to anode (due to the porous structure of polyacrylamide gels) at different speeds based on their molecular weight/size. Finally, the protein in the gel is stained with Coomassie blue or other protein binding stains to visualise the bands. In this thesis, gel electrophoresis was used to examine the degradation of *in vitro* released neurotrophins.

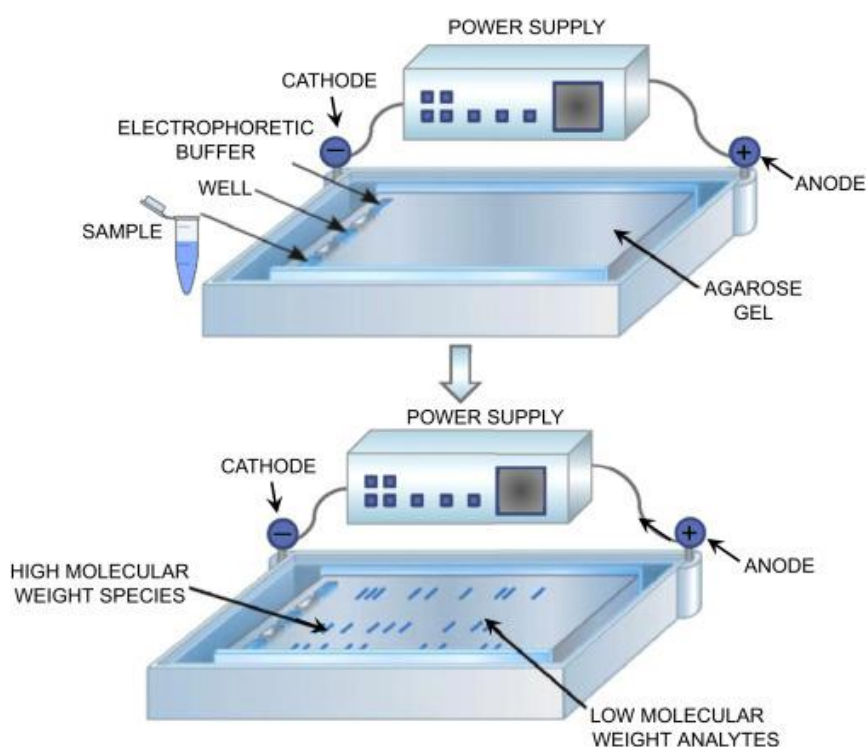


Figure 11. Schematic representation of the process of protein separation using gel electrophoresis.³³

2.11 ZETA-POTENTIAL MEASUREMENT

Zeta (ζ)-potential describes the electric differences between the slipping plane around a colloid particle and the bulk fluid away from the interface.³⁴ It provides a reference to the exact surface charge of a particle. For instance, if a negatively charged particle is dispersed in a solution, positive ions will be attracted to the surface of the particle, which will form electrical double layers around the particle. The ions are strongly bound in the inner region (Stern layer) and loosely associated with the outer (diffuse) layers. When the particle settles down due to the gravity, ions in these regions can move, whereas the ions outside these regions will not be influenced. Furthermore, several factors such as the ionic strength, pH and the solvent influence the value of the ζ -potential, and therefore need to be taken into consideration.

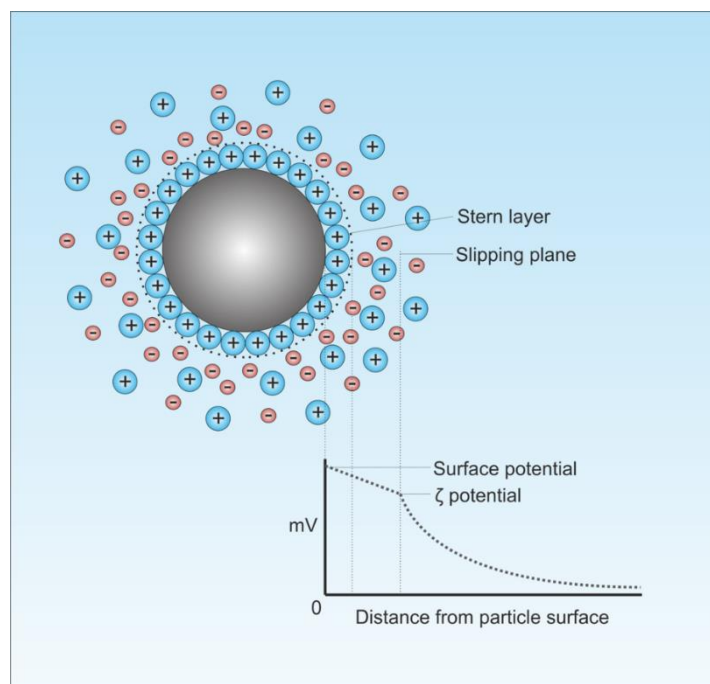


Figure 12. Schematic representation of a negatively charged particle in solution with a Stern layer of ions on the particle surface.³⁵

Microelectrophoresis is a typical technique used to measure ζ -potential values. The particles move as an electric field applied, which will lead to change in the intensity of a laser light and scattering off the particles. The intensity change is associated with the particle velocity and therefore their electrophoretic mobility. The ζ -potential is related to the electrophoretic mobility and can be calculated using Henry's law as follows:

$$U_E = \frac{2\varepsilon\zeta}{3\eta} f(\kappa a) \quad (3)$$

where U_E is the electrophoretic mobility, ε is the dielectric constant, ζ is zeta potential, η is the viscosity of the dispersant and $f(\kappa a)$ is Henry's function.³⁶

In this thesis, the ζ -potential of silica supraparticles was measured to confirm the surface charge of the supraparticles.

2.12 INDUCTIVELY COUPLED PLASMA–OPTICAL EMISSION SPECTROMETRY (ICP-OES)

ICP-OES is a technique used for the detection of elements using plasma and a spectrometer. It is suitable for analysing samples with concentrations ranging from 0.0002 to 1000 ppm. The sample is typically diluted in a solution (mostly water) and sucked by a pump through a nebuliser and into a spray chamber. Then argon gas enters the spray chamber and is fed to the plasma torch. Alternating current flows at the end of the torch generate an alternating magnetic field that accelerates the electrons. Upon collision of the electrons with argon atoms, a considerably hot plasma (6000–10000 K) is generated in which thermal energy is taken up by the electrons. After the excited electrons return to the ground energy level, light is liberated in different colours. Finally, the emission spectrum can be recorded by the detector. Different elements can be analysed at the same time if they have different emission spectra (the peaks do not

overlap). By comparing the spectrum with that of the standard solution, the concentration of each element can be determined. In this thesis, ICP-OES was used to measure the degradation of the silica supraparticles.

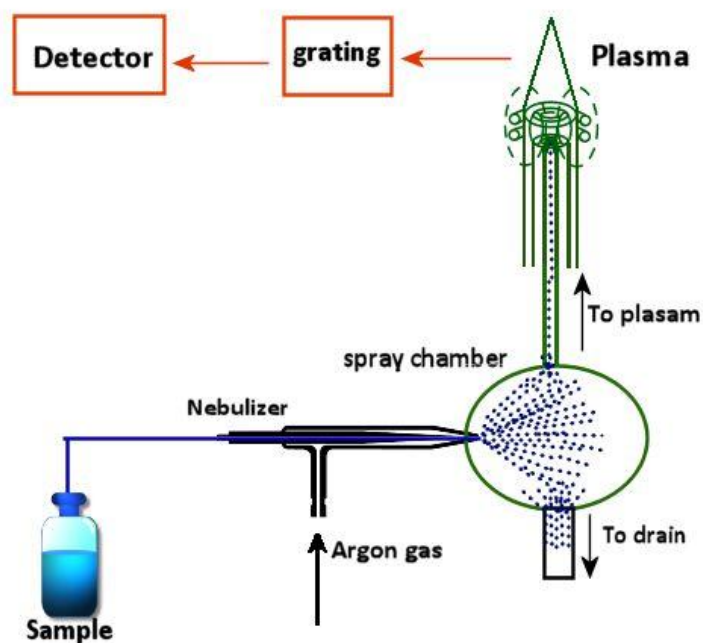


Figure 13. Schematic representation of an ICP-OES instrument.³⁷

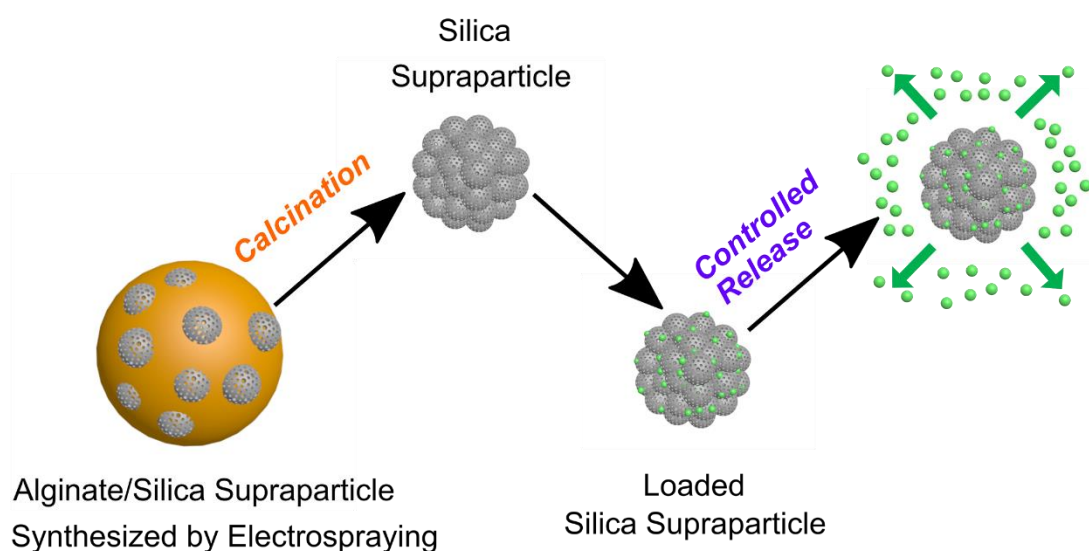
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Chapter 3

Gel-Mediated Electrospray Assembly of Silica Supraparticles for Sustained Drug Delivery



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Gel-Mediated Electrospray Assembly of Silica Supraparticles for Sustained Drug Delivery

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Supporting Information

ABSTRACT: Supraparticles (SPs) composed of smaller colloidal particles provide a platform for the long-term, controlled release of therapeutics in biomedical applications. However, current synthesis methods used to achieve high drug loading and those involving biocompatible materials are often tedious and low throughput, thereby limiting the translation of SPs to diverse applications. Herein, we present a simple, effective, and automatable alginate-mediated electrospray technique for the assembly of robust spherical silica SPs (Si-SPs) for long-term (>4 months) drug delivery. The Si-SPs are composed of either porous or nonporous primary Si particles within a decomposable alginate matrix. The size and shape of the Si-SPs can be tailored by controlling the concentrations of alginate and silica primary particles used and key electrospraying parameters, such as flow rate, voltage, and collector distance. Furthermore, the performance (including drug loading kinetics, loading capacity, loading efficiency, and drug release) of the Si-SPs can be tuned by changing the porosity of the primary particles and through the retention or removal (via calcination) of the alginate matrix. The structure and morphology of the Si-SPs were characterized by electron microscopy, dynamic light scattering, N₂ adsorption–desorption analysis, and X-ray photoelectron spectroscopy. The cytotoxicity and degradability of the Si-SPs were also examined. Drug loading kinetics and loading capacity for six different types of Si-SPs, using a model protein drug (fluorescently labeled lysozyme), demonstrate that Si-SPs prepared from primary silica particles with large pores can load significant amounts of lysozyme (~10 µg per SP) and exhibit sustained, long-term release of more than 150 days. Our experiments show that Si-SPs can be produced through a gel-mediated electrospray technique that is robust and automatable (important for clinical translation and commercialization) and that they present a promising platform for long-term drug delivery.

KEYWORDS: alginate, electrospray, silica supraparticles, porous silica, drug delivery



INTRODUCTION

Colloidal particles can exhibit a range of properties (e.g., electronic, magnetic, optical, and catalytic), making them suitable for application in diverse disciplines such as chemistry, physics, engineering, and biomedicine.^{1–4} Supraparticles (SPs) are assemblies (clusters) of primary colloidal particles, and their size typically ranges from micrometers to millimeters.⁵ As the size and structure of a material can significantly influence

its mechanical and physicochemical properties, SPs can thus exhibit substantially different properties from those of their individual primary (colloidal) particle counterparts.^{6,7} SPs have been applied in various fields, including photonics (as light-

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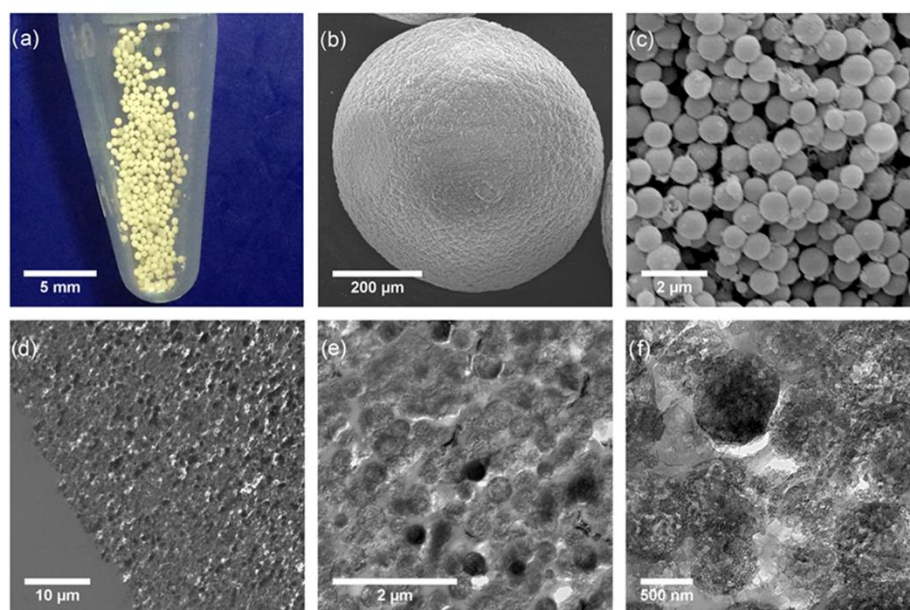


Figure 1. Characterization of $^1\text{Si-SPs}$. (a) Photograph of $^1\text{Si-SPs}$ in a 1.7 mL centrifuge tube. (b) SEM image of a $^1\text{Si-SP}$ and (c) a higher resolution SEM image showing the surface structure of a $^1\text{Si-SP}$. (d–f) Electron microscopy images of a thinly sliced section of a $^1\text{Si-SP}$ at different magnifications.

The FITC-lysozyme loading efficiency was calculated from eq 4

$$\text{loading efficiency (\%)} = \frac{m_0 - m_n}{m_0} \times 100\% \quad (4)$$

where m_0 is the total amount of FITC-lysozyme added to the SPs (μg) and m_n is the amount of free FITC-lysozyme in the supernatant.

In Vitro Release Studies. For the in vitro release studies, $^1\text{Si-SPs}$ and $^1\text{Si-SPs}^{\text{alg}}$ were examined. The FITC-lysozyme-loaded SPs for the in vitro release studies were prepared by sterilizing the SPs in 80% v/v ethanol as described above, followed by incubation of 10 SPs ($^1\text{Si-SPs}$ or $^1\text{Si-SPs}^{\text{alg}}$) in 100 μL of FITC-lysozyme solution (0.2 mg mL^{-1} in Milli-Q water); these two types of SPs gave the highest drug loading. After 3 days of incubation, the supernatant was removed and 100 μL of PBS (pH 7.4) was added to each tube and incubated at 37 $^\circ\text{C}$. At defined time intervals (over 150 days), 90 μL of the solution was collected and replaced with fresh PBS. The fluorescence of the collected samples was measured with an Infinite M200 microplate reader, and the concentration of FITC-lysozyme in the supernatant was calculated using a standard curve of known concentrations of FITC-lysozyme (Figure S5a).

Drug release kinetics was also assessed after loading with higher quantities of the model drug FITC-lysozyme. A loading concentration of 1.0 mg mL^{-1} FITC-lysozyme (in Milli-Q water) was added to 10 $^1\text{Si-SPs}$, and the in vitro drug release behavior of the SPs in PBS was assessed as described above using the standard curve in Figure S5b.

Additionally, the drug release kinetics of the SPs in a hydrogel, as a potential medium for surgical delivery, was assessed. Specifically, in vitro FITC-lysozyme release in PF127 hydrogel was examined using the same protocol as that described above. After 3 days of incubation with FITC-lysozyme, 33.3 μL of PF127 hydrogel (30 wt % in Milli-Q water) was added to a 1.7 mL centrifuge tube, followed by addition of 16.7 μL of PBS buffer. PF127 hydrogel and PBS solution were kept on ice before use. The resulting concentration of PF127 was 20 wt %, which resulted in gelling as the solution was incubated at 37 $^\circ\text{C}$.

For BDNF loading, five sterile $^1\text{Si-SPs}$ were incubated with 50 μL of BDNF solution (1.0 mg mL^{-1} in Milli-Q water). After 3 days of

incubation, the supernatant was removed and 100 μL of PBS (pH 7.4) was added to the tube and incubated at 37 $^\circ\text{C}$. At defined time intervals (over 40 days), 95 μL of the solution was collected and replaced with 95 μL of fresh PBS for the duration of the release studies. The amount of BDNF released from $^1\text{Si-SPs}$ was determined using a BDNF-specific enzyme-linked immunosorbent assay (ELISA) and a standard curve of BDNF samples as per the manufacturer's protocol (Figure S6).

RESULTS AND DISCUSSION

Synthesis and Characterization of Silica Supraparticles. The electrospray setup illustrated in Scheme 1 (and Scheme S1) was used to prepare Si-SPs from a suspension of primary silica particles in alginate. The setup shows that as voltage is applied, liquid containing alginate and primary silica particles is ejected from the nozzle tip, forming a cone of liquid (i.e., a Taylor cone) from which spherical droplets emanate. The droplets are collected in a gelling bath of calcium chloride, which cross-links the alginate to form spherical particles. A number of electrospray parameters can affect the assembly of the SPs, potentially impacting the final size and circularity of the assembled SPs, including nozzle diameter,³³ ionization mode of the electrospray,³⁴ and the viscosity and surface tension of the collection bath, as well as the five main parameters that were systematically varied in this study: (i) the concentration of alginate; (ii) the concentration of primary silica particles; (iii) the flow rate; (iv) the applied voltage; and (v) the distance to collector (distance between the nozzle tip and the surface of the calcium chloride). The sizes of the Si-SPs generated using variations in these five parameters are summarized in Table 1.

The viscosity of the solution, and potentially, its electrical conductivity and surface tension, was largely influenced by the concentration of alginate (Table 1, rows 1–3) and had a

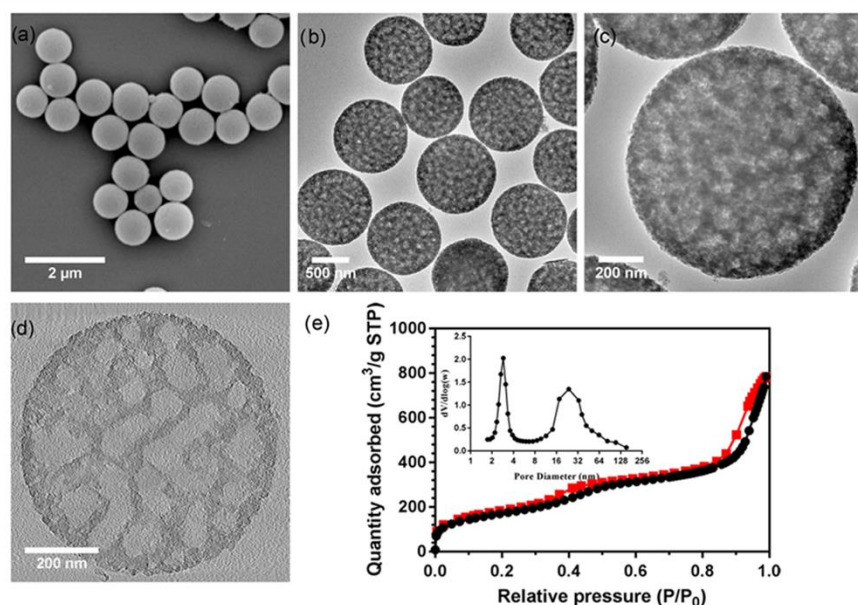


Figure 2. Characterization of primary silica particles with large pores. (a) SEM image of the primary silica particles. (b, c) TEM images of the primary silica particles at different magnifications. (d) Virtual slice through a primary silica particle by cryoelectron tomography (cryo-ET). (e) N_2 adsorption–desorption isotherm of the primary silica particles. Inset in (e) shows the distribution of the pores (<100 nm) of the primary silica particles.

significant impact on Si-SP formation.^{33,35,36} At a relatively low alginate concentration (i.e., 5 mg mL⁻¹), nonspherical and small Si-SPs (<100 μ m in diameter) were obtained, as the low viscosity of the alginate solution resulted in the formation of less stable droplets as the droplet generation transitioned between spraying and dripping.^{37–39} When the concentration of alginate was increased to 30 mg mL⁻¹, the viscosity was relatively higher and the synthesized Si-SPs were more spherical and larger (average diameter of $\sim$ 550 μ m) than those obtained using lower alginate concentrations.^{33,40,41} When the alginate concentration was increased further, the viscosity was too high for the nozzle and syringe pump and the system stalled or clogged. The diameter of the Si-SPs increased as the concentration of the primary silica particles increased (Table 1, rows 3–5). This direct correlation was expected as a large amount of silica in each droplet is expected to result in a large amount of silica remaining after calcination (i.e., after liquid and organic components are removed), compared to those obtained at lower concentrations of primary silica particles, where the lower mass fraction of silica results in larger shrinkage during drying and calcination.

The influence of the applied voltage (between the nozzle and the electrically grounded calcium chloride bath) on the size of the Si-SPs was subsequently investigated (Table 1, rows 3, 6, and 7). At lower applied voltages and field strengths (i.e., 5 kV over 10 cm), the electric field force was too weak to form a stable Taylor cone,^{38,42,43} resulting in the formation of large droplets (“spitting” instead of “dripping”). When the applied voltage was increased from 5 to 13 kV and further to 21 kV, the diameter of the Si-SPs decreased significantly (from $\sim$ 1500 to $\sim$ 240 μ m), as it transitioned from a dripping mode to an electrospray mode. Thus, a stronger electric field force is

generated at higher applied voltages (13 and 21 kV), resulting in the formation of a more stable Taylor cone formation and thus well-controlled droplet generation (Scheme S1).^{44,45}

The collector distance, which was varied from 4 to 15 cm, had little influence on the size of the Si-SPs, with the average diameter of the Si-SPs increasing slightly from $\sim$ 560 to $\sim$ 600 μ m (Table 1, rows 3, 8, and 9). This is consistent with previous studies by Bugarski et al.⁴⁶ and Goosen et al.,⁴⁷ which showed that the collector distance minimally influenced the diameter of alginate beads formed at high applied voltages (>12 kV) that are comparable with those used in our study.

Finally, the effect of the flow rate of the syringe pump on the formation of the Si-SPs was investigated (Table 1, rows 3, 10, and 11). As the flow rate was increased from 3 to 20 mL h⁻¹, there was negligible difference in particle size, with the average SP size shifting from $\sim$ 540 to $\sim$ 560 μ m.

On the basis of these results, Si-SPs prepared under the conditions presented in row 3 (Table 1), which were used subsequently, were considered optimal as they resulted in spherical SPs with a diameter of approximately 550 μ m (Figure 1a,b). For inner ear delivery, this is considered an ideal size as the SPs are large enough to potentially have high drug loading capacity but small enough to be delivered via a cannula to the inner ear. The EISA method previously described for preparing Si-SPs can also lead to a versatile range of SPs in terms of size and circularity,²¹ but this would require extensive manual handling compared with the simple changes in electrospray parameters that are afforded by an automatable electrospray technique.

¹Si-SPs were investigated in detail. From optical images of >130 particles, ¹Si-SPs had a diameter of 544 ± 41 μ m (Figure S7a) and a spherical shape with a circularity of 0.854 ± 0.015

(Figure S7b). The three-dimensional (spherical) morphology of $^{\text{L}}$ Si-SPs was further confirmed using SEM at different tilted angles (Figure S8) and confocal laser scanning microscopy (CLSM) at various z heights (Figure S9). The surface of the Si-SPs was investigated by SEM (Figure 1c), and the results showed that the primary silica particles were arranged in random order (e.g., in contrast to optical opal supraparticles⁸), and some spacing (void volume) between individual primary silica particles was apparent. On the basis of SEM analysis, there were negligible macroscopic differences between $^{\text{L}}$ Si-SPs, $^{\text{S}}$ Si-SPs, and $^{\text{N}}$ Si-SPs (Figure S10).

To investigate the inner structures of $^{\text{L}}$ Si-SPs, electron microscopy of a thin section was obtained (Figure 1d–f). The primary silica particles were closely packed, indicating that the Si-SPs are mechanically robust. This is consistent with the force–fracture testing shown in Figure S11; the average force required to fracture individual $^{\text{L}}$ Si-SPs was 311 ± 93 mN.

SEM and TEM evaluation of the as-prepared primary silica particles with large pores both revealed the spherical morphology of the particles, with a diameter of 800–1000 nm (Figure 2a–c). For comparison, TEM images of nonporous primary silica particles and commercial primary silica particles with small pores are shown in Figure S12. Cryo-ET confirmed the porous structure of the primary particles with large pores and demonstrated the large porosity of the primary silica particles (Figure 2d). The Brunauer–Emmett–Teller method and Barrett–Joyner–Halenda (BJH) model were used to calculate the specific surface area and pore diameter distributions of the primary silica particles (Figure 2e), respectively. The specific surface area of the primary silica particles was $623 \text{ m}^2 \text{ g}^{-1}$, and they had a bimodal porous structure, with smaller pores ranging from 2 to 3 nm and larger pores ranging from 15 to 64 nm. Note that from the cryoelectron tomography (cryo-ET) image analysis (Figure 2d), many larger pores were also evident (>100 nm); the BJH technique primarily measures pores of <100 nm. This requirement also means that the total porosity of the SPs and the total void volume between individual primary particles cannot be measured accurately using the N_2 adsorption–desorption technique. Nevertheless, the high porosity of the primary particles and the Si-SPs is expected to enable high drug loading capacity, which is a vital property for drug carriers aimed for sustained and long-term release.

X-ray photoelectron spectroscopy (XPS) was used to study the elemental composition of $^{\text{L}}$ Si-SPs (Figure S13a). High-resolution XPS spectra of the Si 2p region showed the presence of Si–O–Ca linkages (Figure S13b). These results suggest that upon calcination of $^{\text{L}}$ Si-SPs^{alg}, organic alginate is removed but inorganic calcium remains. The calcination process also causes sintering and formation of Si–O–Si bonds.²¹ Any residual calcium may be introduced into the Si–O–Si network, and thus some Si–O–Ca–O–Si groups are also formed, which may confer additional robustness to the Si-SPs.

In Vitro Cytotoxicity. To investigate the use of $^{\text{L}}$ Si-SPs as biocompatible drug carriers, in vitro cytotoxicity studies were performed. Human brain glioblastoma cells (U87MG cell line) were incubated with $^{\text{L}}$ Si-SPs, and cell viability was assessed using an alamarBlue assay. As shown in Figure 3, $^{\text{L}}$ Si-SPs were nontoxic in this noncontact model even when the number of $^{\text{L}}$ Si-SPs was increased to 10 per well (1×10^4 cells per well). Typical in vivo treatment scenarios of similar particle systems suggest the administration of one to eight SPs per inner ear.²⁰

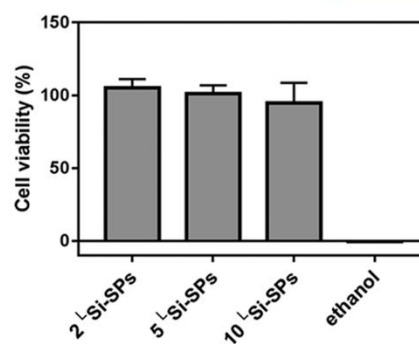


Figure 3. Cell viability of human brain glioblastoma cells (U87MG cell line) incubated with different numbers (2, 5, or 10) of $^{\text{L}}$ Si-SPs for 48 h. Ethanol added to cells with inserts was used as a negative (cytotoxic) control, and untreated cells represent 100% viability. The data presented are the average of four samples, and error bars represent standard deviations.

Drug Loading. To investigate the potential of Si-SPs, particularly $^{\text{L}}$ Si-SPs, as drug carriers for therapeutic proteins in biomedical applications, protein loading studies were conducted with model proteins. FITC-lysozyme was used as a model protein to mimic neurotrophin BDNF because of similarities in their physicochemical properties (lysozyme, molecular weight (M_w) = 14.3 ± 0.5 kDa,⁴⁸ hydrodynamic radius (R_H) = 18.9 ± 0.25 Å,⁴⁸ and isoelectric point (pI) = 11;⁴⁹ BDNF, M_w = 13 kDa,⁵⁰ R_H = 24.0 ± 3.2 Å,⁵¹ and pI = $10^{50,52}$). As shown in Figure 4, Si-SPs exhibited negative ζ

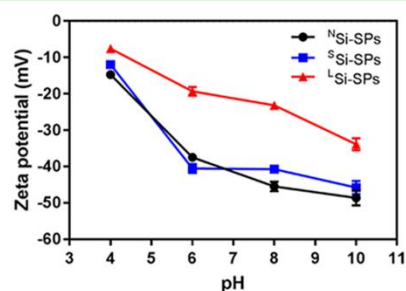


Figure 4. ζ potential of $^{\text{N}}$ Si-SPs, $^{\text{S}}$ Si-SPs, and $^{\text{L}}$ Si-SPs as a function of pH. ζ potential measurements were performed in 10 mM sodium acetate buffer (pH 4), 10 mM phosphate buffer (pH 6), 10 mM HEPES buffer (pH 8), and 10 mM sodium bicarbonate buffer (pH 10).

potentials ranging from approximately -8 to -50 mV as the pH value increased from 4 to 10. Although $^{\text{L}}$ Si-SPs appear less negatively charged than $^{\text{N}}$ Si-SPs and $^{\text{S}}$ Si-SPs, perhaps because of their higher degree of porosity or the in-house method used to prepare the primary silica particles with large pores, positively charged lysozyme and BDNF are likely to be loaded into all Si-SPs, with electrostatic interactions playing a role in the process.

Confocal laser scanning microscopy (CLSM) images of the $^{\text{L}}$ Si-SPs (Figure 5a,b) indicated that FITC-lysozyme associated with the surface of $^{\text{L}}$ Si-SPs. However, as the $^{\text{L}}$ Si-SPs are quite large (hundreds of micrometers in diameter), standard

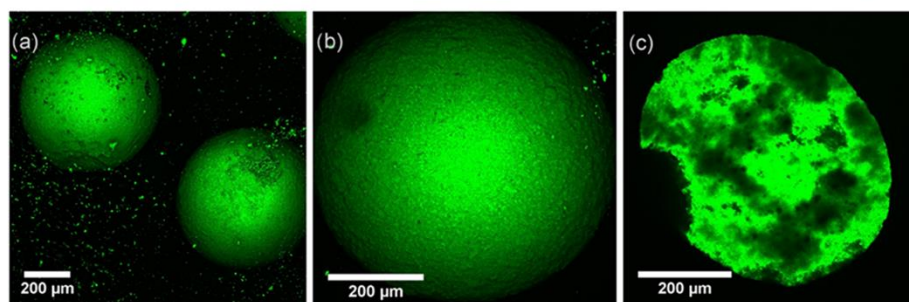
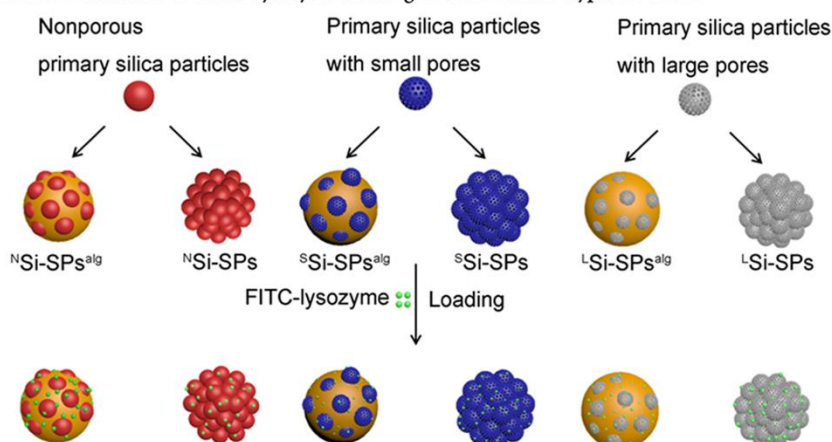


Figure 5. CLSM images of L Si-SPs loaded with FITC-lysozyme (green). (a, b) CLSM images of L Si-SPs at different magnifications. (c) Interior of a fragmented L Si-SP loaded with FITC-lysozyme.

Scheme 2. Schematic Illustration of FITC-Lysozyme Loading in Six Different Types of Si-SPs^a



^aAlginate/nonporous primary silica supraparticles (N Si-SPs^{alg}); alginate/primary silica particles with small pore supraparticles (S Si-SPs^{alg}); alginate/primary silica particles with large pore supraparticles (L Si-SPs^{alg}); silica supraparticles prepared from nonporous primary silica particles (N Si-SPs); silica supraparticles prepared from primary silica particles with small pores (S Si-SPs); and silica supraparticles prepared from primary silica particles with large pores (L Si-SPs).

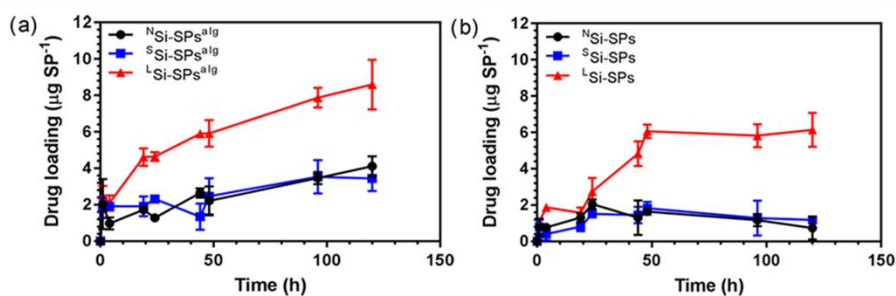


Figure 6. Drug loading kinetics of the different types of Si-SPs prepared. Effect of incubation time on FITC-lysozyme (0.6 mg mL^{-1} in Milli-Q water) loaded into (a) N Si-SPs^{alg}, S Si-SPs^{alg}, and L Si-SPs^{alg}, and (b) N Si-SPs, S Si-SPs, and L Si-SPs. The data are shown as the average of three replicates, each using five SPs, and error bars represent standard deviations.

confocal laser scanning microscopy is not ideally suited to imaging the internal structure of the SPs. Sectioning the L Si-SPs with a scalpel, however, allowed the internal structure of the SPs to be imaged. FITC-lysozyme was observed not only at

the particle surface but also within the porous internal structure of the L Si-SPs (Figure 5c).

Determination of Drug Loading Kinetics, Capacity, and Efficiency. Incubation of lysozyme or neurotrophins with Si-SPs results in protein-loaded Si-SPs. As this process depends

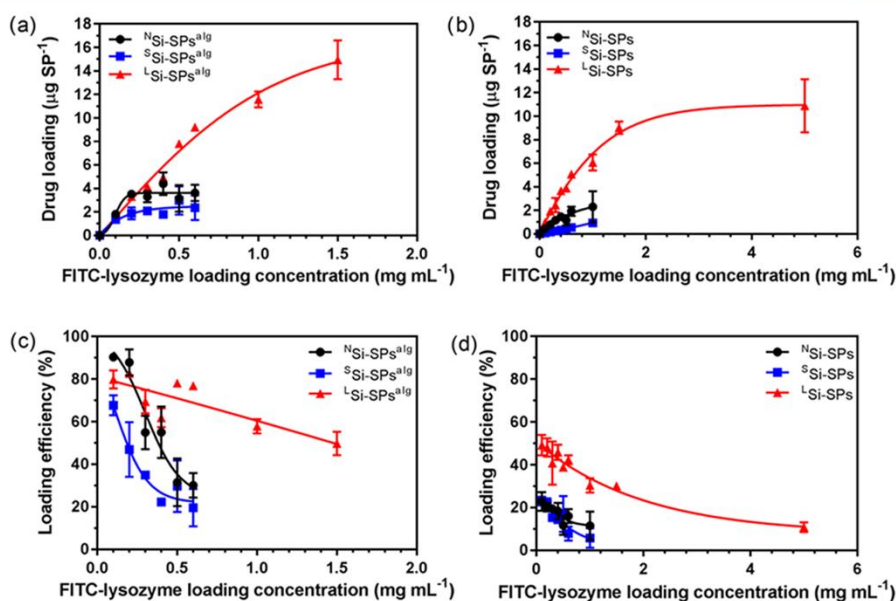


Figure 7. Loading of Si-SPs with FITC-lysozyme at different loading concentrations following a 3 day incubation period. (a) Amount of FITC-lysozyme loaded into Si-SPs^{alg} as a function of FITC-lysozyme loading concentration. (b) Amount of FITC-lysozyme loaded into Si-SPs as a function of FITC-lysozyme loading concentration. The relationship between drug loading and FITC-lysozyme loading concentration from 0 to 1.0 mg mL⁻¹ is shown in Figure S15. FITC-lysozyme loading efficiency of (c) Si-SPs^{alg} and (d) Si-SPs. Data presented are averages of three replicates, each using five SPs, and error bars represent standard deviations.

on several parameters (including incubation time and drug loading concentration), drug loading kinetics was evaluated across a series of loading conditions.⁵³ Drug loading kinetic data were generated for six different types of SPs (NSi-SPs^{alg}, S-Si-SPs^{alg}, L-Si-SPs^{alg}, NSi-SPs, S-Si-SPs, and L-Si-SPs) (Scheme 2). Figure 6 shows the effect of incubation time on FITC-lysozyme loading for the different types of SPs studied. Drug loading is reported as mass of protein per SP; L-Si-SPs have an average weight of 50 μ g, whereas L-Si-SPs^{alg} have an average weight of 95 μ g. Incubation of FITC-lysozyme with NSi-SPs^{alg} and S-Si-SPs^{alg} appeared to reach equilibration after 48 h, whereas saturation of the L-Si-SPs^{alg} with FITC-lysozyme took longer ($\sim$ 96 h) (Figure 6a). In contrast, the drug loading time required to approach saturation for both NSi-SPs and S-Si-SPs was $\sim$ 24 h, whereas that for L-Si-SPs was $\sim$ 48 h. In summary, both L-Si-SPs and L-Si-SPs^{alg} required longer loading times to approach saturation but could load more FITC-lysozyme than the other types of Si-SPs.

On the basis of the results obtained above (Figure 6), an incubation time of 3 days was used in subsequent experiments. Specifically, the loading capacity for the different types of SPs was investigated after a 3 day incubation period with FITC-lysozyme at different loading concentrations (in Milli-Q water) (Figure 7). In general, as the concentration of FITC-lysozyme increased, drug loading increased. Additionally, the alginate-containing Si-SPs (NSi-SPs^{alg}, S-Si-SPs^{alg}, and L-Si-SPs^{alg}) had higher loading capacities than the “alginate-removed” Si-SPs (NSi-SPs, S-Si-SPs and L-Si-SPs) at a given FITC-lysozyme concentration. This is likely due to increased electrostatic interactions between positively charged FITC-lysozyme and negatively charged alginate around neutral pH.²⁴ At low FITC-lysozyme loading concentrations (<0.4 mg mL⁻¹), L-Si-SPs^{alg}

had a drug loading capacity similar to that of NSi-SPs^{alg} and S-Si-SPs^{alg}. In contrast, when the drug loading concentration was higher (>0.4 mg mL⁻¹), a higher amount of FITC-lysozyme could be loaded into L-Si-SPs^{alg} when compared with that achieved for NSi-SPs^{alg} and S-Si-SPs^{alg}. For the “alginate-removed” Si-SPs, a similar trend was observed: L-Si-SPs could load more FITC-lysozyme than NSi-SPs and S-Si-SPs. These results suggest that the large porous structures in L-Si-SPs and L-Si-SPs^{alg} are key factors for improving drug loading, likely by providing additional surface area and hence loading capacity when outer particle surfaces and intraparticle areas (in the SP structures) have been fully saturated with protein.

The experimentally determined maximum loading capacities of FITC-lysozyme into NSi-SPs^{alg} and S-Si-SPs^{alg} were $\sim$ 3 and 2 μ g SP⁻¹, respectively. These values were significantly lower than that observed for L-Si-SPs^{alg}, i.e., 15 μ g of SP⁻¹ (initial FITC-lysozyme concentration was 1.5 mg mL⁻¹, and loading time was 3 days). The experimentally determined maximum loading capacity of FITC-lysozyme into NSi-SPs and S-Si-SPs was $\sim$ 2 and 1 μ g SP⁻¹ respectively, which was also significantly lower than that achieved for L-Si-SPs, i.e., $\sim$ 10 μ g SP⁻¹ (initial FITC-lysozyme concentration was 5.0 mg mL⁻¹, and loading time was 3 days). The reason for using this higher concentration of FITC-lysozyme for L-Si-SPs^{alg} and L-Si-SPs, compared with NSi-SPs^{alg}, S-Si-SPs^{alg}, NSi-SPs, and S-Si-SPs, was that L-Si-SPs^{alg} and L-Si-SPs did not reach drug loading saturation at the lower concentrations. The loading capacity per SP also depends on the diameter of L-Si-SPs where larger L-Si-SPs (1000 μ m) displayed higher loading capacities than the smaller L-Si-SP (200 μ m) counterparts after 3 days of incubation with FITC-lysozyme (Figure S14). This further supports the conclusion that a larger surface area, as a result of

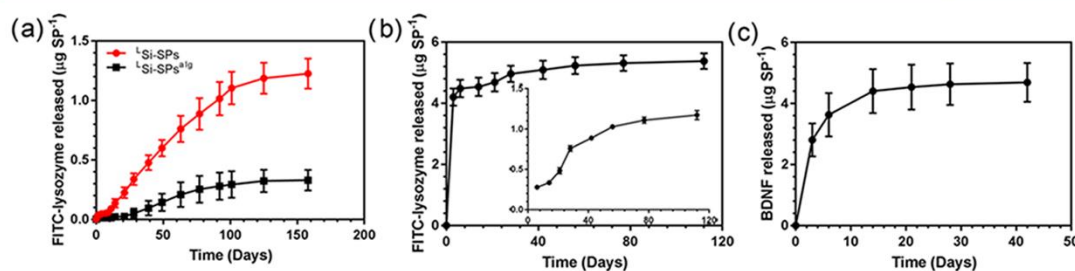


Figure 8. In vitro cumulative drug release profiles. (a) In vitro FITC-lysozyme (loading concentration 0.2 mg mL^{-1} , drug loading for 3 days) release from $^{\text{L}}$ Si-SPs and $^{\text{L}}$ Si-SPs^{alg}. (b) In vitro FITC-lysozyme (loading concentration 1.0 mg mL^{-1} , drug loading for 3 days) release from $^{\text{L}}$ Si-SPs. Inset in (b) shows the in vitro FITC-lysozyme release profile starting from day 6. (c) In vitro BDNF release from $^{\text{L}}$ Si-SPs (loading concentration 1.0 mg mL^{-1} , drug loading for 3 days). BDNF ELISA (Abcam) was used for measuring the BDNF concentration. Data presented in (a–c) are averages of three replicates, each using 10 SPs in (a) and (b) and 1 SP in (c), and error bars represent standard deviations. Release data for individual time points (noncumulative) are available in Figures S17–S19.

both a larger SP diameter and increased porosity, leads to a higher protein loading on the silica surface.

The drug loading efficiency of the six different SPs is summarized in Figure 7c,d. As the loading concentration of FITC-lysozyme increased, greater quantities of FITC-lysozyme could be loaded into SPs. However, overall, the efficiency of loading decreased with increasing FITC-lysozyme concentration.

Drug Release. For the release studies, 10 $^{\text{L}}$ Si-SPs or 10 $^{\text{L}}$ Si-SPs^{alg} were incubated in PBS (pH 7.4) at 37°C . The Si-SPs were initially incubated for 3 days with FITC-lysozyme at a loading concentration of 0.2 mg mL^{-1} . The FITC-lysozyme loading values were 1.89 ± 0.02 and $1.93 \pm 0.01 \mu\text{g SP}^{-1}$ for $^{\text{L}}$ Si-SPs and $^{\text{L}}$ Si-SPs^{alg}, respectively.

As shown in Figure 8a, $>1 \mu\text{g}$ of FITC-lysozyme was released per $^{\text{L}}$ Si-SP over a period of an additional 150 days, with 50% of the protein adsorbed being released at ~ 80 days, as indicated by the first-order release kinetics model in Figure S16. A lower amount of FITC-lysozyme ($\sim 0.4 \mu\text{g}$ per SP) was released from $^{\text{L}}$ Si-SPs^{alg} over the 150 day period. The lower release exhibited by the alginate/silica SPs may indicate blockage of the large pores of the primary silica particles by alginate⁵⁴ or potentially strong interaction between alginate and lysozyme, thereby preventing or retarding lysozyme release. The measured amounts of FITC-lysozyme released at each time point (which were used to prepare the cumulative results presented in Figure 8) are shown in Figure S17.

The FITC-lysozyme and BDNF release profiles from $^{\text{L}}$ Si-SPs were also investigated for particles incubated at a higher concentration (1.0 mg mL^{-1}). The amounts of FITC-lysozyme and BDNF, respectively, loaded at this concentration were 6.49 ± 0.48 and $7.17 \mu\text{g SP}^{-1}$, respectively. Under this higher loading concentration, the release profile for FITC-lysozyme was biphasic. A burst release period was apparent in the first 3 days, followed by sustained release over more than 110 days (Figure 8b). Approximately $4.2 \mu\text{g}$ of FITC-lysozyme was released per SP in the burst stage. After 3 days, $\sim 2.3 \mu\text{g}$ of FITC-lysozyme remained loaded in the SPs and a more sustained release behavior was subsequently observed. The slower kinetics of release overall compared with protein loading is attributed to the strong electrostatic interaction between the silica and lysozyme or BDNF that hinders the release of the protein. The protein is slowly released under physiological conditions (in PBS, at 37°C) as the PBS disrupts

the electrostatic interactions and free protein diffuses out of the porous particle. The amount of FITC-lysozyme released at each time point was approximately $0.07\text{--}4 \mu\text{g}$ per SP (Figure S18). As shown in Figure 8c, the in vitro BDNF release profile was similar to that of FITC-lysozyme, with a burst release observed in the first 3 days, followed by a more sustained release for over 40 days. The amount of BDNF released at each time point again ranged from approximately 0.1 to $3 \mu\text{g}$ per SP (Figure S19). The cumulative released value of the SPs after 40 days was $\sim 4.68 \mu\text{g}$ of drug, and the weight of each SP was $\sim 0.05 \text{ mg}$. Thus, the observed cumulative release was $\sim 93.6 \mu\text{g mg}^{-1}$. Interestingly, this is a 4000-fold improvement compared to what has recently been observed for nanoporous silica nanoparticles,⁵⁵ further highlighting the large difference between individual porous nanoparticles and supraparticle assemblies. The amount of BDNF released was also measured by a Micro BCA assay (Figure S20), and similar results were obtained. The released BDNF is expected to be biologically active, as shown in our previous work that described the implantation of BDNF-loaded Si-SPs (prepared using EISA) in the inner ear of guinea pigs.^{20,21}

To investigate potential methods to further tune drug release behavior and aid surgical delivery to the inner ear, in vitro drug release studies of $^{\text{L}}$ Si-SPs and $^{\text{L}}$ Si-SPs^{alg} incorporated within PF127 hydrogels were performed. PF127 hydrogels form viscous liquids at cold temperatures (e.g., 4°C) and gel at body temperature (37°C).⁵⁶ The results showed that most of the FITC-lysozyme was released from both $^{\text{L}}$ Si-SPs and $^{\text{L}}$ Si-SPs^{alg} that were incorporated within PF127 hydrogel by day 14 (Figure S21), indicating accelerated release kinetics when compared with the drug release behaviors of Si-SPs that were not incorporated within the PF127 hydrogel (Figure 8). This accelerated release rate might be attributed to the surfactant properties of PF127. PF127 is an amphiphilic block copolymer of poly(propylene oxide) and poly(ethylene oxide), which can both reduce interfacial tension and form micellar structures upon dilution. These properties may facilitate desorption and release of loaded proteins. The supraparticle–PF127 system is therefore more suitable when shorter-term drug release is desired (~ 1 week), whereas the PF127-free supraparticle systems are more suitable for longer-term (several months) drug release.

Porous silica particles can be degraded in biological environments into silicic acid, which can be excreted into the urine, and are of interest for diverse biomedical applications.⁵⁷

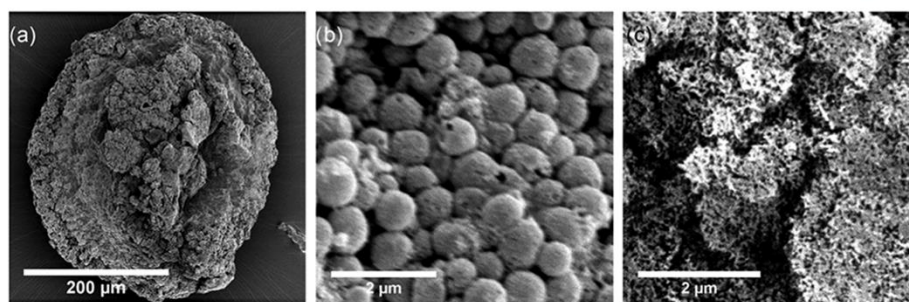


Figure 9. Degradation of ^1Si -SPs after 150 days of in vitro drug release studies. (a) SEM image of ^1Si -SPs after 150 days of incubation in PBS (pH 7.4) at 37 °C. (b, c) SEM images of the surface structure (at different locations) of ^1Si -SPs after 150 days of incubation in PBS (pH 7.4) at 37 °C.

To investigate the degradation behaviors of our system, ^1Si -SPs were incubated in PBS for 150 days (pH 7.4, 37 °C) (Figure 9). The diameter of the ^1Si -SPs decreased by ~55%, and the shape/morphology of the ^1Si -SPs changed substantially. The primary silica particles on the surface of the ^1Si -SPs also changed in both morphology and size (cf. Figures 1 and 9).

CONCLUSIONS

Herein, we presented a rapid and automatable electrospray technique to prepare SPs on the basis of an alginate hydrogel-mediated approach. Thousands of SPs could be prepared within 30 min, which otherwise would take weeks using the conventional EISA approach. The generated SPs were spherical and showed good reproducibility with respect to size and shape. Si-SPs with different sizes were fabricated by adjusting five main system variables, including sample composition and instrumental parameters. Six different SPs were prepared, and among these, ^1Si -SPs showed the highest capability to load therapeutically relevant levels of neurotrophin ($10\ \mu\text{g}\ \text{SP}^{-1}$ when loaded for 3 days with $5.0\ \text{mg}\ \text{mL}^{-1}$ FITC-lysozyme or $7.17\ \mu\text{g}\ \text{SP}^{-1}$ when loaded for 3 days with $1.0\ \text{mg}\ \text{mL}^{-1}$ BDNF). In vitro release profiles demonstrated sustained release of FITC-lysozyme over a period of more than 110 days and BDNF over more than 40 days, as well as a burst release stage (which may provide a loading dose) for both drugs in the first 3 days. ^1Si -SPs, loaded using lower drug loading concentrations ($1.89\ \mu\text{g}\ \text{SP}^{-1}$ when loaded for 3 days with $0.2\ \text{mg}\ \text{mL}^{-1}$ FITC-lysozyme), also exhibited sustained drug release for more than 150 days. Interestingly, the cumulative release from the supraparticles after 40 days was around $94\ \mu\text{g}\ \text{mg}^{-1}$, a 4000-fold improvement compared with recently reported nanoporous silica nanoparticles.⁵⁵ The release kinetics of the supraparticles could be further tuned via the combined use of ^1Si -SPs in block copolymer-based hydrogels. These findings show that ^1Si -SPs assembled through gel-mediated electrospray are promising drug carriers for therapeutics that require long-term delivery of protein drugs such as for the treatment of inner ear diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b10415.

Scheme of cross-linking between calcium chloride and alginate; summary of the six different types of supraparticles examined in the drug loading kinetics, capacity,

and efficiency studies; photographs of the electrospray setup and force–fracture test machine; optical microscopy images of alginate/silica supraparticles; SEM images of ^1Si -SPs at different tilted angles; SEM images of ^8Si -SPs and ^5Si -SPs; TEM images of nonporous primary silica particles and primary silica particles with small pores; calibration curves for FITC-lysozyme and BDNF concentration determination; confocal laser scanning microscopy images of ^1Si -SPs at various thicknesses; size distribution and circularity of ^1Si -SPs; XPS analysis of ^1Si -SPs; FITC-lysozyme loading and release curves of ^1Si -SPs with different diameters; drug loading capacity of SPs as a function of drug loading concentration; amounts of FITC-lysozyme and BDNF released from ^1Si -SPs at given time points; first-order release kinetics of FITC-lysozyme and BDNF release from ^1Si -SPs; BDNF concentration determination using Micro BCA assay; in vitro FITC-lysozyme release data of Si-SPs–PF127 hydrogel system (PDF)

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Notes

The authors declare no competing financial interest. The authors have filed a patent containing some of the findings reported herein.

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Supporting Information

Gel-Mediated Electrospray Assembly of Silica Supraparticles for Sustained Drug Delivery

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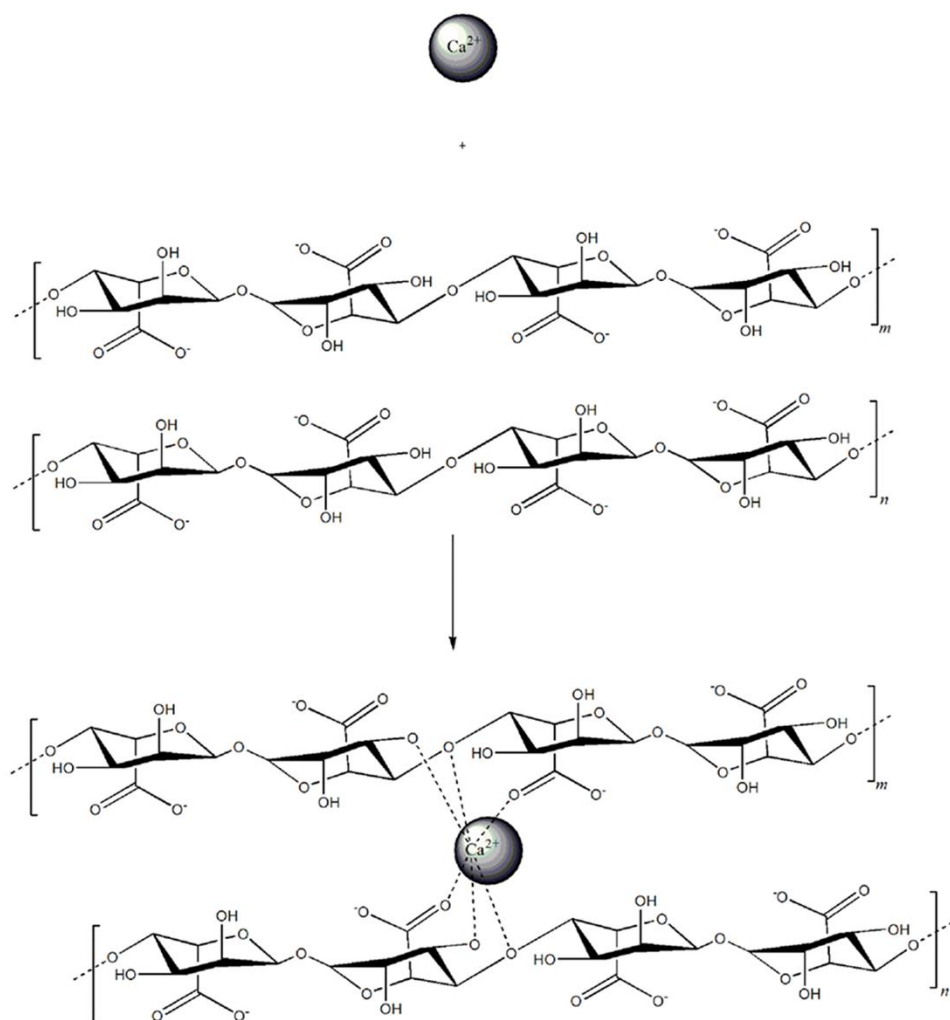


Figure S1. Schematic illustration of crosslinking between calcium ions and alginate.

Table S1. Summary of the Six Different Types of Supraparticles Studied

primary silica particles	company	primary particle diameter	pore diameter	nomenclature of alginate/silica supraparticles (Si-SPs ^{alg})	nomenclature of silica supraparticles (Si-SPs)
nonporous silica particles	microParticles GmbH	~500 nm	nonporous	^N Si-SPs ^{alg}	^N Si-SPs
silica particles with small pores	Sigma-Aldrich	~500 nm	2 nm	^S Si-SPs ^{alg}	^S Si-SPs
silica particles with large pores	synthesized in-house	~1 μ m	2–3 nm, 15–64 nm and >100 nm	^L Si-SPs ^{alg}	^L Si-SPs

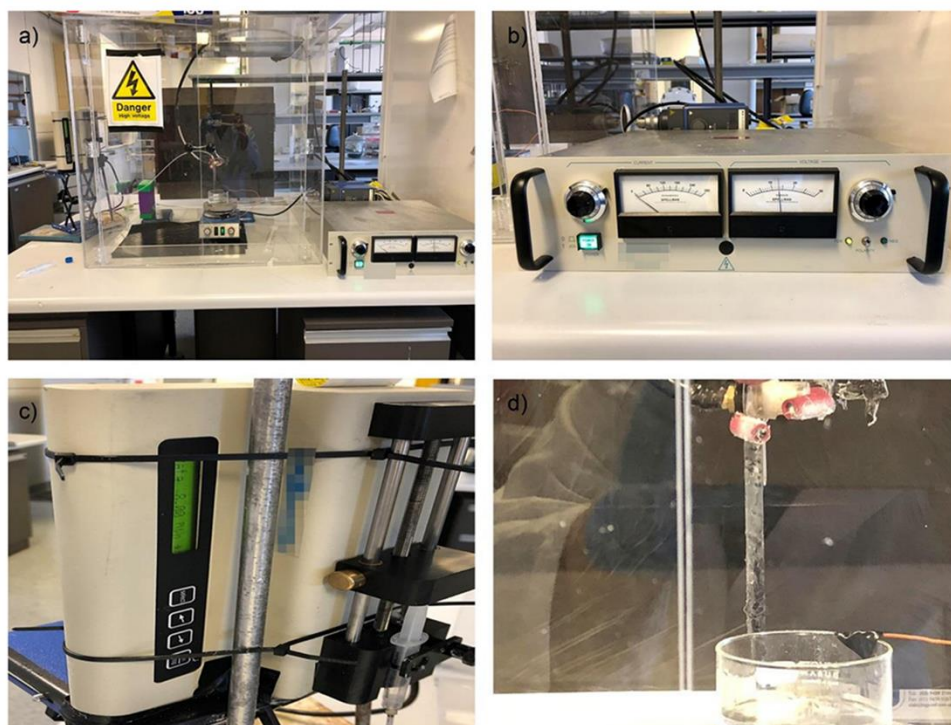


Figure S2. Photographs of (a) the electrospray setup, (b) high voltage power supply, (c) syringe pump, and (d) nozzle and calcium chloride bath.

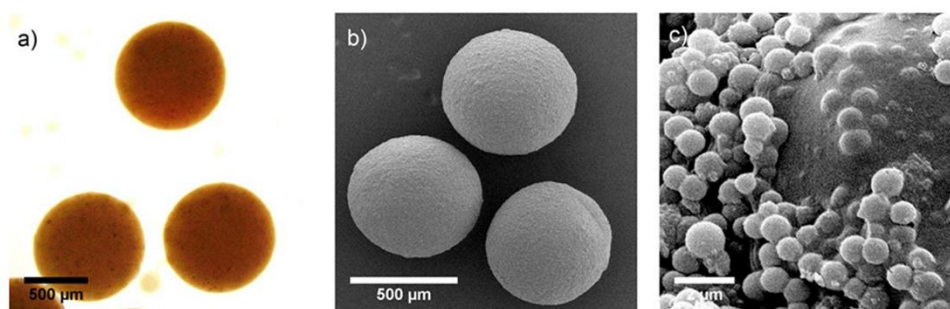


Figure S3. (a) Optical microscopy image of $^{L}\text{Si-SPs}^{\text{alg}}$ in calcium chloride solution. SEM images of (b) $^{L}\text{Si-SPs}^{\text{alg}}$ and (c) the surface structure of $^{L}\text{Si-SPs}^{\text{alg}}$.

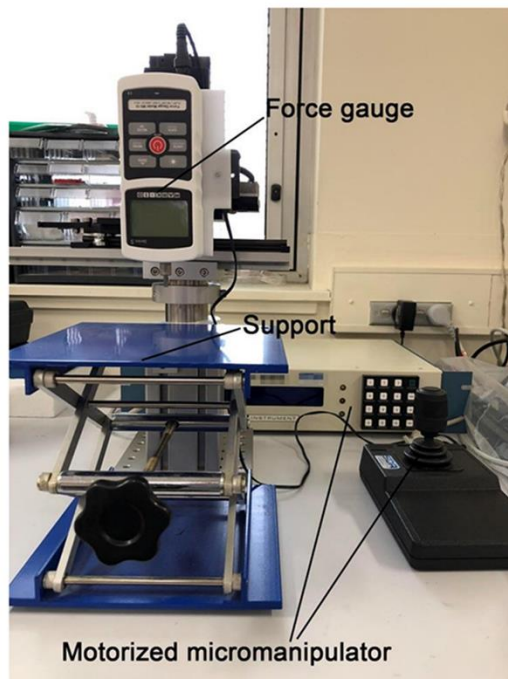


Figure S4. Photograph of the force–fracture test instrument.

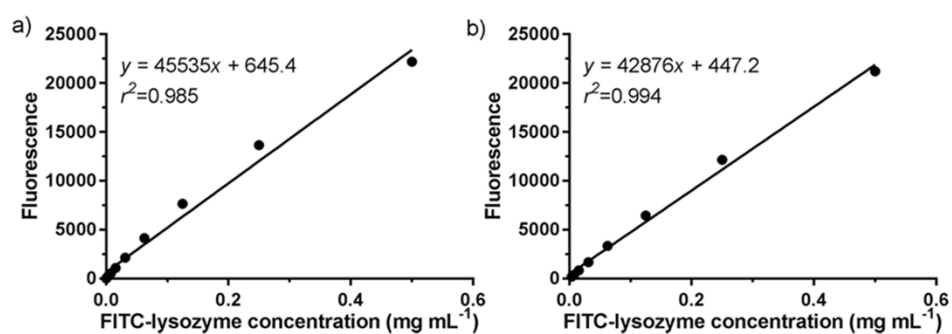


Figure S5. In vitro drug release standard curves for (a) FITC-lysozyme (FITC-lysozyme loading concentration is 0.2 mg mL⁻¹) and (b) FITC-lysozyme (FITC-lysozyme loading concentration is 1.0 mg mL⁻¹).

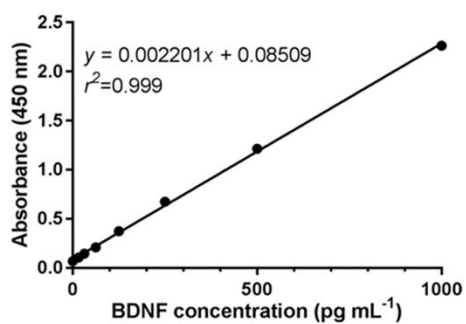
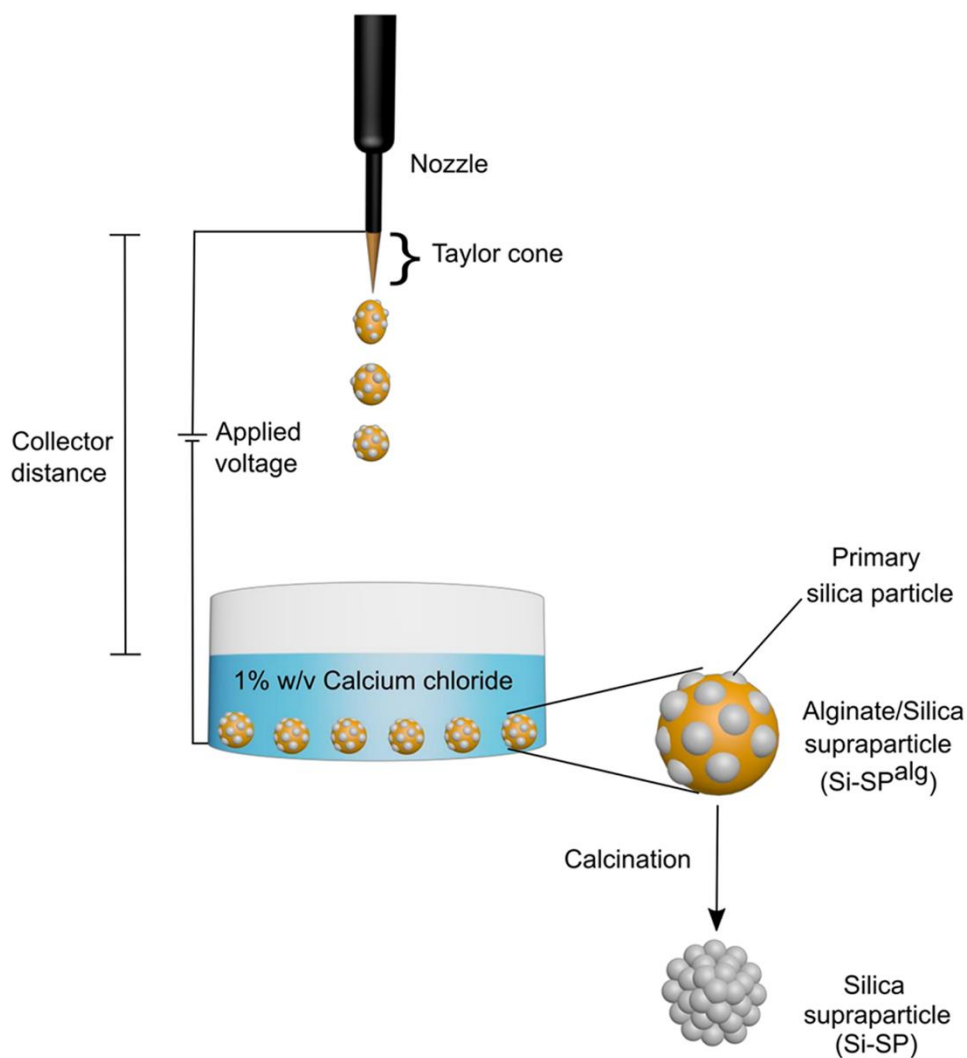


Figure S6. In vitro release standard curve for BDNF.



Scheme S1. Schematic illustration of the formation of a Taylor cone at the nozzle tip of the electrospay setup, subsequently generating droplets of alginate/primary silica particles upon application of voltage. The droplets are collected in a gelling bath containing calcium chloride, which crosslinks the alginate. Silica SPs are obtained following calcination of the Si-SPs^{alg} at 923 K for 30 h.

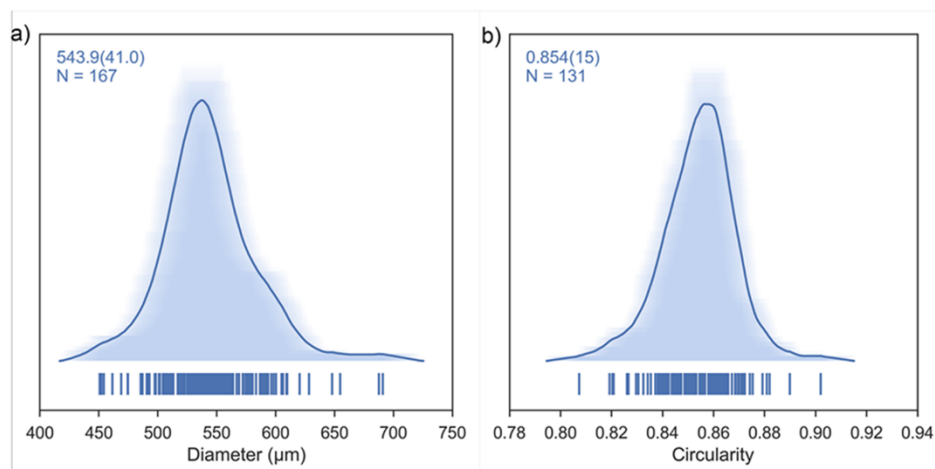


Figure S7. (a) Size distribution and (b) circularity of L Si-SPs. The Si-SPs were synthesized using the following optimized settings: alginate solution, 30 mg mL⁻¹; primary silica particles, 40 mg mL⁻¹; applied voltage, 13 kV; collector distance, 10 cm; and flow rate, 8 mL h⁻¹.

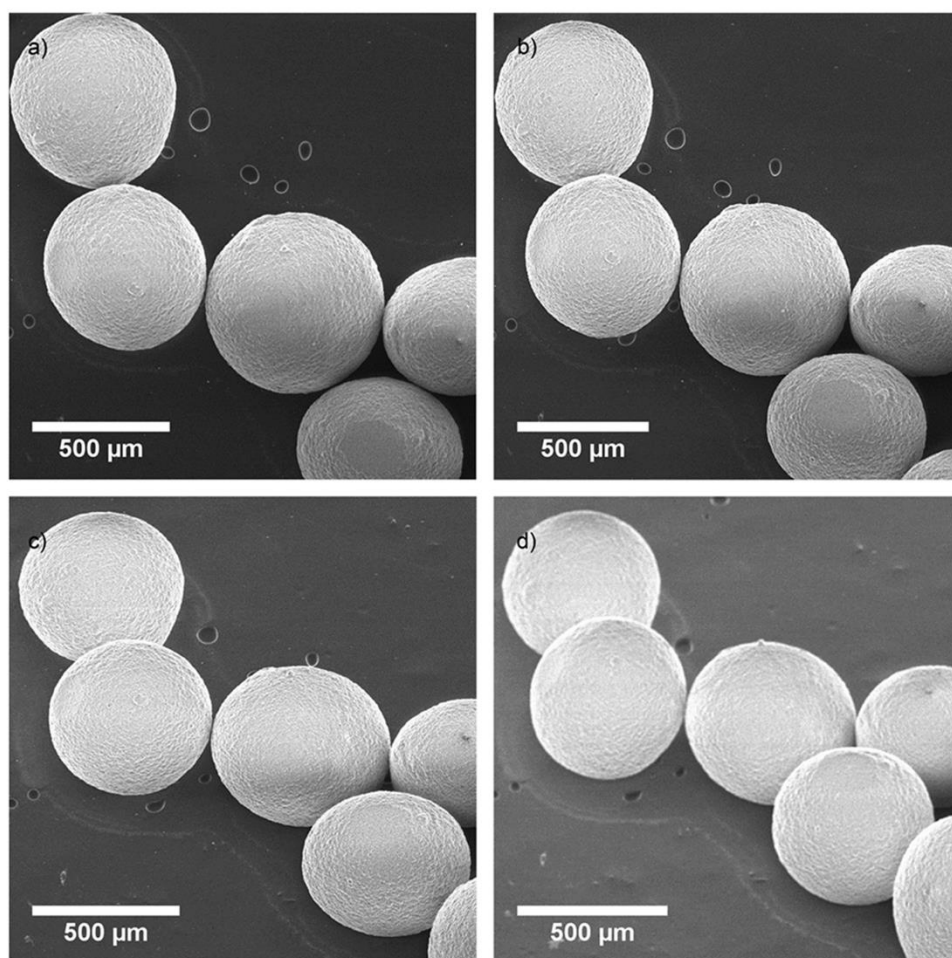


Figure S8. SEM images of ^1Si -SPs captured at different tilted angles: (a) 0, (b) 15, (c) 30, and (d) 45°.

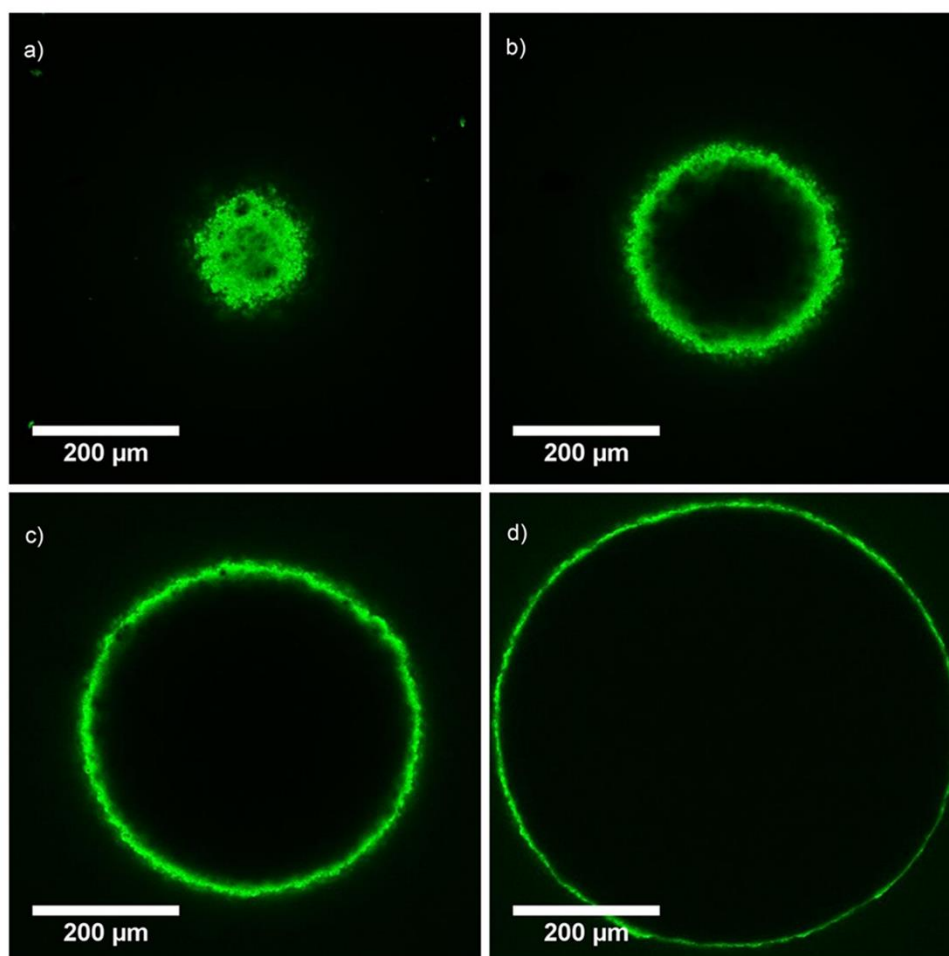


Figure S9. Cross-sectional CLSM images of ^1Si -SPs loaded with FITC-lysozyme (green) at different z heights (a–d).

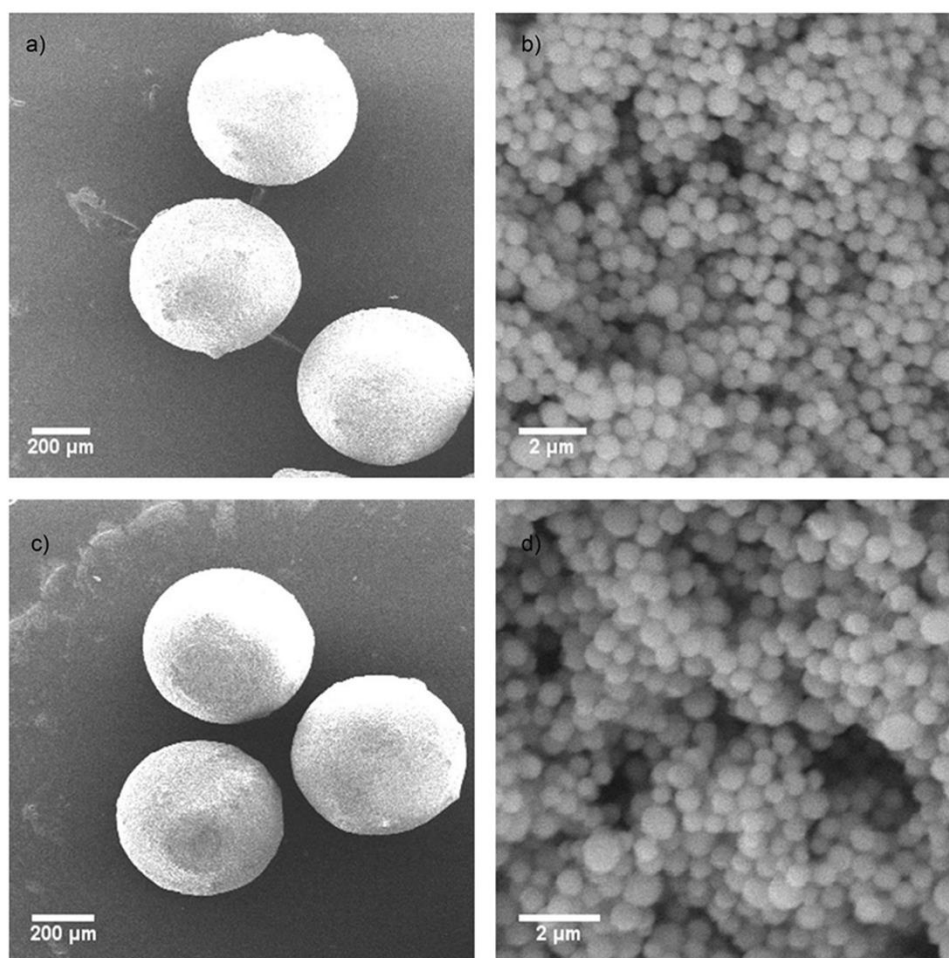


Figure S10. (a) SEM image of $^{\text{N}}\text{Si-SPs}$ and (b) high-resolution SEM image showing the surface structure of $^{\text{N}}\text{Si-SP}$. (c) SEM image of $^{\text{S}}\text{Si-SPs}$ and (d) high-resolution SEM image showing the surface structure of $^{\text{S}}\text{Si-SP}$.

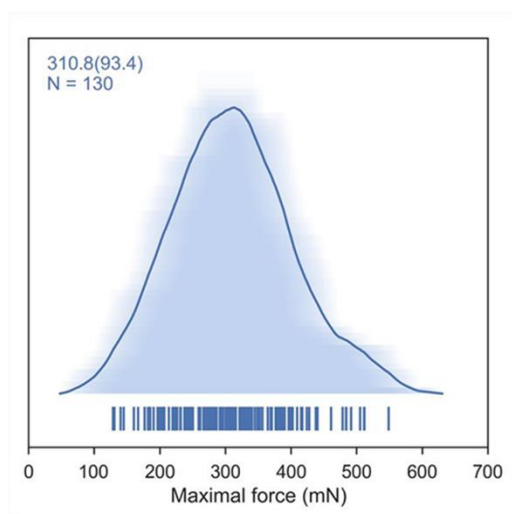


Figure S11. Force required to fracture individual ^LSi-SPs using an indenter.

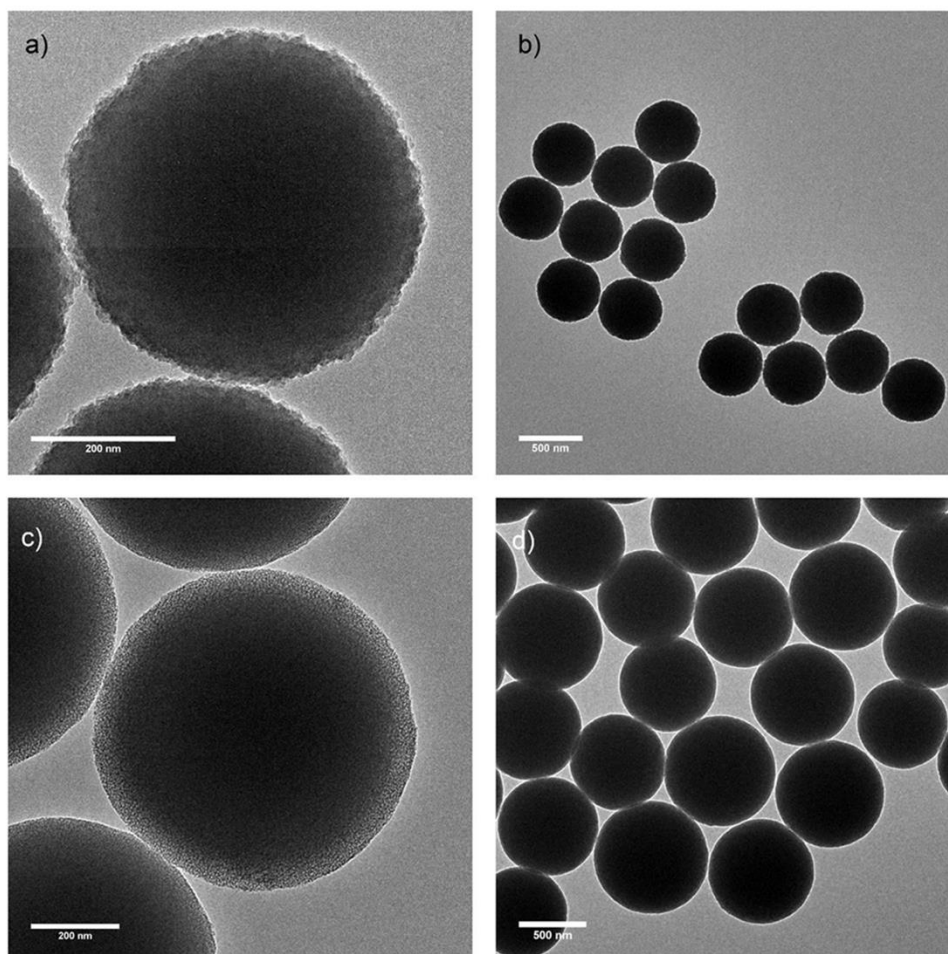


Figure S12. (a, b) TEM images of the nonporous primary silica particles at different magnifications. (c, d) TEM images of the primary silica particles with small pores at different magnifications.

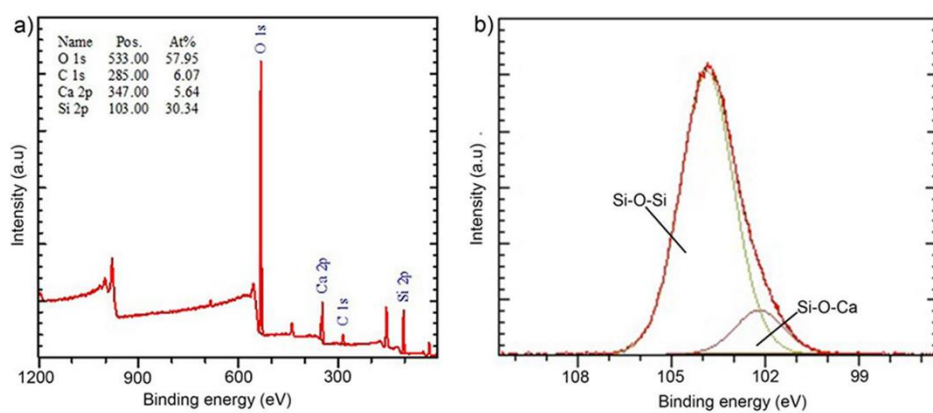


Figure S13. (a) XPS survey spectrum and (b) Si 2p XPS spectra of ^LSi-SPs.

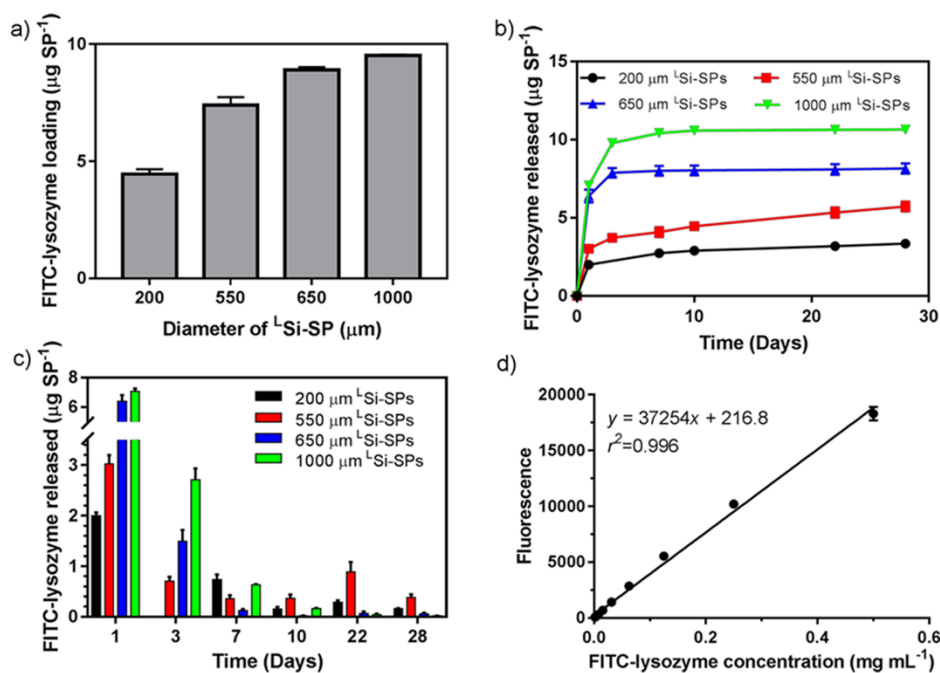


Figure S14. FITC-lysozyme loading and release behavior of LSi-SPs with different diameters (200, 550, 650, and 1000 μm). (a) FITC-lysozyme loading profiles using a FITC-lysozyme loading concentration of 1 mg mL^{-1} in Milli-Q water and 10 SPs per sample. (b) In vitro FITC-lysozyme release profiles. (c) Amount of FITC-lysozyme released from LSi-SPs at a given time point. (d) In vitro FITC-lysozyme release standard curve. The loaded amounts of FITC-lysozyme in the LSi-SPs of varying diameters of 200, 550, 650, and 1000 μm are 4.46 ± 0.2 , 7.40 ± 0.34 , 8.91 ± 0.12 , and 9.52 ± 0.03 $\mu\text{g SP}^{-1}$, respectively.

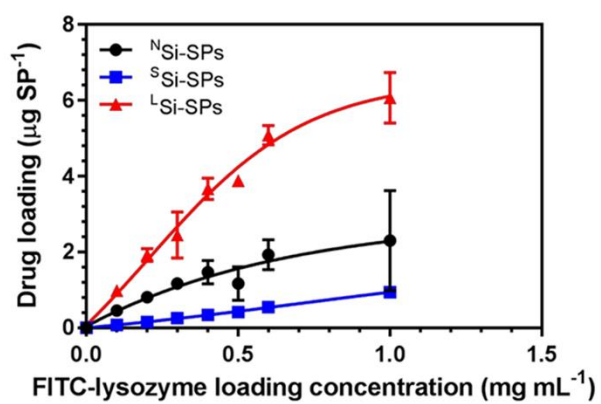


Figure S15. Variations in the amount of drug loaded in Si-SPs as a function of the FITC-lysozyme loading concentration.

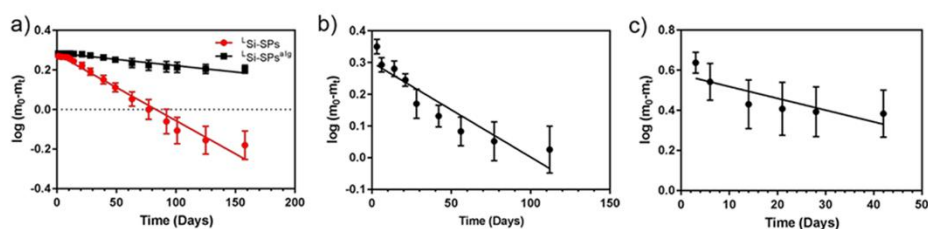


Figure S16. First-order protein release profiles of: (a) FITC-lysozyme (loading concentration 0.2 mg mL^{-1} , drug loading for 3 days) released from $^{L}\text{Si-SPs}$ and $^{L}\text{Si-SPs}^{\text{alg}}$; (b) FITC-lysozyme (loading concentration 1.0 mg mL^{-1} , drug loading for 3 days) released from $^{L}\text{Si-SPs}$; and (c) BDNF released from $^{L}\text{Si-SPs}$ (loading concentration 1.0 mg mL^{-1} , drug loading for 3 days). m_0 is the amount of drug loaded in SP ($\mu\text{g SP}^{-1}$) and m_t is the amount of drug loaded in SPs ($\mu\text{g SP}^{-1}$) at time t (days).

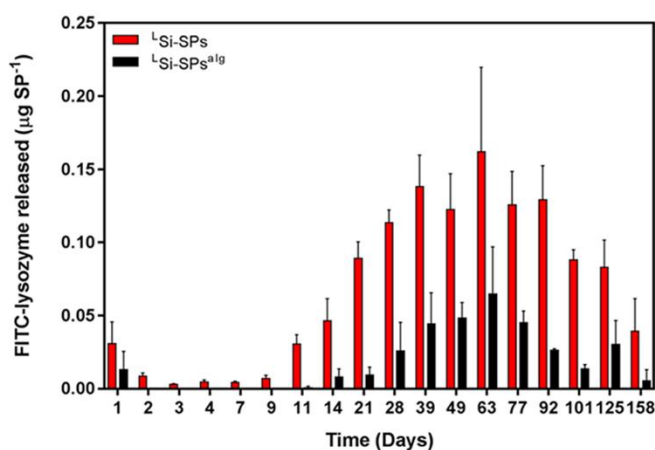


Figure S17. Amount of FITC-lysozyme released from $^{L}\text{Si-SPs}^{\text{alg}}$ and $^{L}\text{Si-SPs}$ at a given time point. Loading conditions: $100 \mu\text{L}$ of 0.2 mg mL^{-1} of FITC-lysozyme and loading time of 3 days. The data presented are averages of three replicates, each using 10 supraparticles, and error bars represent standard deviations.

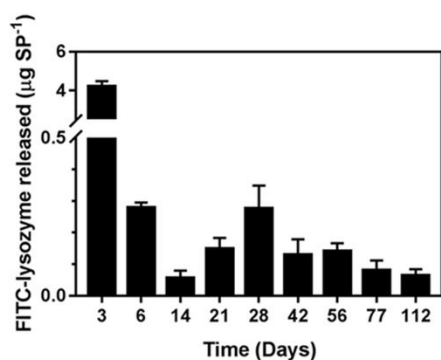


Figure S18. Amount of FITC-lysozyme released from ^LSi-SPs at a given time point. Loading conditions: 100 µL of 1.0 mg mL⁻¹ of FITC-lysozyme and loading time of 3 days. Drug loading was $6.49 \pm 0.48 \mu\text{g SP}^{-1}$. The data presented are averages of three replicates, each using 10 ^LSi-SPs, and error bars represent standard deviations.

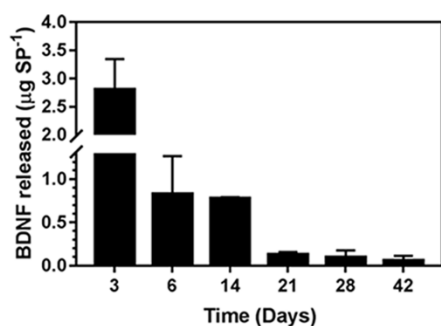


Figure S19. Amount of BDNF released from ¹²⁵I-Si-SP at a given time point, as measured using ELISA. Loading conditions: 1.0 mg mL⁻¹ of BDNF and loading time of 3 days. The data presented are averages of three replicates, each using 1 ¹²⁵I-Si-SP, and error bars represent standard deviations.

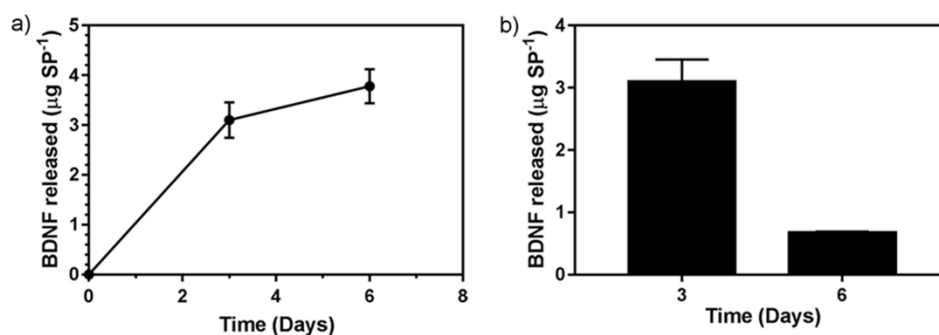


Figure S20. (a) Micro BCA assay to quantify the release of BDNF from L Si-SPs. (b) Amount of BDNF released from L Si-SPs at a given time point. Drug loading was $6.82 \mu\text{g SP}^{-1}$. The data presented are averages of three replicates, and error bars represent standard deviations. (Because the Micro BCA assay has a lower sensitivity than ELISA, it was only used to assess samples with a high concentration of BDNF; thus, release of BDNF from samples was only measured in the first 6 days of the release experiment.)

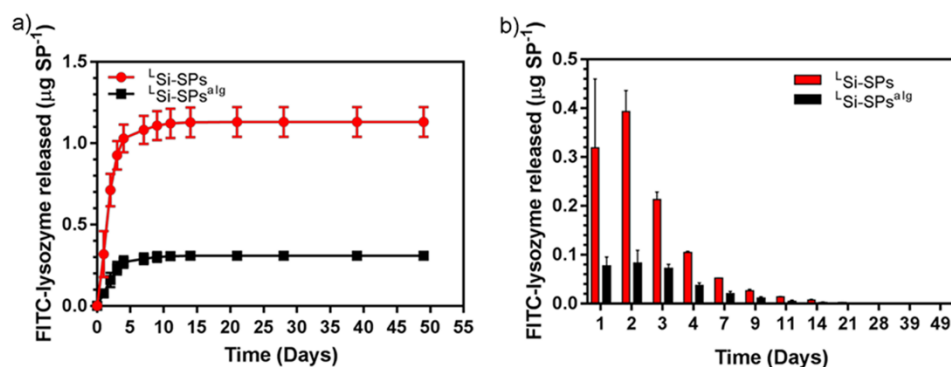
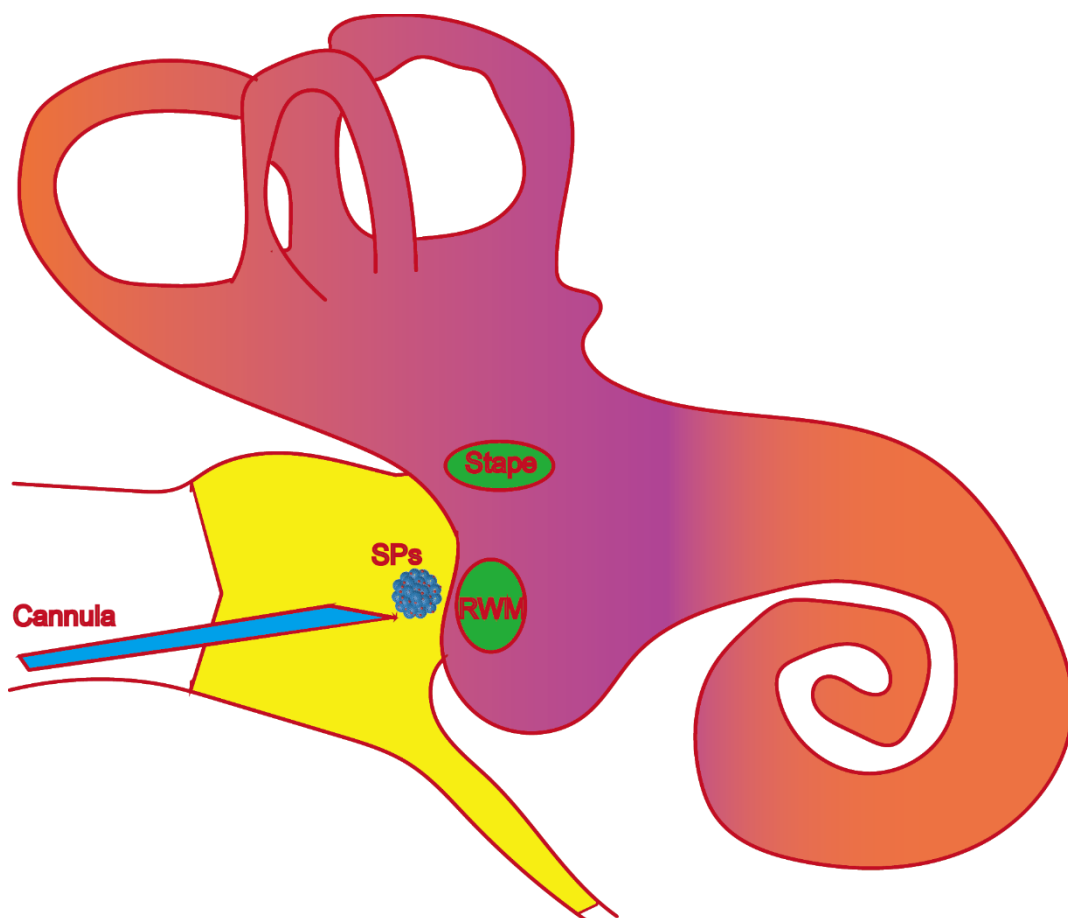


Figure S21. (a) In vitro FITC-lysozyme release profiles of $^{\text{L}}\text{Si-SPs}$ and $^{\text{L}}\text{Si-SPs}^{\text{alg}}$ in PF127 hydrogel system. (b) Amount of FITC-lysozyme released from the $^{\text{L}}\text{Si-SPs}$ -PF127 and $^{\text{L}}\text{Si-SPs}^{\text{alg}}$ -PF127 systems at a given time point (loading condition: 100 μL of 0.2 mg mL^{-1} FITC-lysozyme). Drug loading amounts were 1.89 ± 0.08 and 1.93 ± 0.03 $\mu\text{g SP}^{-1}$ for $^{\text{L}}\text{Si-SPs}$ and $^{\text{L}}\text{Si-SPs}^{\text{alg}}$, respectively. The data presented are averages of three replicates, each using 10 Si-SPs, and error bars represent standard deviations.

Chapter 4

Pharmacokinetics and Inner Ear Transport of Brain-Derived Neurotrophic Factor (BDNF) from Silica Supraparticles



4.1 SUMMARY

In this Chapter, supraparticles (SPs) were examined as a drug delivery carrier for encapsulating brain-derived neurotrophic factor (BDNF) and the SPs were subsequently implanted to either deafened cochlea or the RW membrane (RWM) in a deafened guinea pig model for 3 or 7 days. (i) The pharmacokinetics of BDNF released from SPs in the cochlea were examined and (ii) the concentrations of BDNF released in the cochlea from SP-enabled delivery and an osmotic pump (traditional method of delivering BDNF) were compared. The findings show that the synthesised SPs are a promising platform for long-term BDNF delivery for the treatment of sensorineural hearing loss (SNHL).

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4.2 INTROUDUCTION

Overall the last few decades, it was well-established that local drug delivery to the inner ear can be achieved by either directly injecting drug into the cochlea or injecting drug on the round window membrane (RWM) (i.e., let the drug diffuse through the RWM) to treat hearing impairments.¹ Some drugs are in widespread clinical use for the treatment of inner ear diseases.²⁻⁴ Many new substances and drug delivery systems specific to the inner ear are under development and in some cases are undergoing evaluations in animal experiments and in clinical studies. Of the hearing loss cases reported, 90% is reported as sensorineural hearing loss (SNHL).⁵ SNHL can be treated either through an intratympanic administration route in which the middle ear serves as a drug reservoir or intracochlear administration route in which drug is directly injected into the inner ear. However, the drug could be easily cleared through the Eustachian tube, vasculature or lymphatics when injected into the middle ear.⁶ Further, when drug is directly injected into cochlea, it can rapidly equilibrate with extracellular spaces of the spiral ligament and also pass through the endolymph–perilymph barrier because of less tightly bound adjacent tissue spaces, resulting in an uneven drug distribution.¹

Some factors such as genetic factors, exposure to ototoxic drug, intensive noise and old age can cause damage to the auditory sensory neurons (hair cells or spiral ganglion neurons (SGNs)).⁷⁻⁹ However, research studies have demonstrated that high levels of neurotrophins in the cochlea can effectively protect and repair the synapse between the hair cells and SGNs.¹⁰⁻¹² Further, the exogenous neurotrophins can promote peripheral nerve fibre sprouting without the presence of hair cells.^{13,14} Brain-derived neurotrophic factor (BDNF) is one of the most common neurotrophins that can increase the survival of auditory neurons and the number of auditory nerve fibres.^{15,16} The therapeutic

delivery of BDNF has raised a number of problems related to its pharmacokinetic profile, such as short *in vivo* half-life, uncertain passage through the blood–perilymph barrier and high manufacturing costs.^{17,18} Therefore, it is vital to understand the pharmacokinetics of locally applied neurotrophins (the movement of neurotrophins in the cochlea), especially BDNF, as it involves the interaction of multiple elements.^{1,19}

In Chapter 3, a silica-based supraparticle (SP) system was designed and investigated its use as a carrier to encapsulate BDNF. The SPs (~550 nm) showed sustained *in vitro* BDNF release behaviour. Thus, to evaluate its *in vivo* behaviour, SPs were injected and encapsulated with BDNF, via cannula in either the intracochlear or the RWM. As most of the cochlea structures are in diffusional continuity with the perilymph,²⁰ after the SPs were placed onto the RWM, the BDNF could be released and could diffuse either directly or through the RWM into the organ of Corti, where hair cells, neuronal terminals and other specialised cells could be protected.²¹⁻²³ This chapter describes the distribution of BDNF in the cochlea as BDNF is released from SPs and enters the cochlear fluid. Further, the concentration level of BDNF released from SPs in the cochlea was compared with BDNF delivered via a mini-osmotic pump into the cochlea to evaluate the *in vivo* performance of SPs.^{24,25} This study provides a basic understanding of the mechanism and distribution of encapsulated BDNF in the inner ear, which can provide an insight of the *in vivo* performance of SPs.

4.3 MATERIALS AND METHODS

Materials. Fluorescein isothiocyanate (FITC), lysozyme protein from chicken egg white, alginic acid sodium salt from brown algae (alginate), poly(acrylic acid) (PAA, M_w ~250 kDa, 35 wt% aqueous solution), tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), ammonium hydroxide solution (28–30%),

calcium chloride (CaCl_2) and phosphate-buffered saline (PBS, pH 7.4) were purchased from Sigma-Aldrich (USA). Nitric acid (HNO_3 ; 65% w/v) was bought from Merck (Australia). Hydrogen peroxide (H_2O_2 ; 30% w/v) was obtained from BDH (Australia). Sodium chloride (NaCl) and ethanol were obtained from Chem-Supply (Australia). BDNF was purchased from PeproTech Asia (Israel) and human BDNF ELISA kits were obtained from Abcam (Australia). Ultrapure water (Milli-Q water) with a resistivity of greater than $18 \text{ M}\Omega \text{ cm}$ was used in all experiments and obtained from a three-stage Millipore Milli-Q Plus 185 purification system.

Synthesis of SPs. The silica SPs (Si-SPs) were fabricated using an electrospray set-up, which was published previously.²⁶ Typically, 80 mg of primary silica particles with the diameter of $\sim 1 \text{ }\mu\text{m}$ (protocol to synthesise primary silica particles can be found in reference)²⁷ was poured into 2 mL of aqueous alginate solution (30 mg mL^{-1} 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 another 1 h to ensure that the primary silica particles are distributed evenly in the alginate solution. Subsequently, the solution was poured into a syringe, which was placed in a syringe pump at a constant flow rate of 8 mL h^{-1} . Then the alginate/primary silica particle solution was dripped by the syringe pump into a container (petri dish) with 1% w/v of CaCl_2 solution via electrospray set-up with an applied voltage of 13 kV and 10 cm collector distance between the end of the nozzle and the surface of the CaCl_2 solution. After 30 min, the alginate/silica SPs ($\text{Si-SPs}^{\text{alg}}$) were collected directly from the CaCl_2 bath using a strainer. Lastly, $\text{Si-SPs}^{\text{alg}}$ were calcinated at $650 \text{ }^\circ\text{C}$ for 30 h to remove any of the organic materials (i.e. alginate) to get Si-SPs.

SPs Loading with FITC-Lysozyme/BDNF. *In vitro* loading was conducted using the model drug FITC-lysozyme to characterise the amount of loading and BDNF. To evaluate the loading amount of each individual SP from FITC-lysozyme, Si-SPs were incubated with 100 μL of FITC-lysozyme solution (1 mg mL^{-1} in Milli-Q water). After 3 days of loading, the fluorescence value of the supernatant was measured by a microplate reader. The loading value was calculated by subtracting the amount of FITC-lysozyme in the supernatant from the amount of FITC-lysozyme in the stock solution. For SPs encapsulated with BDNF, after 3 days, these could be readily implanted into deafened guinea pig. For intracochlear implantation, 2 SPs were incubated with 23 μL 1 mg mL^{-1} of BDNF and for RWM implantation, 5 SPs were incubated with 40 μL 1 mg mL^{-1} of BDNF. The loading amount of BDNF in each individual SPs was determined by BDNF ELISA following manufacturer's protocol.

Cannula Preparation Before SPs Injection. Cannula was prepared from a polyethylene tube with OD 0.96 mm and ID 0.58 mm. The two ends of the tube were attached to a blunt needle and a 3 cm polyimide tube with OD 0.16 mm and ID 0.12 mm and sealed with silicone. Before the injection, the BDNF-loaded SPs (in Milli-Q water) were sucked into the cannula and then injected onto the RWM or inside the cochlea. For the osmotic pump delivery, 100 μL of $0.15 \mu\text{g } \mu\text{L}^{-1}$ of BDNF was loaded into a mini osmotic pump. Liquid paraffin was first loaded into the cannula then positioned to a 10 μL Hamilton syringe which is attached to a rate-controlled Microdrive unit.

Injection into Guinea Pig Cochleae. The use of guinea pigs in this study was approved by the St Vincent's Hospital (Melbourne) Animal Ethics Committee and 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).

Prior to injection, guinea pigs were first anaesthetised with a 60 mg kg⁻¹ ketamine and 4 mg kg⁻¹ xylazine mixture via intramuscular injection. A post-auricular incision was performed to separate underlying muscle on the ventral bulla. Then a #14 diamond burr was used to open the bulla and expose the basal turn of the cochlea. The membrane on the top of the basal turn of the cochlea was gently retracted followed by a cochleostomy via a sharpened mental probe. For the BDNF pump delivery, the polyimide side of the cannula was inserted into the cochlea up to the silicon plug and the tapered end inserted and sealed by superglue. When the superglue dried, the BDNF was injected into the cochlea at a constant flow rate at 0.25 $\mu\text{L h}^{-1}$ for 7 days (the osmotic pump could deliver BDNF for 14 days and have premade and sterilised cannulas). Finally, the polyimide end of the cannula was cut and the bulla was sealed with cement.

For implantation at the RWM, using the basal turn of the cochlea for guidance, 5 SPs were placed onto the RWM followed by sealing with superglue. For the intracochlear implantation of SPs, after cochleostomy was performed, 2 SPs were loaded into the cannula and injected into the basal of the cochlea, which was then sealed by superglue. The implantation of SPs on the RWM and cochlea lasts for 3 and 7 days, respectively. The rest of the surgical procedure was as described for the osmotic pump above.

Cochlea Extraction. Three days or 7 days post-injection, guinea pigs were given a 60 mg kg⁻¹ ketamine and 4 mg kg⁻¹ xylazine mixture via intramuscular injection. The apex turn of the cochlea was exposed as described above. Subsequently, a silicone well was built around the apex turn of the cochlea that is coated with Superglue. A small hole

was drilled on the apex turn of the cochlea, followed by the perilymph collection using a capillary tube (**Figure 1**). Approximately 1 μ L of perilymph was collected by each capillary tube for each sample. The samples were kept in the freezer until further analysis, including determining the concentration of BDNF in each perilymph sample using ELISA kits. After sample collection, the animals were treated with a lethal dose of pentobarbitone. Then, the guinea pigs were perfused with 400 mL 0.9 % (w/v) saline (the saline contains 0.025 % (w/v) sodium nitrite and 0.1 % (w/v) heparin sodium). Subsequently, 400 mL neutral buffered formalin was used for the intracardial perfusion followed by harvesting of the bulla. The cochlea was collected with SCs attached. The organs of guinea pigs (heart, liver, intestine, kidney, Ven (ventricles), cochlea nucleus (left and right side, referred as CNL and CNR), inferior colliculus (left and right side, referred as ICL and ICR)) were also collected to determine Si accumulation in each organ.

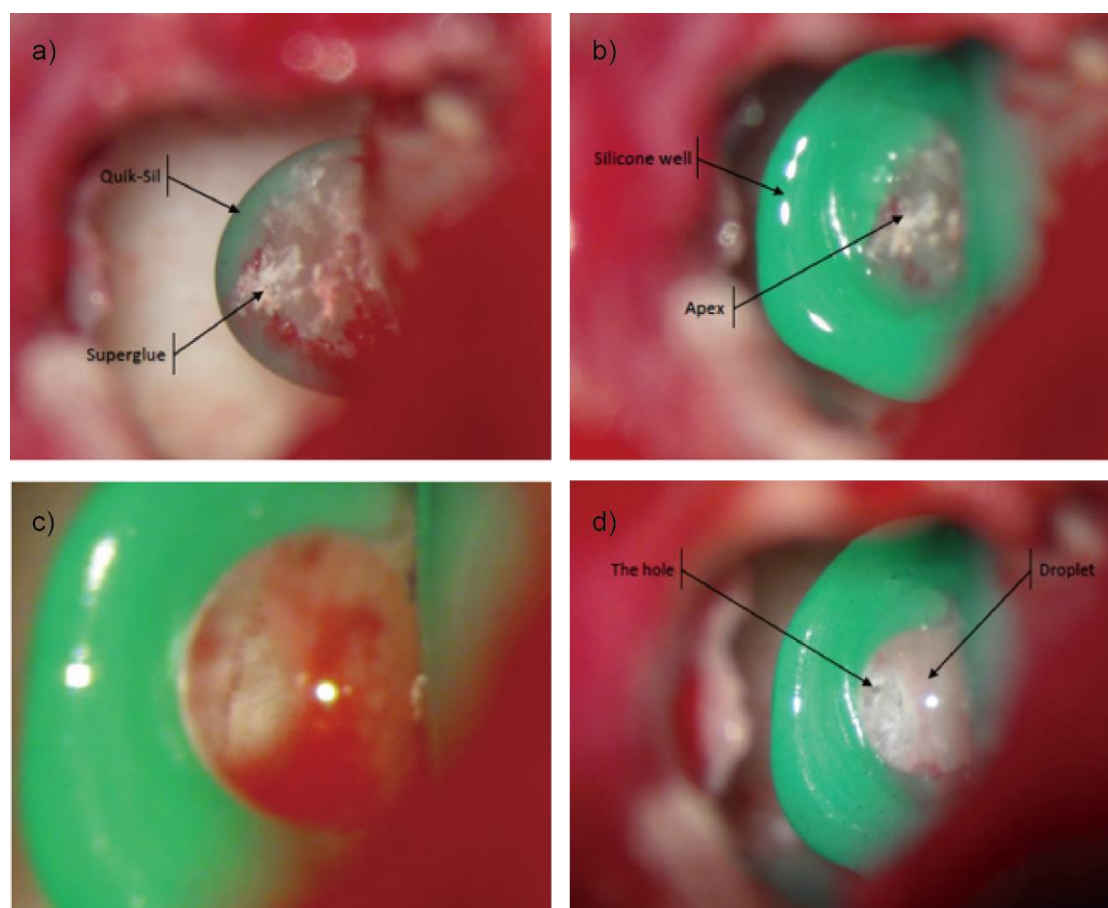


Figure 1. Cochlea sample extraction. (a) Kuik-Sil is injected around the apex that is coated with a thin layer of Superglue. (b) A silicone well (Kwik-Sil) was built at the apex. (c) A droplet of perilymph appears inside the well after drilling a hole at the apex. (d) After taking the first sample of perilymph, the second droplet develops in the well.

Si Distribution in Different Organs. Si distribution in different organs of guinea pigs was determined by ICP-MS (Agilent, USA) under routine multi-element operating conditions using a Helium Reaction Gas cell. The instrument was calibrated using 0, 5, 10, 50, 100 and 500 ppb of certified Si ICP-MS standard calibration solutions for a range of elements. The organs, including heart, liver, intestine, kidney, Ven, CNL, CNR ICL and ICR were directly dissected from guinea pigs and separated into individual tubes. Then the tissue samples were transferred into 5 mL of acid in clear tubes for lyophilising. Then the lyophilised samples were treated with HNO_3 (400 μL for heart,

intestine, Ven, 600 μL for liver and kidney, 200 μL for CNL, CNR, ICL, ICR) overnight. The next day, the digested samples were heated at 90 $^{\circ}\text{C}$ for 20 min using a heating block. Then the samples were removed from the heating block and an equivalent volume of H_2O_2 was added to each sample. After 30 min, the samples were heated at 70 $^{\circ}\text{C}$ for 15 min. The average reduced volume was determined and the samples were further diluted with 1% HNO_3 diluent.

The accumulated Si value ($\mu\text{g g}^{-1}$) was calculated as follows:

$$\mu\text{g g}^{-1} = (\text{raw ppb value} \times \text{dilution factor} \times \text{reduced digest volume}) / (\text{tissue wet weight (unit is 'g')})$$

SEM Images of Implanted SPs on RWM. After the guinea pigs were perfused, implanted SPs on RWM were carefully collected by cannula (**Figure 2**). Scanning electron microscopy (SEM) images of implanted SPs were taken using a Philips XL30 field-emission scanning electron microscope (Philips, Netherlands) with an operating voltage of 5 kV. SEM samples of the SPs were prepared by placing them onto conductive carbon tape. Finally, 20 nm gold was coated on the samples by sputter coating.

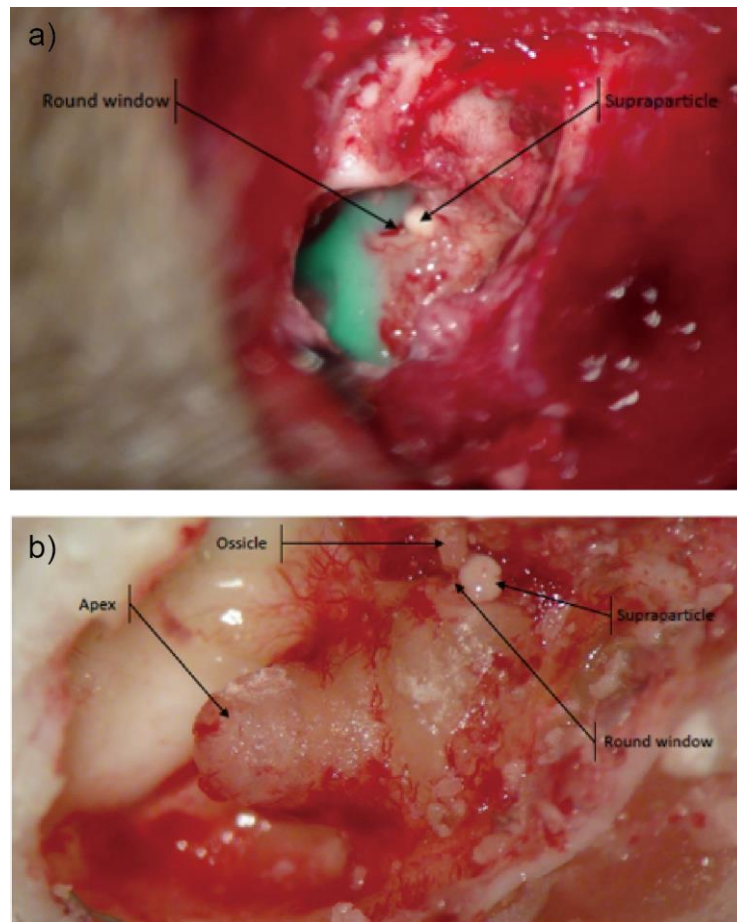


Figure 2. (a) The Kwik-Sil and silicone film at the RWM were removed to reveal the SP. (b) After perfusion, the bone around RWM was removed.

4.4 RESULTS AND DISCUSSION

There are a few strategies to deliver BDNF to the cochlea, including osmotic pumps, viral vectors, cell-based nanocarriers, but the understanding of BDNF distribution in the cochlea after delivery is lacking. In this study, the BDNF delivery efficiency of SPs was compared with delivery using a mini-osmotic pump.

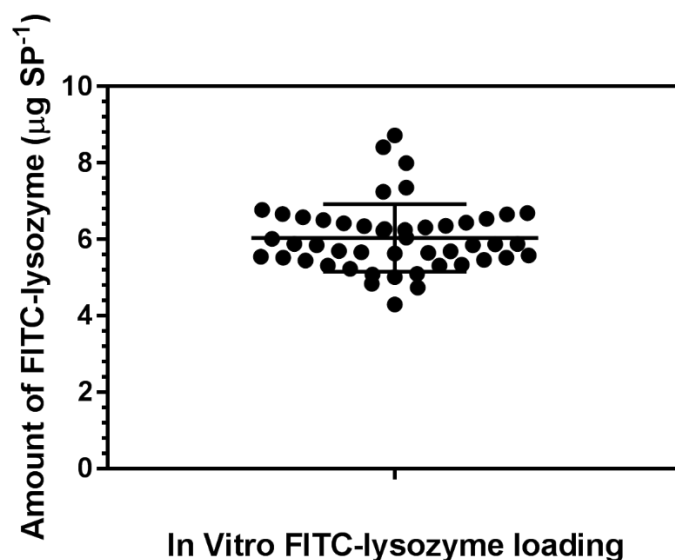


Figure 3. FITC-lysozyme loading amount in each individual SP among different groups.

The SPs were characterised in 47 groups and each group had 5 SPs.

Before encapsulating BDNF into SPs, the loading amount for each individual SP was first determined using model protein FITC-lysozyme to provide a benchmark for the amount of protein loaded in each batch of SPs. On average, the loading amount is $\sim 6.1 \pm 0.7$ μg FITC-lysozyme per SP (**Figure 3**). BDNF was encapsulated in two SPs (~ 5.8 μg of BDNF per SP), injected into the intracochlear of two deafened guinea pig (bilateral injection) and left for 3 days (**Figure 4**). The concentration of BDNF in the apex was higher (~ 0.7 $\text{ng } \mu\text{L}^{-1}$) than that at the basal turn (~ 4 $\text{ng } \mu\text{L}^{-1}$), showing that the BDNF distribution decreases as it diffuses from the apex to the basal turn of the cochlea. Theoretically, after the collection of 5 μL of perilymph, most of the collected fluid was cerebrospinal fluid, thus lower concentration of BDNF was observed.²⁸ As shown in **Figure 4d**, the highest concentration in the apex turn of the cochlea was ~ 0.4 $\text{ng } \mu\text{L}^{-1}$, which is lower than in the other three cochleae (**Figure 4a–c**). As the SPs were injected into both sides of the cochlea of deafened guinea pigs, after the collection of the perilymph from one side of the cochlea, the change in osmotic pressure causes

cochlea perilymph from the other side to flow towards the side in which the perilymph is being collected and thus, a lower concentration of BDNF was observed.

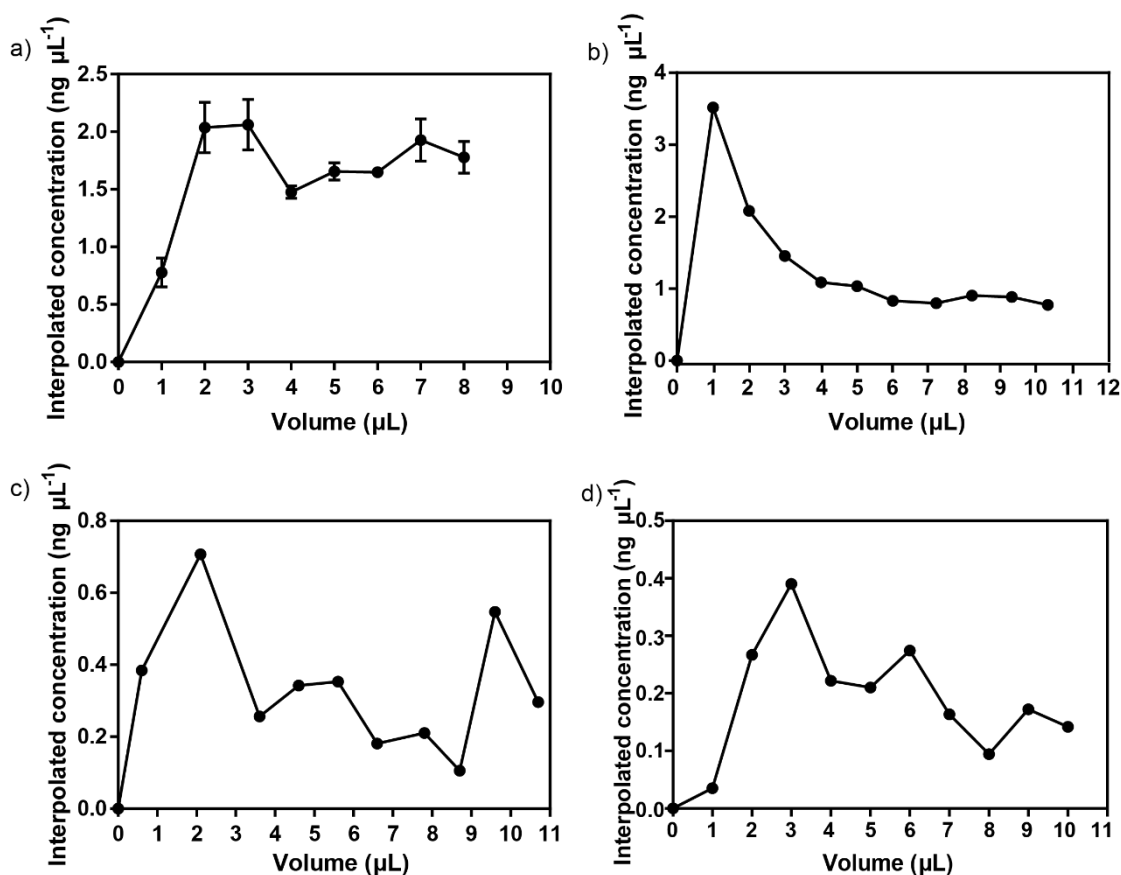


Figure 4. Pharmacokinetics of BDNF released from SPs implanted in the inner ear of 4 guinea pigs (2 SPs was loaded in 23 μL 1 mg mL⁻¹ of BDNF). Perilymph samples were collected 3 days post-implantation from the apex of the cochlea. The x-axis refers to the volume of the perilymph samples collected from the cochlea. The y-axis refers to the concentration of BDNF from the apex turn to the basal turn of the cochlea.

To further investigate the BDNF distribution in the cochlea, two SPs were implanted into two deafened guinea pigs (bilateral injection) for 7 days (**Figure 5**). The BDNF distribution in the cochlea after 7 days was also bell-shaped in which there are higher BDNF levels in the apex of the cochlea than the basal turn. As there was an initial burst

release observed in the *in vitro* release profile of SPs,²⁶ the highest concentration of BDNF after 7 days was expected to be lower than 3 days.

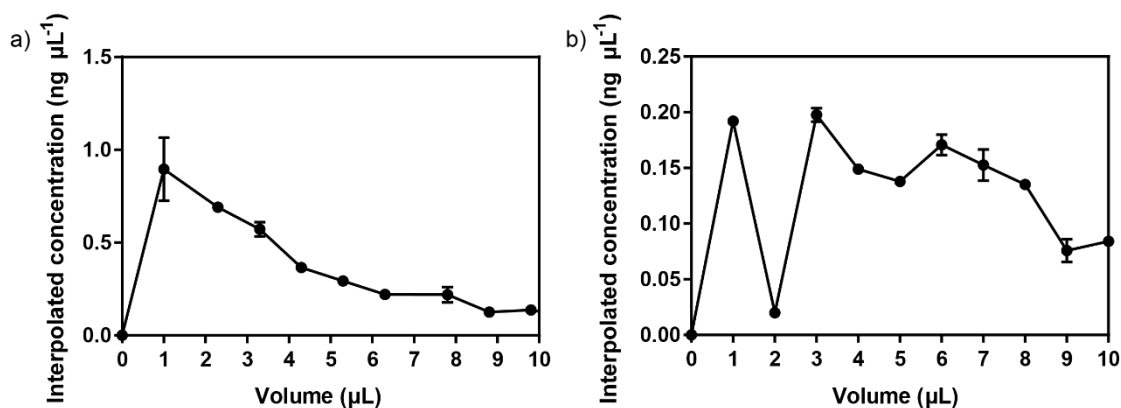


Figure 5. Pharmacokinetics of BDNF released from SPs in the inner ear of two guinea pigs (2 SPs were loaded in 23 μL 1 mg mL^{-1} of BDNF). Perilymph samples were collected 7 days post-implantation from the apex of the cochlea. The x-axis refers to the volume of the perilymph samples collected from the cochlea. The y-axis refers to the concentration of BDNF from the apex turn to the basal turn of the cochlea.

The pharmacokinetics of BDNF in the intracochlear was also studied by implanting five BDNF-encapsulated SPs on the RWM, shown in **Figure 6**. After 7 days, the perilymph sample was collected from the apex turn of the cochlea. In general, a bell-shaped BDNF distribution curve was observed in which the BDNF levels increased followed by a decrease. The highest concentration of BDNF was ~ 0.005 ng μL^{-1} to 0.009 ng μL^{-1} , which is approximately 100 times lower than the intracochlear implantation at 7 days. This is because i) the middle ear environment is drier than the cochlea and hence the drug is not released easily; ii) the released BDNF has to pass through the RWM and iii) there might be drug clearance through the Eustachian tube. It also showed measurable BDNF in the cochlea perilymph sample, which proved that BDNF could successfully diffuse from SPs and pass through the RWM.

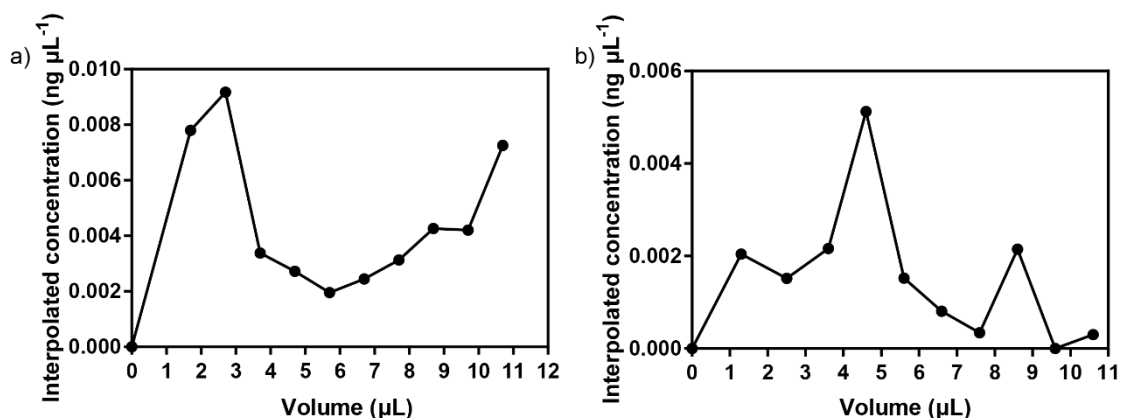


Figure 6. Pharmacokinetics of BDNF released from SPs implanted on the RWM of two guinea pigs (5 SPs were loaded with 40 μL 1 mg mL⁻¹ of BDNF). Perilymph samples 7 days post-implantation were collected from the apex of cochlea. The x-axis refers to the volume of the perilymph samples collected from the cochlea. The y-axis refers to the concentration of BDNF from the apex turn to the basal turn of the cochlea.

Using an osmotic pump delivery system, the BDNF distribution was similar to that obtained from BDNF delivery from SPs (**Figure 7a**). However, the highest BDNF concentration observed was ~0.02 ng μL⁻¹, which is ~40 times less than that achieved by intracochlear implantation of SPs for 7 days. This demonstrates that the drug delivery efficiency of SPs is higher than that achieved by osmotic pump delivery strategy. Only 5 samples were collected for **Figure 7b** (the perilymph could not be collected by the capillary tube). There might be some leakage occurring when the cochlea was opened, thus, the concentration of the BDNF in **Figure 7b** was relatively low compared to **Figure 7a**.

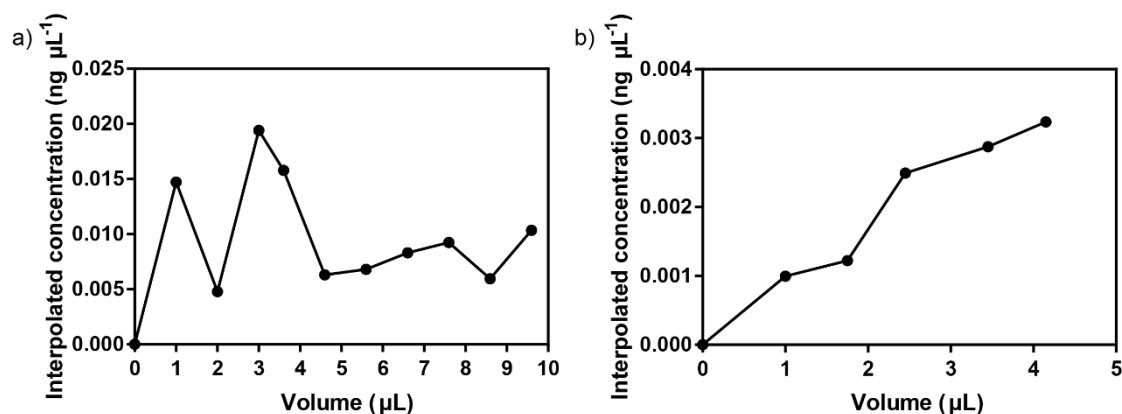


Figure 7. Pharmacokinetics of BDNF delivered in the intracochlear via an osmotic pump of two guinea pigs. Perilymph samples were collected from the apex of the cochlea after 7 days. The x-axis refers to the volume of the perilymph samples collected from the cochlea. The y-axis refers to the concentration of BDNF from the apex turn to the basal turn of the cochlea.

After the implantation of SPs on the RWM for 7 days, the SPs were collected and imaged using SEM as shown in **Figure 8a**. A higher magnification image showed the surface morphology of the implanted SPs (**Figure 8b**). The morphology of the primary silica particles changed significantly compared to the original primary silica particles where distinct spheres were obvious (refer to reference 26) and is due to the degradation of the primary silica particles and effect of the biological environment on the SPs.^{29,30}

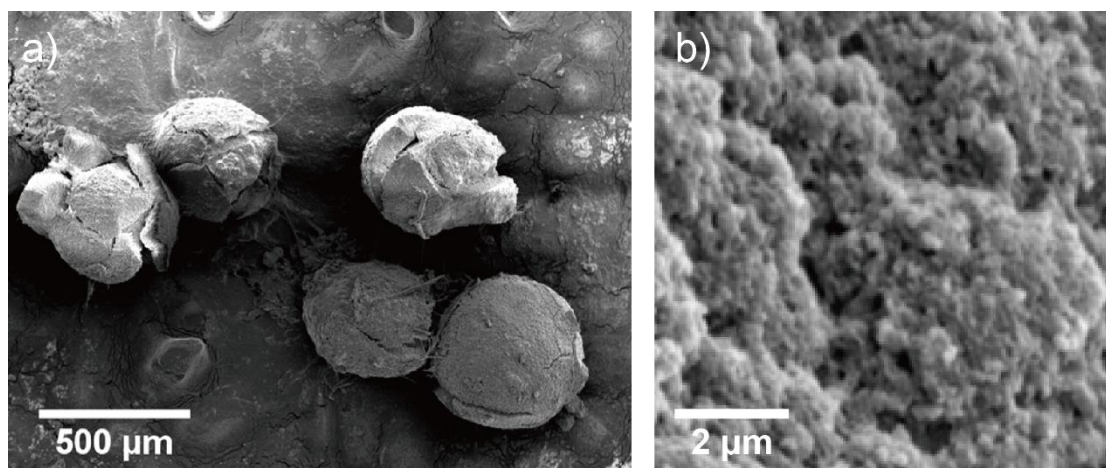


Figure 8. SEM images of implanted SPs on the RWM at (a) low magnification and (b) high magnification on the surface of implanted SPs from (a).

The *in vivo* biodistribution of Si released from silica SPs was investigated by ICP-MS. Different organs, including heart, liver, intestine, kidney, Ven, CNL, CNR, ICL and ICR, from guinea pigs implanted with SPs were collected (after 28 days) to determine Si biodistribution. It was found that all the organs collected had negligible Si accumulation (approximately $\sim 2 \mu\text{g g}^{-1}$ to $\sim 5 \mu\text{g g}^{-1}$) (**Figure 9**). After digesting organs, the measured Si concentration in different organs was below 500 ppb, which could not be measured consistently using an Agilent 7700 ICP-MS. Nevertheless, this study provided insight into relative *in vivo* Si accumulation after SPs implantation.

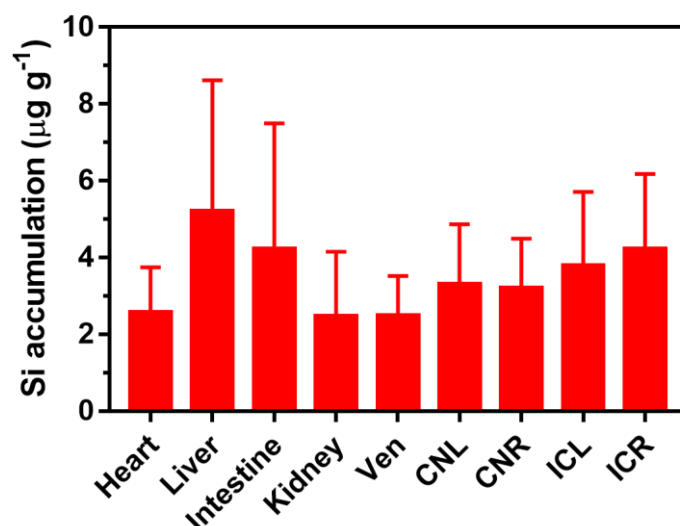


Figure 9. Si distribution in different organs (heart, liver, intestine, kidney, Ven, CNL, CNR, ICL and ICR) of guinea pig after the implantation of SPs for 28 days.

4.5 CONCLUSIONS

This study provides an understanding of the BDNF distribution in the cochlea after implantation of BDNF-loaded SPs via intratympanic administration and intracochlear administration. The biodistribution of Si, as a degradation product of SPs, in different organs and the morphology of SPs after *in vivo* implantation were examined, which support the biocompatibility and biodegradability of SPs. This study not only demonstrates that SPs can achieve higher drug (BDNF) delivery efficiencies via direct intracochlear injection when compared with BDNF delivery via an osmotic pump, but also shows that BDNF can pass through the RWM into the cochlea when the SPs are implanted in the middle ear. The findings provide evidence that the SPs can be used as a promising versatile drug delivery system for future clinical applications.

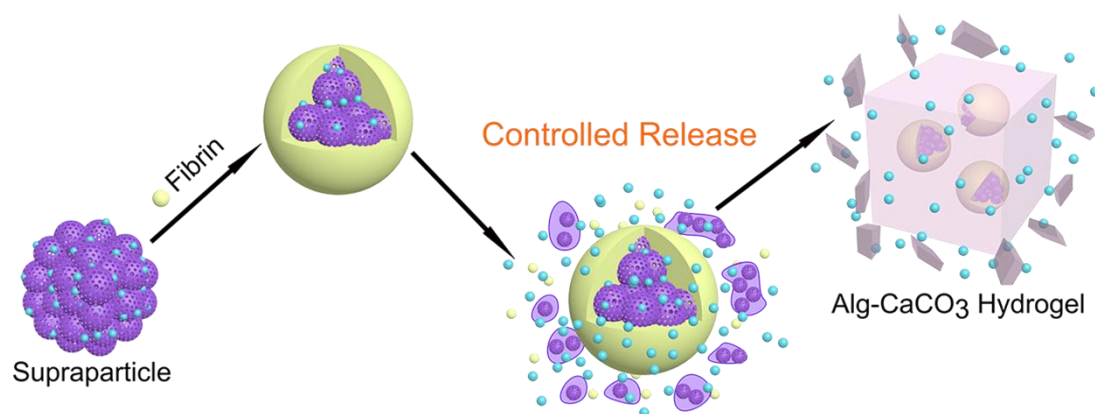
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Chapter 5

Engineering Biocoatings to Prolong Drug Release from Supraparticles



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Article

Engineering Biocoatings To Prolong Drug Release from Supraparticles

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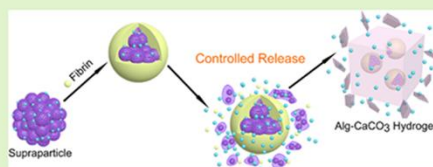
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Supporting Information

ABSTRACT: Supraparticles (SPs) assembled from smaller colloidal nanoparticles can serve as depots of therapeutic compounds and are of interest for long-term, sustained drug release in biomedical applications. However, a key challenge to achieving temporal control of drug release from SPs is the occurrence of an initial rapid release of the loaded drug (i.e., “burst” release) that limits sustained release and potentially causes burst release-associated drug toxicity. Herein, a biocoating strategy is presented for silica-SPs (Si-SPs) to reduce the extent of burst release of the loaded model protein lysozyme. Specifically, Si-SPs were coated with a fibrin film, formed by enzymatic conversion of fibrinogen into fibrin. The fibrin-coated Si-SPs, ^FSi-SPs, which could be loaded with $7.9 \pm 0.9 \mu\text{g}$ of lysozyme per SP, released >60% of cargo protein over a considerably longer period of time of >20 days when compared with the uncoated Si-SPs that released the same amount of the cargo protein, however, within the first 3 days. Neurotrophins that support the survival and differentiation of neurons could also be loaded at $\sim 7.3 \mu\text{g}$ per SP, with fibrin coating also delaying neurotrophin release (only 10% of cargo released over 21 days compared with 60% from Si-SPs). In addition, the effects of incorporating a hydrogel-based system for surgical delivery and the opportunity to control drug release kinetics were investigated—an alginate-based hydrogel scaffold was used to encapsulate ^FSi-SPs. The introduction of the hydrogel further extended the initial release of the encapsulated lysozyme to ~ 40 days (for the same amount of cargo released). The results demonstrate the increasing versatility of the SP drug delivery platform, combining large loading capacity with sustained drug release, that is tailorable using different modes of controlled delivery approaches.



INTRODUCTION

Nanotechnology has been widely applied in the field of drug delivery for a diverse range of diseases such as cancer,^{1,2} viral infections,³ and neurological diseases.^{4,5} In drug delivery, the application of nanoengineered particles, such as liposomes,⁶ polymer particles,⁷ and mesoporous silica particles,⁸ can improve drug stability, prevent drug degradation, and achieve controlled and sustained release of therapeutics.⁹ Sustained release drug delivery systems are of interest, particularly for applications where drug release is required over weeks or months, thereby reducing the frequency and amount of drug administration while eliciting the same (or improved) therapeutic effect. However, early and uncontrolled release of drug, or “burst release”, is a major challenge in developing sustained release drug delivery systems.¹⁰ Burst release results in higher initial local drug concentrations, which, dependent on

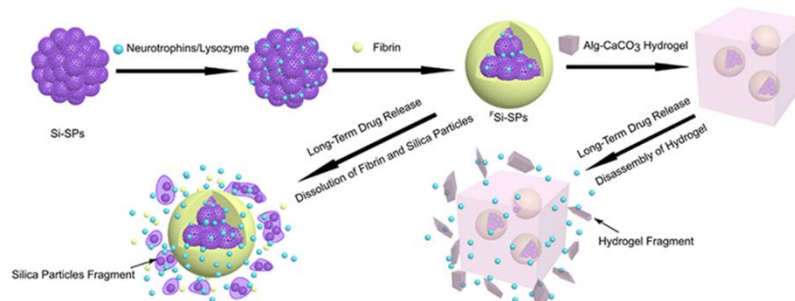
payload, can cause off-target toxicity. A second issue with burst release is the reduction in the effective lifetime of the drug delivery system; this is particularly relevant for nanocarriers where burst release could deplete a significant proportion of encapsulated drug because of short diffusional distances from the core to the surface of a nanoparticle.¹¹ Various approaches have been investigated to minimize the extent of burst release from nanocarriers, including modifying the structure or surface of nanocarriers,^{12–16} conjugating drug and nanocarriers via covalent bonds,¹⁷ and coating the surface of nanocarriers.^{18,19}

Recently, we reported the electrospray assembly of $\sim 550 \mu\text{m}$ silica supraparticles (Si-SPs) composed of thousands of smaller

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Scheme 1. Schematic Illustration of the SP Drug Delivery Systems Developed in the Present Study^a

^aSi-SPs are preloaded with protein therapeutics (neurotrophins or the model protein lysozyme) before coating with fibrin to generate ^FSi-SPs. The coated SPs, ^FSi-SPs, are subsequently embedded in an alg-CaCO₃ hydrogel system. ^FSi-SPs exhibit reduced initial burst release (compared to uncoated Si-SPs). Hydrogel encapsulation further reduces initial burst release. Long-term, sustained release of the preloaded protein is obtained through degradation of fibrin, Si-SPs, and the hydrogel.

porous silica particles. Si-SPs could encapsulate approximately 7 μg of a model drug per SP and exhibited sustained, long-term drug release (>4 months).²⁰ One of our main interests in Si-SPs is for the inner ear delivery of neurotrophins for the treatment of sensorineural hearing loss. Neurotrophins, including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are naturally occurring proteins expressed by certain types of cells within the inner ear including auditory neurons, hair cells, and supporting cells.^{21,22} Neurotrophins are important for supporting the differentiation and survival of auditory neurons and hence are highly relevant drug candidates for encapsulation in Si-SPs for treating hearing loss.^{23,24} BDNF has been loaded in Si-SPs—which can be prepared using evaporation-induced self-assembly,^{25–27} mold-templating approaches,^{28,29} and gel-mediated electrospray assembly²⁰—achieving protein loadings (>100 000 ng mg⁻¹)²⁰ that are several orders of magnitude higher than those achieved by comparable colloidal nanoparticle systems (~20 ng mg⁻¹).³⁰ Importantly, BDNF-loaded Si-SPs have shown improved auditory nerve survival when delivered in the inner ear of guinea pigs.^{27,31} For inner ear drug delivery, it is important to control the release kinetics of neurotrophins in the inner ear to maintain a therapeutically relevant drug level over time. In the central nervous system, high neurotrophin doses can alter neuronal excitability.^{32,33} Reducing burst release to achieve sustained, continuous release over long periods (e.g., months) is critical for future clinical translation of the SP technology.

Herein, we hypothesize that drug release from SPs can be slowed down using engineered coatings. A major consideration is the use of a material/coating that is biologically compatible to enable future clinical use. Thus, a bioinspired strategy, using a biocoating and/or a composite hydrogel, is presented to reduce the extent of burst release of proteins preloaded in Si-SPs (Scheme 1). Specifically, our coating strategy is based on fibrin, an endogenous protein that is vital for blood clotting and hemostasis. Fibrin is a key component of the typical physiological response for the prevention of bleeding/hemorrhaging or blood clot formation: insoluble fibrin is enzymatically formed from fibrinogen, a glycoprotein present in blood plasma, which in the presence of thrombin becomes fibrin.^{34–36} We hypothesize that this bioinspired coating strategy (which mimics aspects of the blood clotting formation) would result in a compact fibrin coating with low permeability, thus delaying drug release from Si-SPs. This is similar to what is

observed for coated surgical sutures.³⁷ Our results showed a substantial delay in initial release: in the presence of uncoated Si-SPs, >60% of drug release occurred in the first 3 days, whereas in the presence of the fibrin-coated Si-SPs (^FSi-SPs) drug release extended to over >20 days (a >6-fold increase). Furthermore, cytotoxicity studies in the U87MG human brain glioblastoma cell line showed no adverse effects, and degradation studies provided further evidence for the biodegradability of the fibrin coating.

To facilitate future clinical use and surgical handling of the present SP drug delivery system, a hydrogel scaffold that gels upon application of external stimuli was developed by incorporating ^FSi-SPs within an injectable, biocompatible, and biodegradable alginate-CaCO₃ particle (alg-CaCO₃) hydrogel system. We previously reported approaches using poloxamer-based hydrogels that accelerated the release rate of drugs encapsulated in Si-SPs (thus increasing the initial drug release).²⁰ In contrast, the alg-CaCO₃ system presented herein reduced the initial drug release rate. Taken together, our results demonstrate that the incorporation of ^FSi-SPs into an alg-CaCO₃ hydrogel matrix is a promising and potentially translatable platform for sustained and controlled protein delivery with substantially reduced burst release compared to conventional SP drug delivery systems.^{27,28} This approach has potential clinical application in the treatment of middle ear or inner ear diseases with neurotrophins or other biomedical applications requiring sustained release.

EXPERIMENTAL SECTION

Materials. Cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), ammonium hydroxide solution (28–30%), poly(acrylic acid) (PAA, $M_w \approx 250$ kDa, 35 wt % aqueous solution), fluorescein isothiocyanate (FITC), lysozyme protein from chicken egg white, alginate sodium salt from brown algae (alginate), calcium chloride (CaCl₂), phosphate-buffered saline (PBS, pH 7.4), fibrinogen from human plasma, phenazine methosulfate (PMS), Dulbecco's PBS (DPBS), sodium hydroxide (NaOH), poly(sodium 4-styrenesulfonate) (PSS), sodium carbonate (Na₂CO₃), D-glucono- δ -lactone (GDL), and silicon standard for the inductively coupled plasma experiments were purchased from Sigma-Aldrich (USA). Thrombin reagent was purchased from Siemens (Australia). Hydrochloric acid, sodium chloride (NaCl), tris(hydroxymethyl)methylamine (Tris), and calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O) were obtained from Chem-Supply (Australia). 2,3-Bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT), the Micro BCA protein

assay kit, and NT-3 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Thermo Fisher Scientific (Australia). The U87MG human brain glioblastoma cell line was purchased from American Type Culture Collection (USA). Biowhittaker Dulbecco's modified Eagle's medium (DMEM) was obtained from Lonza (Australia). BDNF and NT-3 were purchased from PeproTech Asia (Israel), and human BDNF ELISA kits were obtained from Abcam (Australia). Ethanol was purchased from Chem-Supply (Australia). Ultrapure water (Milli-Q water) with a resistivity of greater than 18 MΩ cm was used in all experiments and obtained from a three-stage Millipore Milli-Q Plus 185 purification system. All chemicals were used without further purification.

Synthesis of Primary Silica Particles. Primary silica particles (~1 μm diameter) were synthesized using a modified version of a previously published protocol.³⁸ Briefly, 1.1 g of CTAB was dissolved in 50 mL of Milli-Q water, followed by mixing with 4.3 g of PAA under vigorous stirring at room temperature (i.e., ~22 °C). After 20 min, a clear solution was obtained, to which 3.5 mL of ammonium hydroxide solution (28–30%) was added with vigorous stirring for 20 min, resulting in a milky suspension. TEOS (4.46 mL) was then 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 Teflon-sealed 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 (Jetlow, Australia) and calcined at 550 °C for 30 h to remove any organic materials.

Synthesis of Si-SPs. Si-SPs were assembled using an electrospray method as described previously.²⁰ Briefly, 80 mg of the primary silica particles was dispersed in 2 mL of aqueous alginate solution (30 mg mL⁻¹ in Milli-Q water). After mixing, the suspension was ultrasonicated using 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. Subsequently, the solution was poured into a syringe, which was positioned in a syringe pump set at a constant flow rate of 8 mL h⁻¹. An applied voltage of 13 kV was used in the electrospray setup to drip the alginate/primary silica particle solution into a reservoir of 1% w/v of aqueous CaCl₂. A 10 cm collector distance was set between the end of the nozzle and the surface of the CaCl₂ solution. After 5 min, the alginate/Si-SPs (Si-SPs^{alg}) were collected directly from the CaCl₂ bath using a strainer and washed extensively with Milli-Q water (four times). Finally, the alginate was removed from Si-SPs^{alg} by calcination at 650 °C for 30 h to produce Si-SPs. The density of Si-SPs is ~71.8 μg cm⁻³, calculated using the mass of individual Si-SPs divided by the volume of individual Si-SPs (the mass and diameter of SPs are measured, and then the density is approximated by assuming that the individual Si-SPs are perfect spheres).

Synthesis and in Vitro Fibrin Degradation of ^FSi-SPs. The preparation of ^FSi-SPs is schematically illustrated in Scheme 1. Typically, 10 Si-SPs were placed in a 1.7 mL Eppendorf tube and sterilized with 100 μL of 80% (v/v) ethanol at ~22 °C for 4 h. SPs were then washed with Milli-Q water six times (after sterilization, Si-SPs were handled under aseptic conditions in a biosafety cabinet). Then, 50 μL of 20 mg mL⁻¹ fibrinogen dissolved in 50 mM Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl with pH adjusted to 7.2) was added to Si-SPs and incubated at 4 °C overnight (~16 h). The supernatant was then aspirated, and Si-SPs were incubated with 50 μL of 1.72 mg mL⁻¹ thrombin in 50 mM TBS, containing 40 mM CaCl₂ at ~22 °C for 1 h. Finally, the supernatant was aspirated, and Si-SPs were washed with Milli-Q water three times.

The degradation of fibrin from ^FSi-SPs was assessed by incubation of ^FSi-SPs with 100 μL of PBS (pH 7.4) at 37 °C (with 10 ^FSi-SPs in each sample). At specific time intervals (up to 42 days), 95 μL of the supernatant was collected and stored at -20 °C for further analysis and then replaced with 95 μL of fresh PBS. The concentration of the degraded fibrin in the supernatant was quantified using a Micro BCA protein assay kit based on the manufacturer's protocol. The degradation percentage of fibrin was calculated as follows:

$$\text{Percentage of fibrin remaining} = \frac{m_0 - m_n}{m_0} \times 100\% \quad (1)$$

where m_0 is the initial amount of fibrin on the SPs and m_n is the amount of fibrin in the supernatant at time point n .

Synthesis of CaCO₃ Particles. CaCO₃ particles were synthesized using a previously described protocol³⁹ with modifications. First, 5 g of PSS (M_w 70 kDa) was dissolved in 500 mL of Milli-Q water to form 10 mg mL⁻¹ PSS solution. Then, a precursor solution consisting of 1 mL of 1 M Na₂CO₃, 0.5 mL of 10 mg mL⁻¹ PSS, and 3.5 mL of Milli-Q water was prepared. In a separate flask, 20 mL of 10 mg mL⁻¹ PSS solution was added to 175 mL of Milli-Q water with vigorous stirring. After stirring for 1 min, 5 mL of 1 M Ca(NO₃)₂·4H₂O solution was added with vigorous stirring for 2 min. The precursor solution (5 mL) was then added and the resulting mixture was stirred vigorously for 1 min, after which the stirrer was turned off and the mixture was allowed to stand for 3 min, followed by further vigorous stirring for 1 min. The as-synthesized CaCO₃ particles were dried overnight in an oven at 80 °C and subsequently ground using a mortar and pestle. The fine CaCO₃ powder was then calcined with air flow at 500 °C for 2 h to remove any organic materials.

Preparation and in Vitro Degradation of the Alg-CaCO₃ Hydrogel. To prepare the hydrogel, 1 mL of 20 mg mL⁻¹ alginate solution in Milli-Q water was mixed with 15 μL of 100 mg mL⁻¹ CaCO₃ particles (particle diameter ~1.8 μm, see Figure S1). Following this, 53.4 μL of 100 mg mL⁻¹ GDL was added to achieve a CaCO₃ particle-to-GDL molar ratio of 1:2, resulting in a neutral pH value of the formed hydrogel. The gelation time of the hydrogel can be tuned by adjusting the concentration of the alginate solution and/or the amount of CaCO₃ particles.⁴⁰

To study the degradability of the alg-CaCO₃ hydrogel, the mass (dry weight) of the hydrogel was monitored over time. PBS (500 μL, pH 7.4) was added to the hydrogel (500 μL of 20 mg mL⁻¹ alg-CaCO₃ hydrogel) and incubated at 37 °C. At various time points (0, 3, 7, 14, 21, 28, and 42 days), PBS was removed, and the remaining hydrogel samples were washed with Milli-Q water (five times), frozen, and stored at -20 °C until analysis. For the longer time points, PBS was replaced with 500 μL of fresh PBS, and the sample was returned to the incubator set at 37 °C until the next time point. All remaining samples were lyophilized, and the dry weight of the hydrogel was measured. The weight loss percentage was calculated as follows

$$\text{Weight loss (\%)} = \frac{m_0 - m_n}{m_0} \times 100\% \quad (2)$$

where m_0 is the initial weight of the hydrogel at time point 0 and m_n is the weight of hydrogel at time point n .

Characterization. Scanning electron microscopy (SEM) images of Si-SPs, ^FSi-SPs, and CaCO₃ particles were taken using a Philips XL30 field-emission scanning electron microscope (Philips, Netherlands) at an operating voltage of 5 kV. SEM samples of the SPs were prepared by depositing them onto a conductive carbon tape, followed by sputter coating to obtain a 20 nm gold coating on the samples. SEM-energy-dispersive X-ray spectroscopy (EDX) mapping of ^FSi-SPs was performed on a Philips XL30 field-emission scanning electron microscope (Philips, Netherlands). Prior to analysis, the alg-CaCO₃ hydrogel was lyophilized overnight. The lyophilized sample was then placed directly onto a conductive carbon tape, followed by gold sputter coating for imaging. Fourier-transform infrared (FTIR) spectra of Si-SPs and ^FSi-SPs were recorded using an FTIR spectrophotometer (Bruker, Australia). The thickness and surface roughness of the fibrin coating were characterized by atomic force microscopy (AFM; JPK NanoWizard II BioAFM) in contact mode. Briefly, a glass microscope slide was cleaned using a plasma cleaner. Fibrinogen solution was added onto the glass slide, incubated overnight at 4 °C, and washed with Milli-Q water. For the preparation of the fibrin coating, thrombin solution was added to the fibrinogen film and incubated for 1 h at room temperature (22 °C), followed by washing with Milli-Q water. To measure the thickness, a scalpel blade was used to scratch the dry fibrinogen or fibrin film and images were taken at several points on the edge of the scratch. Film thickness and surface roughness analyses were

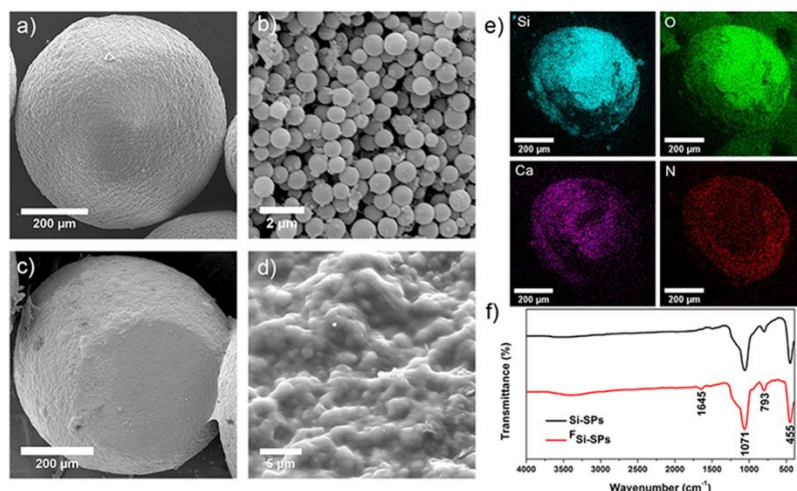


Figure 1. Characterization of Si-SPs and $^{\text{F}}$ Si-SPs. (a) SEM image of Si-SPs and (b) high-resolution SEM image displaying the surface of Si-SPs. (c) SEM image of $^{\text{F}}$ Si-SPs and (d) high-resolution SEM image displaying the surface of $^{\text{F}}$ Si-SPs. (e) Elemental distribution of $^{\text{F}}$ Si-SPs. (f) FTIR spectra of Si-SPs and $^{\text{F}}$ Si-SPs.

carried out using the JPKSPM data processing software. The thickness and surface roughness values obtained were averaged and reported as mean $\pm$ standard deviation.

In Vitro Release Studies. In vitro release studies were conducted using either the model drug FITC-lysozyme or neurotrophin (BDNF or NT-3). Following sterilization of Si-SPs with 80% v/v ethanol and the synthesis of $^{\text{F}}$ Si-SPs (as described earlier), 10 Si-SPs were incubated with 100 μL of FITC-lysozyme solution (1 mg mL^{-1} in Milli-Q water) in a 1.7 mL Eppendorf tube and mixed for 3 days at room temperature (22 $^{\circ}\text{C}$). After incubation, the supernatant was aspirated to remove unbound lysozyme, and the particles were washed with 100 μL of Milli-Q water three times. FITC-lysozyme-loaded Si-SPs were coated with fibrin as described above. To study FITC-lysozyme release, 100 μL of fresh PBS (as the elution buffer) was added to the loaded Si-SPs or $^{\text{F}}$ Si-SPs. At defined time intervals (over 150 days), 95 μL of the supernatant was collected (and stored at -20°C until analysis) and replaced with 95 μL of fresh PBS. The fluorescence of the collected samples was measured using an Infinite M200 microplate reader (Tecan, Switzerland), and the concentration of FITC-lysozyme in the supernatant was calculated using a FITC-lysozyme standard curve (Figure S2). FITC-lysozyme release from $^{\text{F}}$ Si-SPs was also assessed as a function of fibrinogen concentration (2, 20, and 40 mg mL^{-1}) at a given concentration (1.72 mg mL^{-1}) of thrombin (the resulting samples are referred to as $^{2\text{F}1.72}$ Si-SPs, $^{20\text{F}1.72}$ Si-SPs, and $^{40\text{F}1.72}$ Si-SPs, respectively) and as a function of thrombin concentration (0.1, 0.5, 1.72, and 5 mg mL^{-1}) at a given concentration (20 mg mL^{-1}) of fibrinogen (the resulting samples are denoted as $^{20\text{F}0.1}$ Si-SPs, $^{20\text{F}0.5}$ Si-SPs, $^{20\text{F}1.72}$ Si-SPs, and $^{20\text{F}5}$ Si-SPs, respectively). A description, including nomenclature, of the particles prepared in the present study is given in Table S1.

For neurotrophin (BDNF or NT-3) loading, four sterilized Si-SPs were incubated with 30 μL of BDNF or NT-3 solution (1.0 mg mL^{-1} in Milli-Q water). 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). For loading $^{\text{F}}$ Si-SPs, the neurotrophin was loaded into Si-SPs, followed by fibrin coating as mentioned above. Then, 100 μL of PBS (pH 7.4) was added to each tube and incubated at 37 $^{\circ}\text{C}$. At defined time points (~ 28 days), 95 μL of the supernatant was collected (and stored at -20°C until analysis) and replaced with 95 μL of fresh PBS. This sampling method was repeated at each time point over the course of the release studies. The amount of BDNF or NT-3 released from Si-SPs or $^{\text{F}}$ Si-SPs was determined using a BDNF- or NT-

3-specific ELISA using a standard curve of BDNF or NT-3 as per the manufacturer's protocol (Figure S3).

The release of FITC-lysozyme from $^{\text{F}}$ Si-SPs embedded in the hydrogel was determined as follows. Alginate solution was mixed with CaCO_3 particles as described above. Prior to gelation, 10 $^{\text{F}}$ Si-SPs (preloaded with FITC-lysozyme for 3 days) were added to the alginate- CaCO_3 mixture. To induce gelation, GDL was added at the same concentration and volume as described above. After ~ 2 min, the hydrogel formed. To study the release in PBS, 100 μL of PBS (pH 7.4) was added, and the sample was incubated at 37 $^{\circ}\text{C}$. At defined time points (over 110 days), 95 μL of the supernatant was collected (and stored at -20°C until analysis) and replaced with fresh PBS. The fluorescence of the collected samples was measured with an Infinite M200 microplate, and the corresponding FITC-lysozyme concentration was determined using a standard curve (Figure S4).

Note that for all comparisons presented in individual figures, release experiments were performed simultaneously and fluorescence measurement of the collected samples was performed on the same day using the same standard curve.

In Vitro Si-SPs and $^{\text{F}}$ Si-SP Degradation Studies. Si-SPs were first sterilized with 100 μL of ethanol (80% v/v) at $\sim 22^{\circ}\text{C}$ for 4 h and then washed with Milli-Q water four times (following sterilization, SP handling was performed in a biosafety cabinet using aseptic technique). Subsequently, $^{\text{F}}$ Si-SPs were prepared as described above. SP degradation was studied by immersing Si-SPs or $^{\text{F}}$ Si-SPs (20 SPs per sample tested) in 1 mL of 10 mM PBS (pH 7.4) at 37 $^{\circ}\text{C}$. At defined time points (over 70 days), 1 mL of the supernatant was collected and replaced with 1 mL of fresh PBS. The presence of silica degradation product in the supernatant was measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES). Sodium hydroxide (8 M, 500 μL) was added to 500 μL of each collected supernatant to dissolve any silica fragments. The samples were incubated at 95 $^{\circ}\text{C}$ for 30 min, followed by 30 min of sonication. The samples were then incubated overnight at 37 $^{\circ}\text{C}$. The dissolved sample (1 mL) was mixed with 4 mL of Milli-Q water, resulting in a 10-fold sample dilution from the originally collected supernatant. The amount of silicon in each sample (corresponding to the released silicon from the SPs) was measured by ICP-OES using a silicon standard curve (Figure S5). In addition to these measurements, the morphology of Si-SPs and $^{\text{F}}$ Si-SPs after long-term incubation in PBS was observed by SEM.

Cytotoxicity. First, Si-SPs were sterilized with 100 μL of ethanol (80% v/v) at $\sim 22^{\circ}\text{C}$ for 4 h, followed by washing sequentially with Milli-Q water (three times) and cell culture medium (three times).

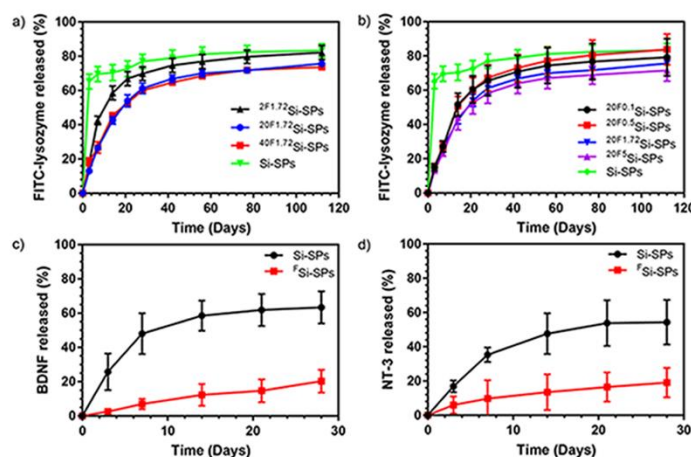


Figure 2. In vitro cumulative release profiles. (a) FITC-lysozyme release from Si-SPs and $^{\text{F}}$ Si-SPs (fibrin coating was obtained at varying concentrations of fibrinogen (2, 20, and 40 mg mL^{-1}) while maintaining the concentration of thrombin at 1.72 mg mL^{-1} ; the samples are respectively denoted as 2F1.72Si-SPs, 20F1.72Si-SPs, and 40F1.72Si-SPs). (b) FITC-lysozyme release from Si-SPs and $^{\text{F}}$ Si-SPs (fibrin coating was obtained at varying concentrations of thrombin (0.1, 0.5, 1.72, and 5 mg mL^{-1}), while keeping the concentration of fibrinogen constant at 20 mg mL^{-1} ; the samples are respectively denoted as 20F0.1Si-SPs, 20F0.5Si-SPs, 20F1.72Si-SPs, and 20F5Si-SPs). Release profiles of (c) BDNF and (d) NT-3 from Si-SPs and $^{\text{F}}$ Si-SPs. Data presented in (a–d) are averages of triplicates with error bars, indicating standard deviations, each measuring release from 10 SPs in (a,b) and 1 SP in (c,d). Release percentage data for individual time points are shown in Figures S8, S9, S11, and S12.

After sterilization, Si-SPs were handled in a biosafety cabinet using an aseptic technique. To determine the cytotoxicity of the degradation products of the SPs, 1 mL of cell culture media (DMEM supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin, 100 mg mL^{-1} streptomycin) was added to Si-SPs or $^{\text{F}}$ Si-SPs at varying quantities. At defined time intervals, the cell medium was aspirated (and stored at $-20\text{ }^{\circ}\text{C}$ for future cell analysis) and replaced with fresh cell media.

Brain-derived U87MG glioblastoma cells were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified incubator ($37\text{ }^{\circ}\text{C}$, 5% CO_2). U87MG cells were chosen as they are a human brain cell line, which is relevant for potential applications of the SPs in drug delivery to the human inner ear. U87MG cells were seeded in a 96-well plate at 2×10^4 cells per well in 100 μL of the medium and incubated overnight. Subsequently, 100 μL of the sample (degradation products from Si-SPs and $^{\text{F}}$ Si-SPs incubated in cell culture media and collected at different time points as described above) was added to each well and incubated ($37\text{ }^{\circ}\text{C}$, 5% CO_2). After incubation for 48 h, the cell culture medium was aspirated, and 100 μL of XTT/PMS solution was added to each well to measure cell viability. After incubation for 4 h, the absorbance of the solution was measured using an Infinite M200 microplate reader at a wavelength of 475 nm and a reference wavelength of 675 nm. Differential absorbance was calculated by subtracting the absorbance at 675 nm from the absorbance at 475 nm. XTT/PMS solution was prepared by mixing 135 μL of 2 mM PMS in DPBS solution with 10.8 mg of XTT in 54 mL of DMEM supplemented with 10% fetal bovine serum. Sample wells containing only medium (i.e., no cells) were used as background controls for the absorbance measurements. Cells exposed only to fresh DMEM were termed untreated controls and used for the normalization of cell toxicity.

Minimum Information Reporting in Bio–Nano Experimental Literature. The studies conducted herein, including material characterization, biological characterization, and experimental details, conform to the minimum information reporting in bio–nano experimental literature (MIRIBEL) reporting standard for bio–nano research,⁴¹ and we include a companion checklist of these components in the Supporting Information.

RESULTS AND DISCUSSION

Synthesis of $^{\text{F}}$ Si-SPs. Si-SPs synthesized by gel-mediated electrospray assembly have been demonstrated to exhibit a high

loading capacity and a sustained drug release for over 110 days.²⁰ However, it was also noted that $\sim 60\%$ of FITC-lysozyme preloaded in Si-SPs was released within the first 3 days.²⁰ To delay this burst release, a bioconjugation strategy was developed herein to prepare $^{\text{F}}$ Si-SPs. As observed in Figure 1a–d, Si-SPs and $^{\text{F}}$ Si-SPs were spherical and consisted of thousands of smaller porous silica particles. Incubation of Si-SPs with fibrinogen, followed by the enzyme thrombin, results in the enzymatic conversion of fibrinogen into a mesh of fibrin on the Si-SP surface. Fibrin is a fibrous nonglobular protein that is involved in the process of blood clotting.^{34–36} SEM was used to examine the surface structure of SPs following fibrin coating. At low resolution, there is little observable difference in morphology between Si-SPs and $^{\text{F}}$ Si-SPs (Figure 1a,c), but at higher resolution, SEM imaging revealed distinct differences in the surface morphology of the SPs (Figure 1b,d), indicating the successful coating of Si-SPs with fibrin, as evidenced by the film coverage of the intraparticle pores within the SPs. SEM–EDX elemental mapping confirmed the presence of silicon (Si), oxygen (O), calcium (Ca), and nitrogen (N) uniformly distributed on the surface of $^{\text{F}}$ Si-SPs (Figure 1e). FTIR spectroscopy of Si-SPs confirmed the presence of Si–O–Si bonds with stretching vibrations of Si–O–Si observed at 1071 cm^{-1} , bending vibrations of O–Si–O observed at 793 cm^{-1} , and rocking vibrations of Si–O–Si observed at 455 cm^{-1} (Figure 1f, black spectrum).⁴² $^{\text{F}}$ Si-SPs displayed a comparable FTIR spectrum (Figure 1f, red spectrum) except for the presence of an additional absorption peak observed at 1645 cm^{-1} , which may be assigned to the amine I band, because of the stretching vibration of C=O and C–N bands from fibrin in $^{\text{F}}$ Si-SPs.^{43,44} The thickness and surface roughness of the fibrin coating were measured using AFM. Before the addition of thrombin, the fibrinogen coating had a thickness of $0.9 \pm 0.4\text{ }\mu\text{m}$ with a root-mean-square roughness of $86 \pm 42\text{ nm}$. After exposure to thrombin and subsequent conversion to fibrin, the thickness increased to $2.3 \pm 0.3\text{ }\mu\text{m}$ and the roughness also increased to $331 \pm 152\text{ nm}$ (Figure S6). Taken together, these results

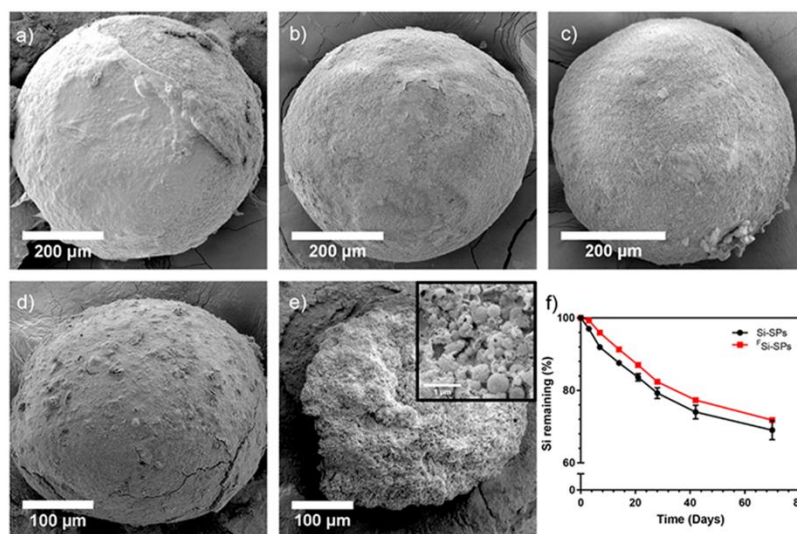


Figure 3. SEM images of ^{29}Si -SPs after incubation in PBS (pH 7.4) at 37 °C for (a) 3, (b) 14, (c) 21, (d) 42, and (e) 70 days. The inset in (e) shows the surface structure of ^{29}Si -SPs after incubation for 70 days in PBS (pH 7.4) at 37 °C. (Representative images of ^{29}Si -SPs before incubation are shown in Figure 1 and images of degraded Si-SPs are shown in Figure S13.) (f) ICP-OES analysis to quantify the Si released from Si-SPs and ^{29}Si -SPs throughout an incubation period of 70 days. ^{29}Si -SPs were prepared using 20 mg mL⁻¹ of fibrinogen and 1.72 mg mL⁻¹ of thrombin.

confirm the successful preparation of Si-SPs and the formation of a fibrin coating on Si-SPs.

In Vitro Drug Release. Preloading a model protein therapeutic (FITC-lysozyme) into Si-SPs (Scheme 1) followed by coating with a compact layer of fibrin as a “sealant” was hypothesized to slow the release of the entrapped “drug” from ^{29}Si -SPs. To evaluate FITC-lysozyme release profiles of ^{29}Si -SPs, FITC-lysozyme-loaded ^{29}Si -SPs were incubated in PBS (pH 7.4) at 37 °C, and the supernatant was collected for protein content analysis at varying time points. A range of parameters for ^{29}Si -SP synthesis were examined, including varying the concentration of fibrinogen (while keeping the concentration of thrombin constant) and the concentration of thrombin (while keeping the concentration of fibrinogen constant). FITC-lysozyme was loaded in Si-SPs (incubation for 3 days at ~22 °C) to achieve an average loading amount of 7 μg per SP. FITC-lysozyme loading in Si-SPs prior to fibrin coating under different fibrinogen and thrombin concentrations is shown in Figure S7. The percentage of FITC-lysozyme released from the different SPs at each time point is shown in Figures S8 and S9, and the respective release profiles are shown in Figure 2a,b. Figure S10 shows the data fitted according to the first-order release kinetics model. As noted in Figure 2a,b, both Si-SPs and ^{29}Si -SPs showed sustained FITC-lysozyme release profiles over 110 days. However, fibrin coating (^{29}Si -SPs) resulted in a reduced initial release. Specifically, >60% of the protein was released from uncoated Si-SPs within 3 days. In comparison, release from ^{29}Si -SPs (Si-SPs coated using 2 mg mL⁻¹ of fibrinogen and 1.72 mg mL⁻¹ of thrombin) occurred on a longer timescale of >20 days to achieve the same extent of release. The release period was further extended to 28 days when using $^{20}\text{F}^{1.72}\text{Si}$ -SPs and $^{40}\text{F}^{1.72}\text{Si}$ -SPs (^{29}Si -SPs prepared using higher concentrations of fibrinogen) (Figure 2a). Similarly, by applying different concentrations of thrombin, the release of FITC-lysozyme from ^{29}Si -SPs was slowed down, although the actual thrombin concentration (from 0.1 to 5 mg mL⁻¹) had little effect on the

early release of the protein (in the first 12 days). This is reasonable as thrombin is an enzyme that converts soluble fibrinogen into insoluble (“sticky”) fibrin, and the amount of thrombin (within a reasonable range) is not likely to limit the final quantity of fibrin fibers formed. As noted in Figure 2a,b, the cumulative drug release amount was lower than 100%, likely due to the photobleaching of FITC that occurs over long periods of time and/or the presence of a firmly adsorbed protein on Si-SPs.⁴⁵ Nevertheless, these results demonstrate that fibrin coating can substantially reduce the initial burst release of the model cargo FITC-lysozyme from Si-SPs.

This study was extended to clinically relevant neurotrophins, BDNF and NT-3, to assess the potential application of Si-SPs for inner ear neurotrophin delivery. BDNF or NT-3 was loaded in Si-SPs (incubation for 3 days at ~22 °C), achieving an average loading of 7.3 μg per SP. Fibrin coating of BDNF- and NT-3-loaded Si-SPs was performed using 20 mg mL⁻¹ of fibrinogen and 1.72 mg mL⁻¹ of thrombin. These parameters were chosen (and were used in all subsequent experiments), as they provided optimal delayed burst release based on the FITC-lysozyme studies discussed above. Figure 2c,d shows the cumulative release profiles of BDNF and NT-3 of Si-SPs and ^{29}Si -SPs in PBS (pH 7.4) at 37 °C. The release of both BDNF and NT-3 from ^{29}Si -SPs shows decreased release rates compared with the release profile of uncoated Si-SPs, which is further demonstrated from the percentage of BDNF or NT-3 released from ^{29}Si -SPs at each time point (Figures S11 and S12). At day 21, ~60% of the loaded BDNF or NT-3 was released from uncoated Si-SPs, whereas only ~10% of the loaded BDNF or NT-3 was released from ^{29}Si -SPs (Figure 2c,d), indicating that the fibrin coating strategy can also delay the release of preloaded BDNF and NT-3. The lower amount of BDNF/NT-3 released from ^{29}Si -SPs when compared with the amount of FITC-lysozyme released from ^{29}Si -SPs might be due to the stronger binding of BDNF/NT-3 to the fibrin matrix, resulting in extended BDNF and NT-3 retention within ^{29}Si -SPs when compared with FITC-lysozyme reten-

tion.⁴⁶ The released neurotrophins are expected to maintain their biological activity, as shown in our previous work after implantation of the SPs in the inner ear of guinea pigs.^{27,31}

Degradation of Si-SPs and ^FSi-SPs. The release of lysozyme and BDNF/NT-3 from ^FSi-SPs in PBS (in vitro) or the inner ear fluid (in vivo) is expected to occur partly as a result of concurrent dissolution of silica and degradation of the biodegradable fibrin coating. Silica has been generally recognized as safe by the U.S. Food and Drug Administration for over 50 years and is used as a food additive in various products.⁴⁷ Silica-based nanomaterials can be degraded into nontoxic components (e.g., silicic acid or poly(silicic acid), which can be excreted through urine) and are of interest for diverse biomedical applications.^{47–49} Yamada et al. have demonstrated that the degradation of silica particles depends on the porosity and colloidal dispersity of the particles.⁴⁸ In addition, the presence of calcium ions can accelerate the degradation of silica particles.⁴² In vitro degradation studies of Si-SPs were performed by incubating Si-SPs or ^FSi-SPs in PBS (pH 7.4, 37 °C). SEM analysis revealed a progressive decrease in the size of Si-SPs (Figure S13) and ^FSi-SPs (Figure 3a–e) over time and distinct degradation—^FSi-SPs had a cracked and collapsed morphology after incubation for 42 days (Figure 3d). After incubation for 70 days in PBS at 37 °C, the diameter of ^FSi-SPs decreased by ~40% and substantial changes in SP morphology were apparent when comparing ^FSi-SPs at day 3 (Figure 3a) and ^FSi-SPs at day 70 (Figure 3e). Higher-magnification SEM imaging of the surface of ^FSi-SPs after incubation for 70 days (inset in Figure 3e) showed the appearance of the underlying primary silica particles, which also appeared fragmented, thus confirming the degradability of the fibrin coating. This was further confirmed by measuring the amount of fibrin remaining on ^FSi-SPs (Figure S14)—a near-complete degradation of fibrin was evident after incubation for 42 days. At 28 days, which was the time needed to release 60% of the loaded FITC-lysozyme, only about 5% of fibrin remained on ^FSi-SPs, after which the drug release profiles of Si-SPs and ^FSi-SPs became comparable (Figure 2a,b).

To quantify the release of Si from SPs, ICP–OES was performed using samples collected at different time points (3, 7, 14, 21, 28, 42, and 70 days) (Figure 3f). After incubation of SPs for 70 days in PBS at 37 °C, approximately 30% of Si was released from both Si-SPs and ^FSi-SPs. The extent of degradation of Si during the first 7 days appeared to be slightly lower for ^FSi-SPs than for Si-SPs, with ~95% of silica remaining after 7 days for ^FSi-SPs compared to ~90% for Si-SPs (Figure S15)—this indicates that the presence of the fibrin coating on the surface of ^FSi-SPs may hinder the degradation of the primary silica particles. After 14 days, ^FSi-SPs and Si-SPs displayed comparable degradation profiles (Figures 3f and S15), as expected—only around less than 25% of fibrin remained on ^FSi-SPs after 14 days (Figure S14) and most of the primary silica particles are therefore directly exposed in PBS (i.e., uncoated).

In Vitro Cytotoxicity. In our previous study,²⁰ we showed, using an alamar Blue live/dead cell assay, that Si-SPs do not cause cytotoxicity to brain-derived U87MG glioblastoma cells after exposure to the cells for 48 h. However, as Si-SPs and ^FSi-SPs are envisaged for use as drug depots for long-term, sustained drug release (>110 days), additional cytotoxicity experiments were undertaken herein to evaluate the release of any toxic components from Si-SPs and ^FSi-SPs over longer timescales. The in vitro cytotoxicity of the degradation products of ^FSi-SPs was examined using U87MG cells. The supernatant containing

fragmented Si and fibrin coating was collected over 77 days from 2, 5, 10, or 15 ^FSi-SPs and added directly to cells, and the viability of the cells was measured after incubation for 2 days using the XTT assay (Figure 4). No cytotoxicity arising from the

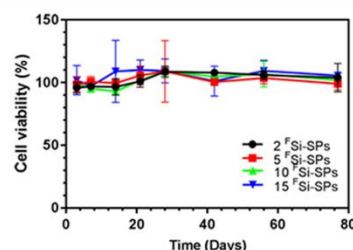


Figure 4. Cell viability of human brain glioblastoma cells after exposure for 48 h to cell media containing degradation products from 2, 5, 10, or 15 ^FSi-SPs; ^FSi-SPs were incubated in cell media for up to 77 days under sterile conditions. Each data point represents the average of triplicate wells with error bars, indicating the standard deviation. Untreated cells (i.e., cells exposed to standard culture media only) were used to define 100% viability.

degradation products of ^FSi-SPs was observed even as the number of ^FSi-SPs was increased from 2 to 15. Similar results were observed for Si-SPs (Figure S16). These results support the biocompatibility of Si and fibrin and their corresponding breakdown products, and although the brain-derived U87MG cell line is a cancer cell line, the results are consistent with our previous study that demonstrated the biocompatibility of silica-based SPs in the cochlea of guinea pigs.³¹

In Vitro Drug Release of ^FSi-SPs Encapsulated in an Alg-CaCO₃ Hydrogel. Hydrogel scaffolds, such as chitosan-glycerophosphate hydrogel,⁵⁰ gel foam,⁵¹ and peptide hydrogels,⁵² are commonly used as drug delivery systems for potential treatment of middle ear or inner ear diseases to aid in the local retention of drug within the ear and increase drug residence time,⁵³ as well as to control pharmacokinetics in the ear.^{51,54} ^FSi-SPs embedded in a hydrogel scaffold may provide additional control of neurotrophin release, as well as aid in the surgical implantation and local retention of neurotrophin-loaded SPs in the target location.²⁰ In the present study, a hydrogel system formed by cross-linking alginate and Ca²⁺ was investigated as an approach to achieve additional control of the release of protein therapeutics from Si-SPs. Alginate is a natural polysaccharide and has been shown to be biocompatible in the inner ear over long durations of implantation.⁵⁵ In the present work, porous CaCO₃ particles (~1.8 μm diameter) served as a source of Ca²⁺ to cross-link alginate into a hydrogel; cross-linking is initiated upon addition of GDL, a common food additive, which releases Ca²⁺ from CaCO₃ particles. By administering drug-loaded Si-SPs in a liquid mixture containing alginate and CaCO₃ particles, followed closely by the addition of GDL to solidify the hydrogel into place, a triggered hydrogel-based controlled drug release platform can be realized. Owing to the high surface area of the porous CaCO₃ particles and the mild hydrolysis conditions associated with GDL, gelation occurred within ~2 min following addition of GDL (Figure S17). SEM images revealed the porous structure of the resulting hydrogel material (Figure 5).

FITC-lysozyme-loaded ^FSi-SPs were encapsulated within the alg-CaCO₃ hydrogel, and the release of FITC-lysozyme when incubated in PBS (pH 7.4) at 37 °C was assessed (Figure Sd,e). The FITC-lysozyme loading values for the SP systems used in

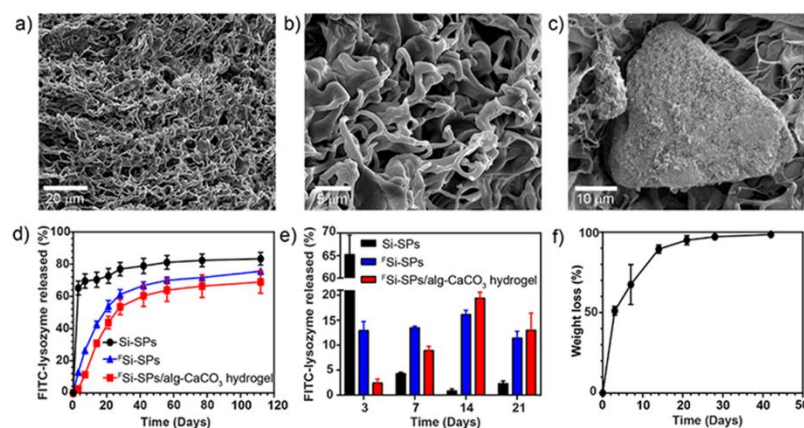


Figure 5. SEM images of the cross section of the lyophilized alg-CaCO₃ hydrogel (synthesized using 2 wt % alginate solution and 1.5 mg of CaCO₃ particles) at (a) low and (b) high magnification. (c) SEM image of a fragment of ¹⁹F-Si-SPs within the lyophilized alg-CaCO₃ hydrogel. (d) In vitro cumulative FITC-lysozyme release profiles of Si-SPs, ¹⁹F-Si-SPs, and ¹⁹F-Si-SPs/alg-CaCO₃ hydrogel. (e) Percentage of FITC-lysozyme released (noncumulative) from Si-SPs, ¹⁹F-Si-SPs, and ¹⁹F-Si-SPs/alg-CaCO₃ hydrogel at a given time point within the first 21 days. (f) Degradation of the alg-CaCO₃ hydrogel in PBS (pH 7.4) at 37 °C monitored over 42 days. Data presented are averages of triplicates with error bars representing standard deviations.

this study (Si-SPs, ¹⁹F-Si-SPs, and ¹⁹F-Si-SPs encapsulated in the hydrogel or ¹⁹F-Si-SPs/alg-CaCO₃ hydrogel) are shown in Figure S18. As demonstrated earlier, burst release can be delayed by applying a fibrin coating on Si-SPs, and Figure 5d shows that it can be delayed further in the presence of a hydrogel, with ~60% of FITC-lysozyme released over 40 days. The difference in the amount of FITC-lysozyme released is more pronounced over the first 7 days—~25% of FITC-lysozyme had been released from free ¹⁹F-Si-SPs compared with ~12% released from ¹⁹F-Si-SPs embedded in the hydrogel. After 7 days, the release rates for samples with or without a hydrogel (compare red and blue bars in Figure 5e) appear to be similar. This can be explained by the degradation of the hydrogel, which was >80% degraded by day 14 (Figure 5f). Individual FITC-lysozyme release data (percentage release) for ¹⁹F-Si-SPs and ¹⁹F-Si-SPs/alg-CaCO₃ hydrogel are shown in Figure S19. The observed reduction in release rate (Figure 5d) is in contrast to our previous report where the drug release rate was accelerated by using a poloxamer-based hydrogel system.²⁰ These results demonstrate that different hydrogel systems can be used to either accelerate or delay protein release from loaded Si-SPs. Furthermore, the in vitro release profiles of Si-SPs/alg-CaCO₃ hydrogels were investigated using varying amounts of CaCO₃ particles and concentrations of alginate solution (Figure S20). The protein loading values are shown in Figure S21. No large differences in the FITC-lysozyme release profile from the different Si-SP platforms examined were observed (Figure S20). Taken together, these results show that an alg-CaCO₃ hydrogel system, wherein gelation is triggered by the addition of GDL, can encapsulate drug-loaded Si-SPs with robust release profiles. The intended use is as simple as a two-component, “mix-and-inject” system where a clinician or surgeon can place the drug delivery system at the intended location, such as in the middle ear or inner ear.

CONCLUSIONS

We demonstrated the engineering of ¹⁹F-Si-SPs with a high loading capacity (~7 μg per SP; ~140 μg mg⁻¹) that can act as a long-term drug delivery system with high biocompatibility and

degradability and improved drug release performance. A bioinspired coating approach involving the endogenous protein fibrin (enzymatically converted from fibrinogen) was used to considerably reduce the initial rapid release (i.e., burst release) observed for Si-SPs to overcome the problem of rapid initial loss of drug from the reservoir and potential side effects associated with high initial dosing. The uncoated Si-SPs released >60% of the cargo protein in the first 3 days, whereas release from ¹⁹F-Si-SPs occurred over a longer period of >20 days, representing a >6-fold improvement. The fibrin coating is biodegradable (<25% of coating remained after 20 days) and sustained release of the encapsulated protein from ¹⁹F-Si-SPs was observed over 110 days. The release behavior of Si-SPs could be modified further by encapsulating the drug-loaded ¹⁹F-Si-SPs into an alg-CaCO₃ hydrogel—encapsulation into a hydrogel resulted in reduced initial release of the loaded protein, while also enabling future clinical use by facilitating SP localization and retention. Interestingly, the alg-CaCO₃ hydrogel system presented here has an opposite effect on the SP release rate (i.e., retards instead of accelerates) when compared with our previous report using poloxamer-based hydrogels.²⁰ Our results provide further evidence of the versatility of SP drug delivery systems and present strategies (biocoatings and hydrogel platforms, of which the gelation can be triggered by external stimuli) to tailor the drug release profiles of Si-SPs to facilitate their future potential clinical application in long-term, protein drug delivery.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.9b00710.

SEM images of CaCO₃ particles; calibration curves for FITC-lysozyme, BDNF, and NT-3 concentration determination; calibration curves for silicon concentration determination; FITC-lysozyme, BDNF, and NT-3 release percentage data from Si-SPs or ¹⁹F-Si-SPs at individual time points; FITC-lysozyme release percentage data from ¹⁹F-Si-

SPs/alg-CaCO₃ hydrogel at individual time points; FITC-lysozyme-loading amount in Si-SPs before coating with different concentrations of fibrinogen or thrombin; in vitro fibrin degradation curve; SEM images of the degradation of Si-SPs; in vitro Si degradation of Si-SPs and ¹⁸F-Si-SPs; cell viability of Si-SPs over 77 days; photographs of the formation of the alg-CaCO₃ hydrogel; FITC-lysozyme loading amounts in different SPs; in vitro FITC-lysozyme release profiles of Si-SPs and ¹⁸F-Si-SPs prepared by incorporation in the alg-CaCO₃ hydrogel at varying concentrations of alginate or amounts of CaCO₃ particles; AFM characterization of the thickness and roughness of fibrin coating; and MIRIBEL checklist including material characterization, biological characterization, and experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Engineering Biocoatings to Prolong Drug Release from Supraparticles

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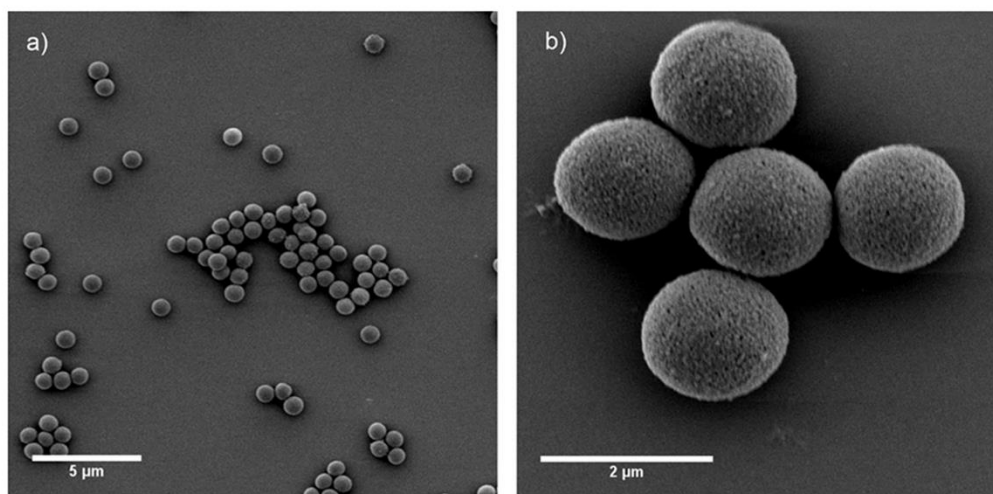


Figure S1. SEM images of CaCO_3 particles at (a) low and (b) high magnification.

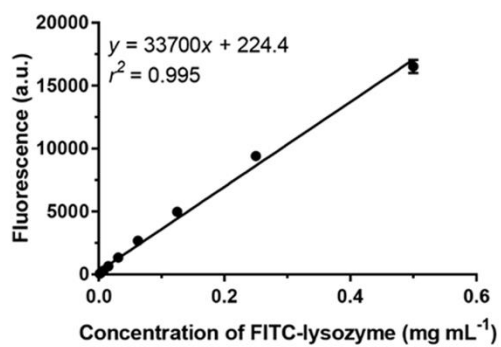


Figure S2. Drug release standard curve for *in vitro* FITC-lysozyme release profiles of FSi-SPs presented in Figure 2a, b.

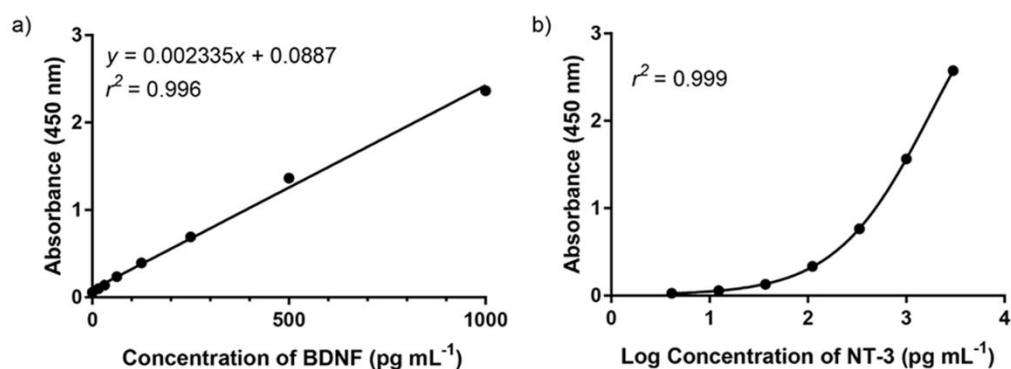


Figure S3. *In vitro* release standard curve for (a) BDNF or (b) NT-3 in the ELISA assay.

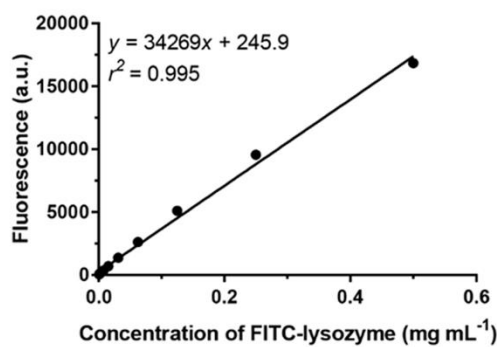


Figure S4. Drug release standard curve for *in vitro* FITC-lysozyme release profile of ^FSi-SPs/alg-CaCO₃ hydrogel.

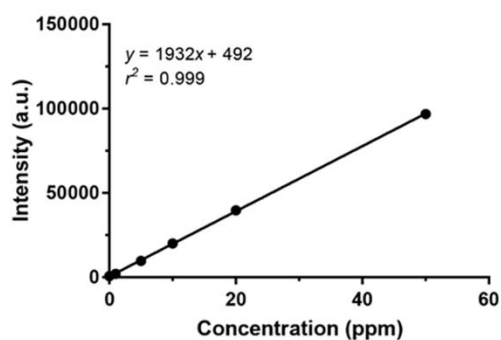


Figure S5. ICP silicon standard curve for Si concentration determination.

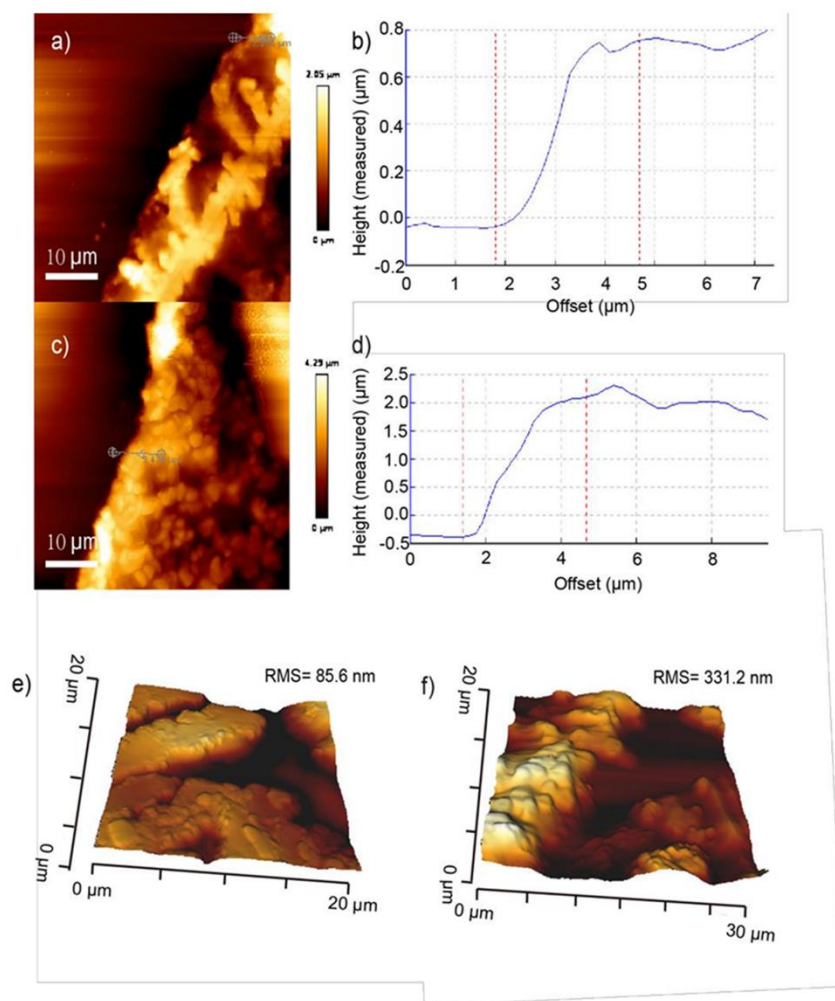


Figure S6. AFM characterization of the thickness of the (a, b) fibrinogen coating and (c, d) resulting fibrin coating after incubation with thrombin. Height measurements are shown in (b) and (d) at the corresponding point at the edge of a scalpel-scratched film. The surface roughness of (e) fibrinogen and (f) fibrin was also characterized by AFM.

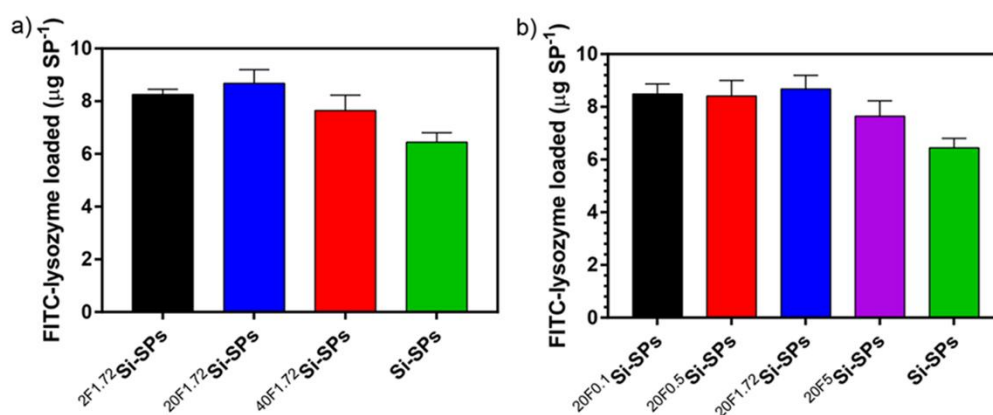


Figure S7. Amount of FITC-lysozyme loaded in individual Si-SPs before coating with different concentrations of (a) fibrinogen or (b) thrombin.

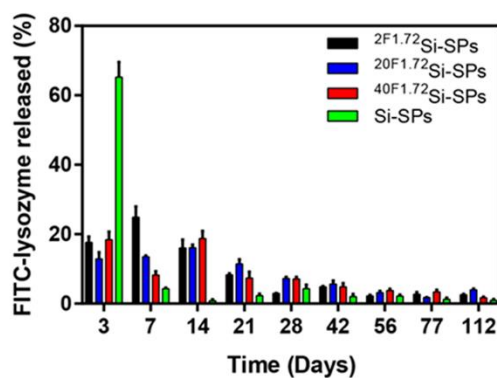


Figure S8. Percentage of FITC-lysozyme released from Si-SPs and different ^FSi-SPs at given time points. Different fibrin coatings were formed by varying the concentration of fibrinogen (2, 20, and 40 mg mL⁻¹), while keeping the concentration of thrombin constant at 1.72 mg mL⁻¹. Samples are denoted as ^{2F1.72}Si-SPs, ^{20F1.72}Si-SPs, and ^{40F1.72}Si-SPs, respectively. Data presented are averages of triplicates with error bars indicating standard deviations, each using 10 SPs.

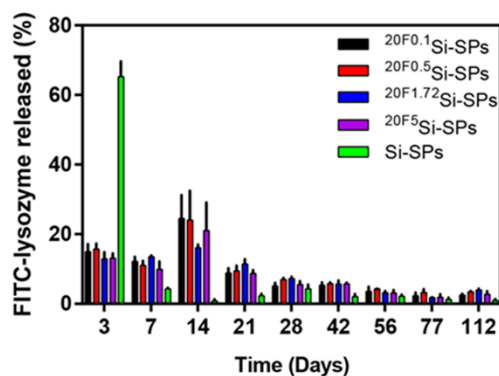


Figure S9. Percentage of FITC-lysozyme released from Si-SPs and different ^FSi-SPs at given time points. Different fibrin coatings were formed by varying the concentration of thrombin (0.1, 0.5, 1.72, and 5 mg mL⁻¹), while keeping the concentration of fibrinogen fixed at 20 mg mL⁻¹. Samples are denoted as ^{20F0.1}Si-SPs, ^{20F0.5}Si-SPs, ^{20F1.72}Si-SPs, and ^{20F5}Si-SPs, respectively. Data presented are averages of triplicates with error bars indicating standard deviations, each using 10 SPs.

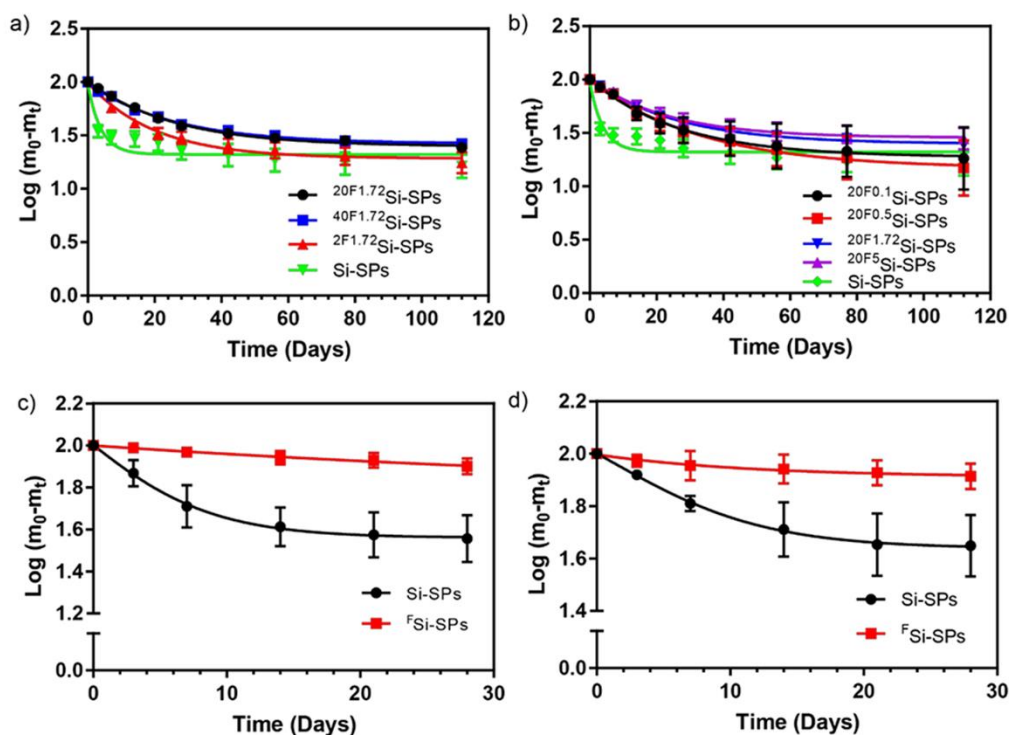


Figure S10. First order protein release profiles of (a) FITC-lysozyme release from Si-SPs and $^{\text{F}}\text{Si-SPs}$ (fibrin coating was obtained at varying concentrations of fibrinogen (2, 20, and 40 mg mL^{-1}), while maintaining the concentration of thrombin at 1.72 mg mL^{-1} ; samples are respectively denoted as $^{2}\text{F}^{1.72}\text{Si-SPs}$, $^{20}\text{F}^{1.72}\text{Si-SPs}$, and $^{40}\text{F}^{1.72}\text{Si-SPs}$). (b) FITC-lysozyme release from Si-SPs and $^{\text{F}}\text{Si-SPs}$ (fibrin coating was obtained at varying concentrations of thrombin (0.1, 0.5, 1.72, and 5 mg mL^{-1}), while keeping the concentration of fibrinogen constant at 20 mg mL^{-1} ; samples are respectively denoted as $^{20}\text{F}^{0.1}\text{Si-SPs}$, $^{20}\text{F}^{0.5}\text{Si-SPs}$, $^{20}\text{F}^{1.72}\text{Si-SPs}$, and $^{20}\text{F}^5\text{Si-SPs}$). (c) BDNF and (d) NT-3 released from Si-SPs or $^{\text{F}}\text{Si-SPs}$. m_0 is the amount of drug loaded in SPs ($\mu\text{g SP}^{-1}$) and m_t is the amount of drug loaded in SPs ($\mu\text{g SP}^{-1}$) at time t (days).

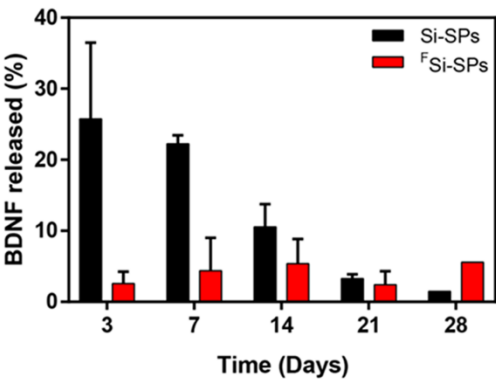


Figure S11. Percentage of BDNF released from Si-SPs and ^FSi-SPs at given time points, as measured using ELISA. Data presented are averages of triplicates with error bars indicating standard deviations, with each triplicate using 1 SP.

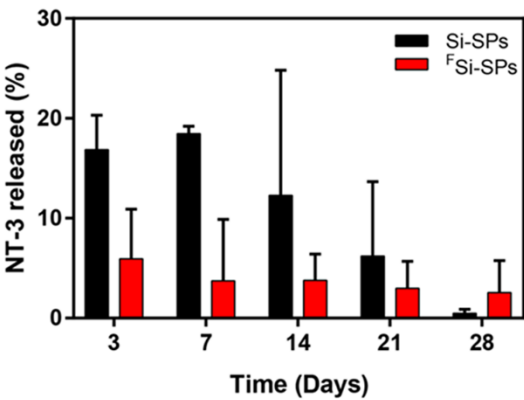


Figure S12. Percentage of NT-3 released from Si-SPs and ^FSi-SPs at given time points, as measured using ELISA. Data presented are averages of triplicates with error bars indicating standard deviations, with each triplicate using 1 SP.

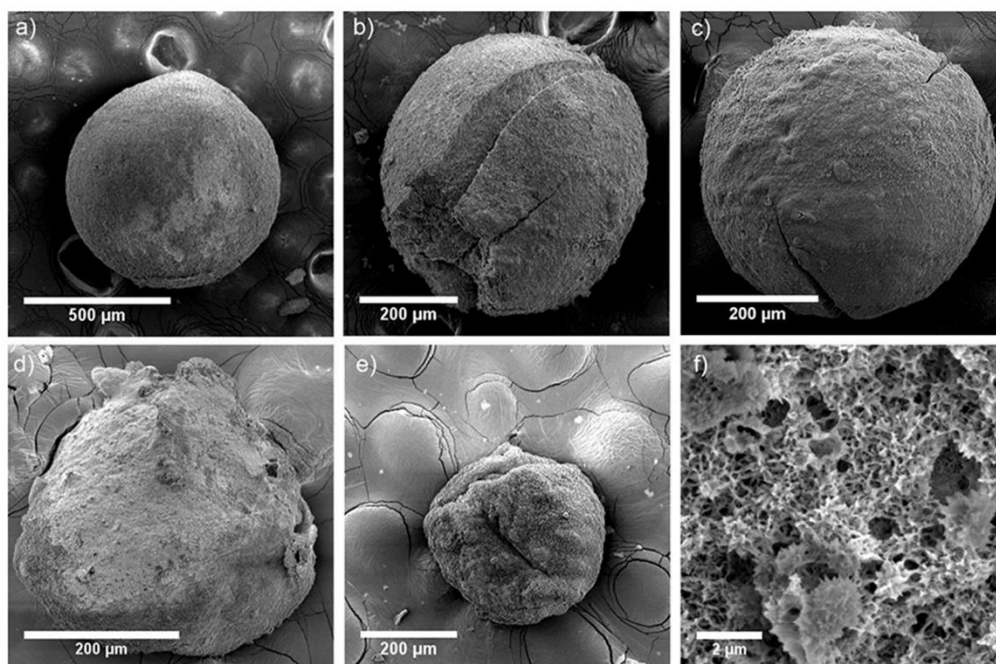


Figure S13. SEM images of Si-SPs after incubation in PBS (pH 7.4) at 37 °C for (a) 3, and (b) 14, (c) 21, (d) 42, and (e) 70 days. (f) SEM image of the surface structure of Si-SPs after incubation in PBS (pH 7.4) at 37 °C for 10 weeks. Comparison of Figure 1b and Figure S13f reveals drastic changes in the size and morphology of primary silica particles on the surface of the SPs.

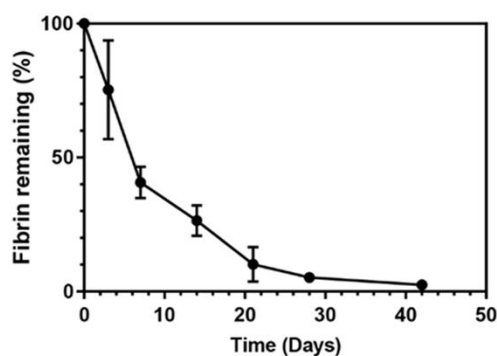


Figure S14. *In vitro* cumulative percentage degradation of fibrin from ^FSi-SPs (20 mg mL⁻¹ fibrinogen and 1.72 mg mL⁻¹ was used for fibrin coating). The amount of fibrin was measured using a Micro BCA protein assay kit. Data presented are averages of triplicates with error bars indicating standard deviations, each using 10 SPs.

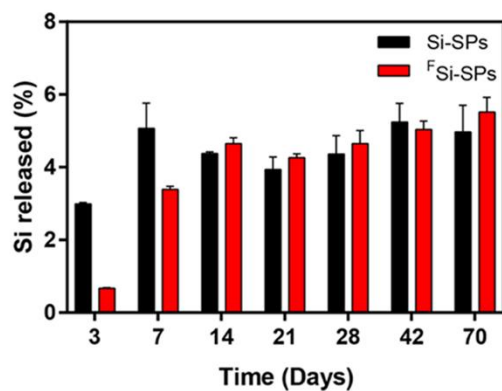


Figure S15. Measured percentage release of Si at given time points. The data presented here were used to plot the data in Figure 3f.

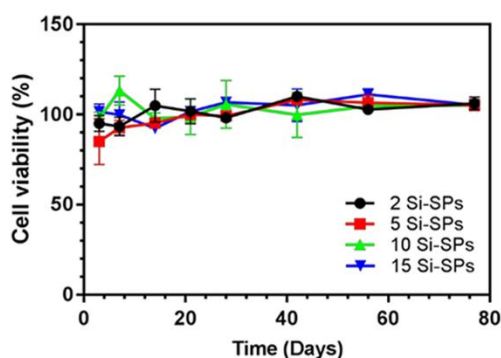


Figure S16. Viability of U87MG human brain glioblastoma cells exposed to cell media containing degradation products from 2, 5, 10, or 15 SPs. The SPs were incubated in cell media for varying amounts of time at 37 °C. Each data point represents the average of triplicates with error bars indicating the standard deviation. Untreated cells (i.e., cells exposed to standard culture media only) were used to define 100% viability.

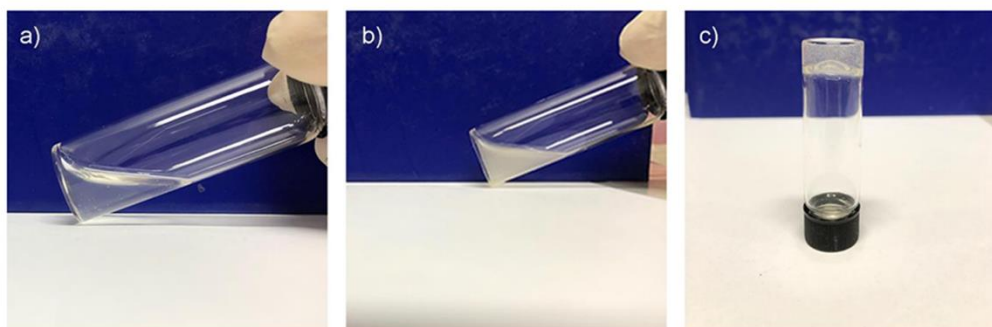


Figure S17. Formation of alg- CaCO_3 hydrogel. Photographs of (a) 2 wt% alginate solution, (b) 2 wt% alginate solution mixed with CaCO_3 particles, and (c) hydrogel formed after addition of D-glucono- δ -lactone (hydrogel formation occurred within ~2 min).

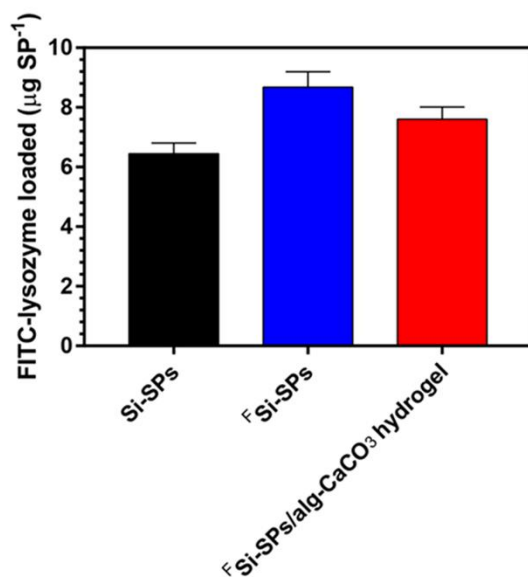


Figure S18. Amount of FITC-lysozyme loaded in individual Si-SPs, $^{\text{F}}$ Si-SPs, and $^{\text{F}}$ Si-SPs/alg- CaCO_3 hydrogel. Associated release profiles are shown in Figure 5.

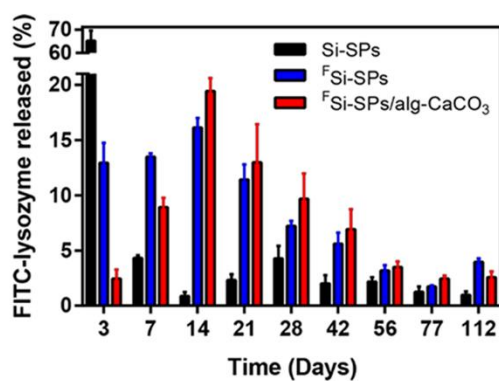


Figure S19. Percentage of FITC-lysozyme released from Si-SPs, $^{\text{F}}$ Si-SPs, and $^{\text{F}}$ Si-SPs/alg- CaCO_3 hydrogel.

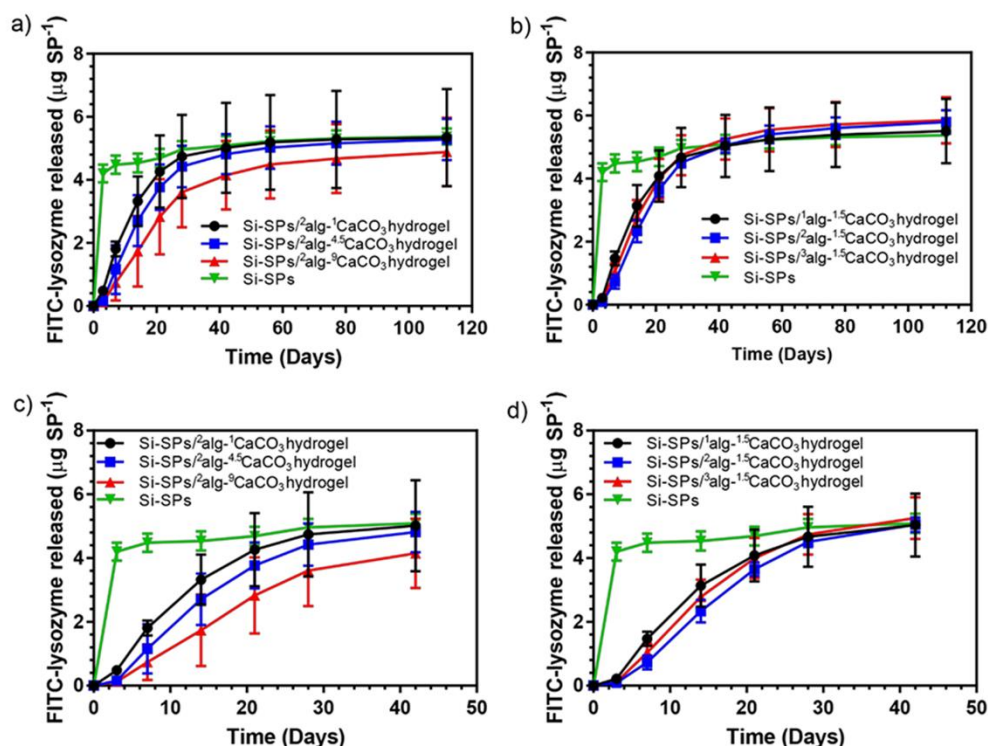


Figure S20. (a) *In vitro* FITC-lysozyme release profiles of Si-SPs@alg-CaCO₃ hydrogels prepared using 2 wt% alginate solution and varying amounts of CaCO₃ particles (1, 4.5, or 9 mg); the samples are denoted as Si-SPs/²alg-¹CaCO₃ hydrogel, Si-SPs/²alg-^{4.5}CaCO₃ hydrogel, and Si-SPs/²alg-⁹CaCO₃ hydrogel, respectively. The green line represents the *in vitro* release profile of free Si-SPs (i.e., not embedded within a hydrogel matrix). (b) *In vitro* FITC-lysozyme release profiles of Si-SPs/alg-CaCO₃ hydrogels prepared using 1.5 mg of CaCO₃ and varying concentrations of alginate solution (1, 2, or 3 wt%). The samples are denoted as Si-SPs/¹alg-^{1.5}CaCO₃ hydrogel, Si-SPs/²alg-^{1.5}CaCO₃ hydrogel, and Si-SPs/³alg-^{1.5}CaCO₃ hydrogel, respectively. The green line represents the *in vitro* release profile of free Si-SPs. The *in vitro* drug release profiles focusing on the first 42 days are respectively shown in (c) and (d). Figure S20c, d shows that the alg-CaCO₃ hydrogel can delay the burst release of FITC-lysozyme from Si-SPs to 28 days.

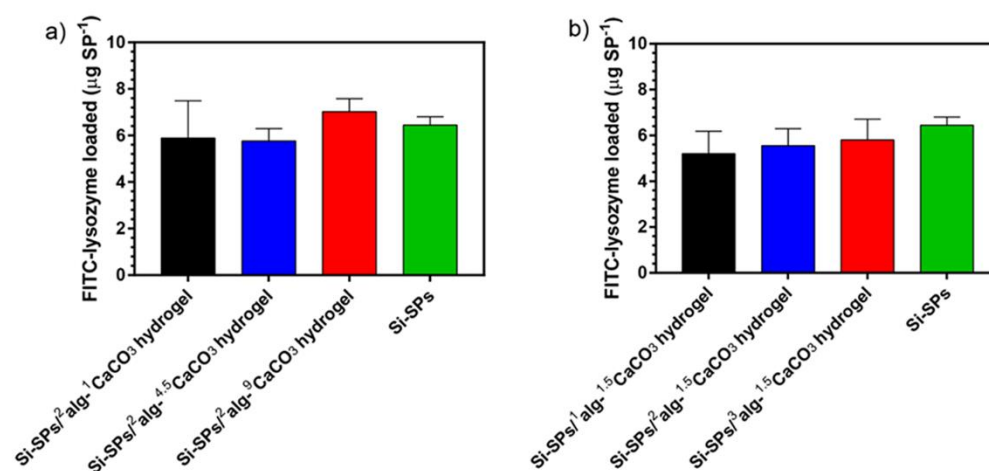


Figure S21. FITC-lysozyme loading amounts in different Si-SPs. Associated release profiles are shown in Figure S20.

Table S1. Particle systems used in this study (see Scheme 1 for graphical overview of the preparation procedure)

Name	Description
SP	supraparticle, assembly of primary silica particles
Si-SPs	silica supraparticle
^F Si-SPs	fibrin-coated silica supraparticles
^{xFy} Si-SPs	fibrin-coated silica supraparticles prepared with $x \text{ mg mL}^{-1}$ fibrinogen and $y \text{ mg mL}^{-1}$ thrombin
alg-CaCO ₃	Hydrogel used for encapsulating the SPs; hydrogel is obtained by gradual dissolution of CaCO ₃ particles

Checklist

Minimum Information Reporting in Bio–Nano Experimental Literature

The MIRIBEL guidelines were introduced here: <https://doi.org/10.1038/s41565-018-0246-4>

The development of these guidelines was led by the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology: <https://www.cbns.org.au/>. Any updates or revisions to this document will be made available here: <http://doi.org/10.17605/OSF.IO/SMVTF>. This document is made available under a CC-BY 4.0 license: <https://creativecommons.org/licenses/by/4.0/>.

The MIRIBEL guidelines were developed to facilitate reporting and dissemination of research in bio–nano science. Their development was inspired by various similar efforts:

- MIAME (microarray experiments): *Nat. Genet.* **29** (2001), 365; <http://doi.org/10.1038/ng1201-365>
- MIRIAM (biochemical models): *Nat. Biotechnol.* **23** (2005) 1509; <http://doi.org/10.1038/nbt1156>
- MIBBI (biology/biomedicine): *Nat. Biotechnol.* **26** (2008) 889; <http://doi.org/10.1038/nbt.1411>
- MIGS (genome sequencing): *Nat. Biotechnol.* **26** (2008) 541; <http://doi.org/10.1038/nbt1360>
- MIQE (quantitative PCR): *Clin. Chem.* **55** (2009) 611; <http://doi.org/10.1373/clinchem.2008.112797>
- ARRIVE (animal research): *PLOS Biol.* **8** (2010) e1000412; <http://doi.org/10.1371/journal.pbio.1000412>
- *Nature*'s reporting standards:
 - Life science: <https://www.nature.com/authors/policies/reporting.pdf>; e.g., *Nat. Nanotechnol.* **9** (2014) 949; <http://doi.org/10.1038/nnano.2014.287>
 - Solar cells: <https://www.nature.com/authors/policies/solarchecklist.pdf>; e.g., *Nat. Photonics* **9** (2015) 703; <http://doi.org/10.1038/nphoton.2015.233>
 - Lasers: <https://www.nature.com/authors/policies/laserchecklist.pdf>; e.g., *Nat. Photonics* **11** (2017) 139; <http://doi.org/10.1038/nphoton.2017.28>
- The “TOP guidelines”: e.g., *Science* **352** (2016) 1147; <http://doi.org/10.1126/science.aag2359>

Similar to many of the efforts listed above, the parameters included in this checklist are **not** intended to be definitive requirements; instead they are intended as ‘points to be considered’, with authors themselves deciding which parameters are—and which are not—appropriate for their specific study.

This document is intended to be a living document, which we propose is revisited and amended annually by interested members of the community, who are encouraged to contact the authors of this document. Parts of this document were developed at the annual International Nanomedicine Conference in Sydney, Australia: <http://www.oznanomed.org/>, which will continue to act as a venue for their review and development, and interested members of the community are encouraged to attend.

After filling out the following pages, this checklist document can be attached as a “Supporting Information” document during submission of a manuscript to inform Editors and Reviewers (and eventually readers) that all points of MIRIBEL have been considered.

Supplementary Table 1. Material characterization*

Question	Yes	No
1.1 Are “ best reporting practices ” available for the nanomaterial used? For examples, see <i>Chem. Mater.</i> 28 (2016) 3535; http://doi.org/10.1021/acs.chemmater.6b01854 and <i>Chem. Mater.</i> 29 (2017) 1; http://doi.org/10.1021/acs.chemmater.6b05235		X
1.2 If they are available, are they used ? If not available, ignore this question and proceed to the next one.		
1.3 Are extensive and clear instructions reported detailing all steps of synthesis and the resulting composition of the nanomaterial? For examples, see <i>Chem. Mater.</i> 26 (2014) 1765; http://doi.org/10.1021/cm500632c , and <i>Chem. Mater.</i> 26 (2014) 2211; http://doi.org/10.1021/cm5010449 . Extensive use of photos, images, and videos are strongly encouraged. For example, see <i>Chem. Mater.</i> 28 (2016) 8441; http://doi.org/10.1021/acs.chemmater.6b04639	X	
1.4 Is the size (or dimensions , if non-spherical) and shape of the nanomaterial reported?	X	
1.5 Is the size dispersity or aggregation of the nanomaterial reported?	X	
1.6 Is the zeta potential of the nanomaterial reported?		Reported in original study (see Ma et al. <i>ACS Appl. Mater. Interfaces</i> 2018 , <i>10</i> , 31019)
1.7 Is the density (mass/volume) of the nanomaterial reported?	X	
1.8 Is the amount of any drug loaded reported? ‘Drug’ here broadly refers to functional cargos (e.g., proteins, small molecules, nucleic acids).	X	
1.9 Is the targeting performance of the nanomaterial reported, including amount of ligand bound to the nanomaterial if the material has been functionalised through addition of targeting ligands?		not applicable
1.10 Is the label signal per nanomaterial/particle reported? For example, fluorescence signal per particle for fluorescently labelled nanomaterials.		not applicable
1.11 If a material property not listed here is varied, has it been quantified ?		not applicable

1.12 Were characterizations performed in a fluid mimicking biological conditions ?	X (PBS and cell culture media)	
1.13 Are details of how these parameters were measured/estimated provided?	X	
Explanation for No (if needed):		

*Ideally, material characterization should be performed in the same biological environment as that in which the study will be conducted. For example, for cell culture studies with nanoparticles, characterization steps would ideally be performed on nanoparticles dispersed in cell culture media. If this is not possible, then characteristics of the dispersant used (e.g., pH, ionic strength) should mimic as much as possible the biological environment being studied.

Supplementary Table 2. Biological characterization*

Question	Yes	No
2.1 Are cell seeding details , including number of cells plated , confluency at start of experiment , and time between seeding and experiment reported?	X	
2.2 If a standardised cell line is used, are the designation and source provided?	X	
2.3 Is the passage number (total number of times a cell culture has been subcultured) known and reported?		X
2.4 Is the last instance of verification of cell line reported? If no verification has been performed, is the time passed and passage number since acquisition from trusted source (e.g., ATCC or ECACC) reported? For information, see <i>Science</i> 347 (2015) 938; http://doi.org/10.1126/science.347.6225.938		X
2.5 Are the results from mycoplasma testing of cell cultures reported?	X	
2.6 Is the background signal of cells/tissue reported? (E.g., the fluorescence signal of cells without particles in the case of a flow cytometry experiment.)		not applicable
2.7 Are toxicity studies provided to demonstrate that the material has the expected toxicity, and that the experimental protocol followed does not?	X	
2.8 Are details of media preparation (type of media , serum , any added antibiotics) provided?	X	
2.9 Is a justification of the biological model used provided? For examples for cancer models, see <i>Cancer Res.</i> 75 (2015) 4016; http://doi.org/10.1158/0008-5472.CAN-15-1558 , and <i>Mol. Ther.</i> 20 (2012) 882; http://doi.org/10.1038/mt.2012.73 , and <i>ACS Nano</i> 11 (2017) 9594; http://doi.org/10.1021/acsnano.7b04855	X	
2.10 Is characterization of the biological fluid (<i>ex vivo/in vitro</i>) reported? For example, when investigating protein adsorption onto nanoparticles dispersed in blood serum, pertinent aspects of the blood serum should be characterised (e.g., protein concentrations and differences between donors used in study).		not applicable
2.11 For animal experiments , are the ARRIVE guidelines followed? For details, see <i>PLOS Biol.</i> 8 (2010) e1000412; http://doi.org/10.1371/journal.pbio.1000412		not applicable
Explanation for No (if needed):		

*For *in vitro* experiments (e.g., cell culture), *ex vivo* experiments (e.g., in blood samples), and *in vivo* experiments (e.g., animal models). The questions above that are appropriate depend on the type of experiment conducted.

Supplementary Table 3. Experimental details*

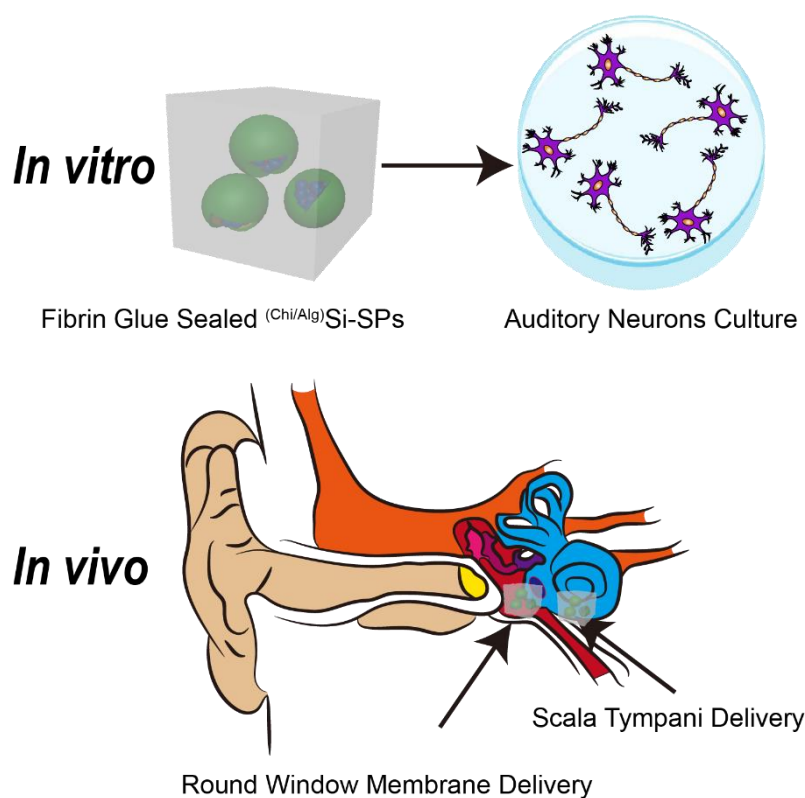
Question	Yes	No
3.1 For cell culture experiments: are cell culture dimensions including type of well , volume of added media , reported? Are cell types (i.e.; adherent vs suspension) and orientation (if non-standard) reported?	X	
3.2 Is the dose of material administered reported? This is typically provided in nanomaterial mass, volume, number, or surface area added. Is sufficient information reported so that regardless of which one is provided, the other dosage metrics can be calculated (i.e. using the dimensions and density of the nanomaterial)?	X	
3.3 For each type of imaging performed, are details of how imaging was performed provided, including details of shielding , non-uniform image processing , and any contrast agents added?	X	
3.4 Are details of how the dose was administered provided, including method of administration , injection location , rate of administration , and details of multiple injections ?		not applicable
3.5 Is the methodology used to equalise dosage provided?		not applicable
3.6 Is the delivered dose to tissues and/or organs (in vivo) reported, as % injected dose per gram of tissue (%ID g ⁻¹)?		not applicable
3.7 Is mass of each organ/tissue measured and mass of material reported?		not applicable
3.8 Are the signals of cells/tissues with nanomaterials reported? For instance, for fluorescently labelled nanoparticles, the total number of particles per cell or the fluorescence intensity of particles + cells, at each assessed timepoint.		not applicable
3.9 Are data analysis details , including code used for analysis provided?		Data analysis details provided, but not code.
3.10 Is the raw data or distribution of values underlying the reported results provided? For examples, see <i>R. Soc. Open Sci.</i> 3 (2016) 150547; http://doi.org/10.1098/rsos.150547 , https://opennessinitiative.org/making-your-data-public/ , http://journals.plos.org/plosone/s/data-availability , and https://www.nature.com/sdata/policies/repositories		X

Explanation for **No** (if needed):

* The use of protocol repositories (e.g., *Protocol Exchange* <http://www.nature.com/protocolexchange/>) and published standard methods and protocols (e.g., *Chem. Mater.* **29** (2017) 1; <http://doi.org/10.1021/acs.chemmater.6b05235>, and *Chem. Mater.* **29** (2017) 475; <http://doi.org/10.1021/acs.chemmater.6b05481>) are encouraged.

Chapter 6

Layer-by-Layer Coating of Silica Supraparticles for Controlled Drug Delivery to the Inner Ear



A manuscript with the results presented in this chapter is being prepared to be submitted for publication.

6.1 SUMMARY

In this Chapter, a bioengineering coating on silica supraparticles (Si-SPs) obtained via a layer-by-layer technique is presented to slow the initial burst release of the uncoated Si-SPs. The *in vitro* release behaviour can be tuned by adjusting the thickness of the coating layers. A new surgical strategy is also developed using a clinically approved gelation system comprising thrombin and fibronectin; this strategy affords the successful implantation of the supraparticles (SPs) on the round window membrane (RWM). Moreover, the bioactivity of the released neurotrophins was studied using spiral ganglion neurons (SGNs) culture and neurite length analysis. Functional hearing after SP implantation was also evaluated by recording auditory brainstem responses (ABRs) in deafened cats. These studies indicate that Si-SPs with bioengineered coatings can serve as promising candidates for the delivery of neurotrophins for treating sensorineural hearing loss.

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6.2 INTRODUCTION

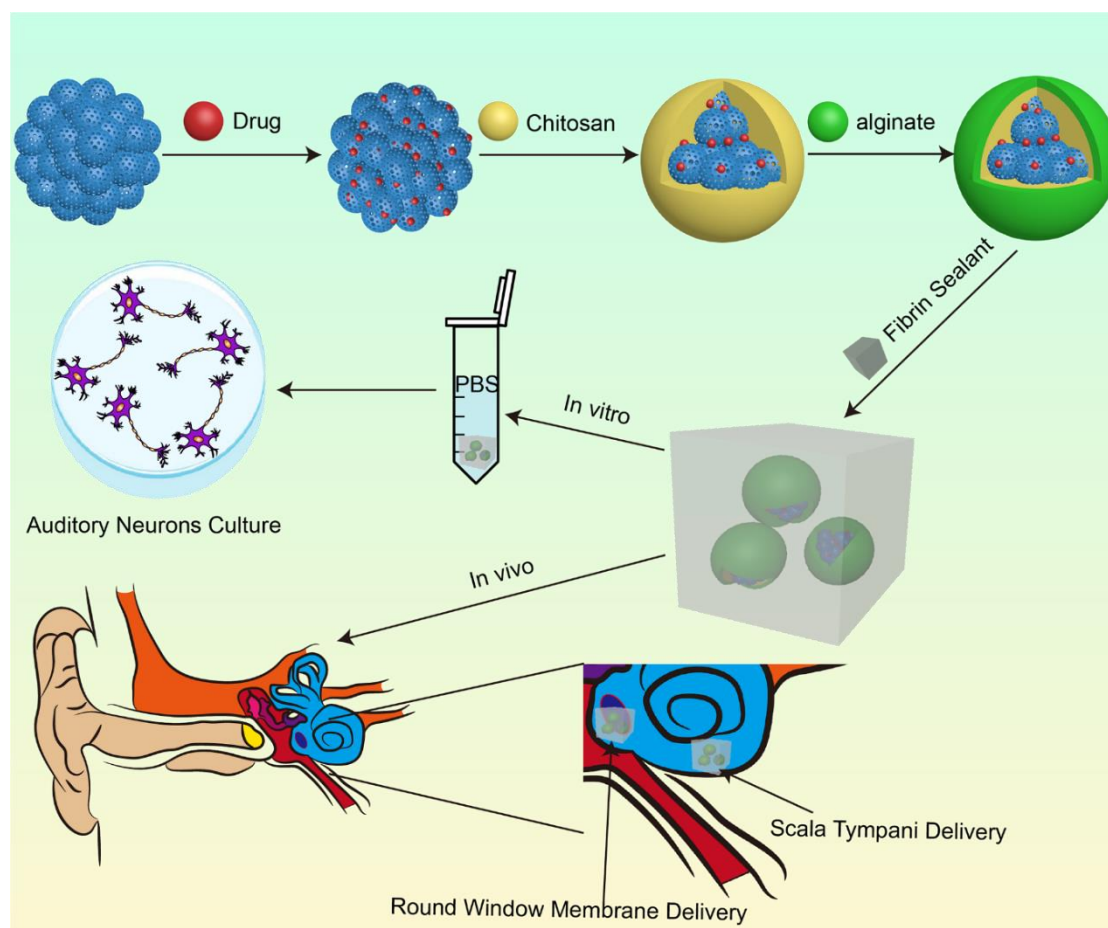
Sensorineural hearing loss, mainly caused by degeneration of sensory pathway in the cochlea, including the hair cells, the primary auditory neurons and the ribbon synapse, is the leading cause of hearing impairment in developed countries.¹⁻³ Exposure to intense noise, viral infections, inherited and autoimmune disorders, or old age, can damage the auditory neuronal cells in the cochlea, resulting in hearing impairment.^{1,4} Neurotrophins, which are naturally occurring proteins expressed by certain types of sensory neurons, including hair cells and supporting cells,^{5,6} have been verified as an effective therapeutic against neurodegenerative disorders of hearing loss.⁷ Consequently, several therapeutic approaches based on intratympanic and intracochlear administration route have been developed to treat impaired sensory neuronal cells in the cochlea efficiently and directly, including hydrogel,⁸ mini-osmotic pumps,⁹ viral vectors,^{10,11} modified stem cells,^{12,13} and nanoparticles.¹⁴ These drug delivery systems can serve as carriers to provide exogenous neurotrophins to improve the survival of sensory neurons.^{7,15} However, most of these drug delivery techniques can only achieve short-term neurotrophin delivery. The use of osmotic pumps is limited by space inside the cochlea, which is circumvented by the use of small-sized nanoparticles or viral vectors but also presents the potential risk of the nanoparticles being delivered through the cochlear aqueduct into the brain and introducing infection with pumping drug in solution into the cochlea, given that the fluids are connected to the cerebrospinal fluid via cochlear aqueduct.^{16,17}

To address the potential risk of nanoparticles being delivered to the brain, large supraparticles (SPs) were developed as a depot for the sustained release of neurotrophins. SP drug delivery systems have been synthesised via evaporation-

induced self-assembly, a mould-templating approach, or gel-mediated electrospray technique. With their large size ($\sim 550\text{ }\mu\text{m}$) and high loading capacity, SPs can address the safety issues from nanoparticle or viral vectors delivery systems, but also achieve long-term drug release over 4 months.¹⁸⁻²⁰ Also, for the inner ear drug delivery, controlling the therapeutic doses of neurotrophins is important because the higher concentration of neurotrophins (due to the initial burst release of drug carriers) might cause off-target toxicity and reduce the effective lifetime of supraparticles.^{21,22}

Herein, Biodegradable chitosan and alginate were employed as a thin film material to coat on SPs to encapsulate neurotrophins or the model protein (lysozyme). Multilayered thin film formation on the SPs was achieved via the layer-by-layer (LbL) technique, where chitosan and alginate were alternately deposited on the surface of pre-loaded SPs. Chitosan and alginate are two biocompatible polysaccharides; the carboxyl group from alginate with a net negative charge can interact electrostatically with amine groups on chitosan, which gives it a net positive charge. By varying the thickness of the coating via the layer number, different release profiles were observed. The *in vitro* cytotoxicity studies based on U87MG human brain glioblastoma cell line and degradation studies demonstrated that the chitosan-alginate coated silica supraparticles ($^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$) are excellent candidates for long-term controlled drug delivery due to their biocompatible and degradable properties. Further, the studies *in vitro* using spiral ganglion neurons (SGNs) culture and by neurite length analysis indicated that the released neurotrophins from $^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$ have higher bioactivities compared to native neurotrophins without loading into SPs. Then $^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$ were implanted with injectable commercial viable fibrin sealant matrix into either the RWM, in which neurotrophins diffuse through the RWM (i.e., the boundary between the middle ear and inner ear) into the cochlea or scala tympani of the cochlea of cats (the overall procedures are schematically illustrated in

Scheme 1). Taken together, our results demonstrate that neurotrophin-loaded $(\text{Chi}/\text{Alg})\text{Si}$ -SPs combined with an injectable and biodegradable fibrin sealant matrix is a promising platform for the clinical treatment of inner ear diseases.



Scheme 1. Schematic illustration showing the drug loading, coating, and *in vitro* and *in vivo* drug release and efficacy of encapsulated neurotrophin. SPs are preloaded with a model protein drug lysozyme or neurotrophin, followed by repeated LbL deposition cycles of chitosan and alginate. The coated SPs, $(\text{Chi}/\text{Alg})\text{Si}$ -SPs, were embedded in a commercial viable fibrin sealant and studied for cargo release in PBS. *In vitro* released neurotrophins were incubated with SGNs for cell culture to determine the bioactivity of released neurotrophins. Finally, $(\text{Chi}/\text{Alg})\text{Si}$ -SPs embedded in fibrin sealant matrix were implanted onto either RWM or scala tympani of cats by a cannula.

6.3 MATERIALS AND METHODS

Materials. Tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), ammonium hydroxide solution (28–30%), poly(acrylic acid) (PAA, $M_w \sim 250$ kDa, 35 wt% aqueous solution), fluorescein isothiocyanate (FITC), lysozyme protein from chicken egg white, alginic acid sodium salt from brown algae (alginate), calcium chloride (CaCl_2), phosphate-buffered saline (PBS, pH 7.4), phenazine methosulfate (PMS), Dulbecco's phosphate-buffered saline (DPBS), silicon standard for the inductively coupled plasma experiments and chitosan with medium molecular weight were purchased from Sigma-Aldrich (USA). Sodium chloride (NaCl) and ethanol were obtained from Chem-Supply (Australia). BDNF and NT-3 were purchased from PeproTech Asia (Israel), recombinant anti-S100 beta antibody (# ab52642) and human BDNF ELISA kits were obtained from Abcam (Australia). The U87MG human brain glioblastoma cell line was purchased from American Type Culture Collection (USA). Biowhittaker Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Lonza (Australia). 2,3-Bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT), NT-3 enzyme-linked immunosorbent assay (ELISA) kits and TrypLE were purchased from Thermo Fisher Scientific (Australia). Polyornithine/laminin coated 8-well culture slides was obtained from Greiner-Bio One (Austria). Primary antibodies Tuj1 (# 801201) was purchased from Biolegend (USA). DAPI was obtained from Vector Laboratories (USA). Ultrapure water (Milli-Q water) with a resistivity of greater than $18 \text{ M}\Omega \text{ cm}$ was used in all experiments and obtained from a three-stage Millipore Milli-Q Plus 185 purification system.

Synthesis of Primary Silica Particles. Primary silica particles with the diameter of $\sim 1 \mu\text{m}$ were synthesised using a modified version of a previously published protocol.²³

Specifically, 1.1 g of CTAB was first dissolved in 50 mL of Milli-Q water followed by adding PAA (4.3g) 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 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 (Jetlow, Australia) and calcined at 550 °C for 30 h to remove the organic materials.

Synthesis of Silica SPs. The silica SPs (Si-SPs) were produced by electrospraying as described previously.¹⁸ Briefly, primary silica particles (80 mg) were added to 2 mL of aqueous alginate solution (30 mg mL⁻¹ 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⁻¹. With an applied voltage of 13 kV, alginate/primary silica particle droplets were electrosprayed into a reservoir of 1% w/v of aqueous CaCl₂ to crosslink the alginate. The distance between the end of the nozzle and the surface of the CaCl₂ solution was set to 10 cm. Subsequently, the alginate/silica SPs (Si-SPs^{alg}) were collected directly from the CaCl₂ bath using a strainer and washed extensively in Milli-Q water (four times). Finally, to obtain Si-SPs, Si-SPs^{alg} were calcinated at 650 °C for 30 h to remove the organic materials (i.e. alginate).

Synthesis of Chitosan-Alginate Layer-by-Layer Silica SPs. The preparation procedure of LbL-coated supraparticles using chitosan and alginate (^(Chi/Alg)Si-SPs) is shown in Scheme 1. Briefly, Si-SPs were sterilised with 100 μ L of 80% (v/v) ethanol at ~ 22 °C for 4 h, followed by rinsing with sterile Milli-Q water six times. Chitosan (50 μ L 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 Si-SPs. After 15 min of incubation, the supernatant was aspirated and the Si-SPs were washed with Milli-Q water 3 times. Then, chitosan-coated Si-SPs were incubated with 50 μ L 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 until the desired number of layers was achieved.

Synthesis of Fluorescein Isothiocyanate (FITC)-Labelled Chitosan. FITC-labelled chitosan (FITC-chitosan) was synthesised using a previously described protocol.²⁴ 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. Scanning electron microscopy (SEM) images of Si-SPs and ^(Chi/Alg)Si-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)Si-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 $\mu\text{L min}^{-1}$ 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)Si-SPs was also characterised using a confocal laser-scanning microscope (Leica, Germany) equipped with an argon laser. Fourier transform infrared (FTIR) spectra of Si-SPs and ^(Chi/Alg)Si-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, Si-SPs and ^(Chi/Alg)Si-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^{-1} .

***In Vitro* ^(Chi/Alg)Si-SPs Degradation Studies.** Si-SPs were first sterilised with 100 μL of ethanol (80% v/v) at ~ 22 °C for 4 h, followed by washing with sterile Milli-Q water four times to wash off excess ethanol. Subsequently, ^(Chi/Alg)Si-SPs were prepared as described above. SP degradation was studied by immersing Si-SPs and ^(Chi/Alg)Si-SPs (10 SPs) per sample in 1 mL of 10 mM PBS (pH 7.4) at 37 °C. At defined time points (over 40 days), 1 mL of the supernatant was removed, kept in the fridge and replaced with 1 mL of fresh PBS. The amount of silica degraded and released from Si-SPs, and

(Chi/Alg)Si-SPs in the supernatant was measured using inductively coupled plasma–optical emission spectroscopy (ICP-OES). For the analysis, 500 μL of 8 M sodium hydroxide was mixed with 500 μL of the collected degradation samples (supernatant) and incubated at 95 °C for 30 min, followed by 30 min of sonication in an ultrasonic water bath. The samples were then placed at 37 °C overnight. 4 mL of Milli-Q water was added to the samples prior to analysis.

In Vitro Cytotoxicity. Si-SPs were sterilised by incubating with 100 μL of ethanol (80% v/v) at ~22 °C. After 4 h, they were washed with sterile Milli-Q water (three times) and cell culture medium (three times). 1 mL of cell culture media (DMEM supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin, 100 mg mL^{-1} streptomycin) was added to varying quantities (2, 5 or 10) of (Chi/Alg)Si-SPs. At defined time points, the cell media was aspirated (and stored at –20 °C for future cell analysis) and replaced with fresh cell media. SPs were handled in a biosafety cabinet.

U87MG human brain-derived glioblastoma cell line was chosen for the cytotoxicity experiment since they are potentially relevant for inner ear delivery. The U87MG cells were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified incubator (37 °C, 5% CO_2). For cytotoxicity experiments, U87MG cells were seeded in a 96-well plate at 2×10^4 cells per well in 100 μL of medium. After 48 h, 100 μL of sample (degradation products collected at different time points from (Chi/Alg)Si-SPs incubated in DMEM cell culture media) was added to each well in triplicates and incubated (37 °C, 5% CO_2) for 48 h. Then, the cell culture medium was aspirated and 100 μL of XTT/PMS solution was added to each well. After 4 h, the absorbance of the solution was measured at a wavelength of 475 nm and a reference wavelength of 675 nm using an Infinite M200 microplate reader. Differential absorbance was calculated by subtracting the absorbance at 675 nm from the absorbance at 475 nm. XTT/PMS

solution was prepared by mixing 10.8 mg XTT in 54 mL DMEM supplemented with 10% fetal bovine serum with 135 μ L of 2 mM PMS in DPBS solution. Sample wells containing only medium (i.e., no cells) were used as background controls for the absorbance measurements. Cells treated only with fresh DMEM were used for the normalisation of cell toxicity.

SDS-PAGE. For the SDS-PAGE analysis, 10 μ L of lysozyme, BDNF and NT-3 samples released from Si-SPs and ^(Chi/Alg)Si-SPs *in vitro* were mixed with 30 μ L of NuPAGE 4 $\times$ LDS sample buffer and heated to 90 °C for 3 min prior to loading in a 4–15% polyacrylamide gel (Bio-Rad Laboratories, Inc.). Native lysozyme, BDNF and NT-3 samples were loaded for comparison. Protein bands were detected using SimplyBlue Safe Stain (Thermo Fisher Scientific) following the manufacturer's instructions.

Circular Dichroism. Circular dichroism (CD) spectra of lysozyme, BDNF and NT-3 solutions released from Si-SPs and ^(Chi/Alg)Si-SPs were recorded at room temperature (~22 °C) using a 1 cm quartz cell with a 260–195 nm measurement range, at 1 nm intervals with a bandwidth of 1 nm. The CD spectra contributions from the PBS buffer were subtracted from each collected sample.

NT-3 Labelling. NT-3 was labelled with ¹²⁵I as reported previously.²⁵ Unbound (free) ¹²⁵I was separated from the NT-3 bound ¹²⁵I using Bio-Gel P-6DG columns (Bio-Rad). The ¹²⁵I labelled NT-3 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 desired concentration of ¹²⁵I NT-3 (1 mg mL⁻¹ in Milli-Q water) was adjusted by mixing unlabelled NT-3 and ¹²⁵I NT-3 with the volume ratio 100:1.

In Vitro Loading and Release Studies. *In vitro* release studies were conducted using either the model drug FITC-lysozyme or neurotrophin (BDNF or NT-3, ^{125}I NT-3). To load SPs with FITC-lysozyme, ten Si-SPs or $^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$ were incubated with 100 μL of FITC-lysozyme solution (1 mg mL^{-1} in Milli-Q water) for 3 days at room temperature ($\sim 22\text{ }^{\circ}\text{C}$). After loading, 100 μL of fresh PBS was added to the loaded Si-SPs or $^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$ and kept in a $37\text{ }^{\circ}\text{C}$ incubator. At defined time intervals, 95 μL of the supernatant was collected (stored in the freezer until further analysis) and replaced with 95 μL of fresh PBS. The fluorescence of the collected samples with 490 nm excitation wavelength and 525 nm emission wavelength was measured using an Infinite M200 microplate reader (Tecan, Switzerland). FITC-lysozyme release from $^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$ was also evaluated as a function of chitosan/alginate concentration (0.1, 0.5, 1 and 2 wt% for both chitosan and alginate) and chitosan/alginate bilayer number (2, 4 and 6 bilayers, with one bilayer consisting of one chitosan layer and one alginate layer). The resulting samples are referred to as $^{x(\text{Chi}/\text{Alg})}\text{Si-SPs}$ (x represents the concentration of chitosan/alginate, y represents the cycles). A description, including nomenclature, of the particles prepared in the present study is given in Table S1. FITC-lysozyme release from Si-SPs embedded in 50 μL of fibrin sealant (a commercially viable hydrogel) was also assessed, using the same protocol above.

For neurotrophin (BDNF, NT-3 or ^{125}I NT-3) loading, 28 μL of 1.0 mg mL^{-1} BDNF, NT-3 or ^{125}I NT-3 was mixed with four sterilised Si-SPs. After incubation for 3 days, the supernatant was removed and the Si-SPs were placed in separate 1.7 mL microcentrifuge tubes (one SP per tube). Neurotrophin-loaded Si-SPs were coated with chitosan and alginate as described above to monitor the release from $^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$. Then, 100 μL of PBS (pH 7.4) was added to each tube and incubated at $37\text{ }^{\circ}\text{C}$. At defined time intervals (3, 7, 14, 21, 28 days), 95 μL of the supernatant was collected

(and stored for further analysis) and replaced with 95 μ L of fresh PBS. The amount of BDNF or NT-3 released from Si-SPs or ^(Chi/Alg)Si-SPs was analysed using a BDNF- or NT-3-specific ELISA as per the manufacturer's protocol. The release profile of ¹²⁵I NT-3 from ^(Chi/Alg)Si-SPs embedded in fibrin sealant was also assessed (six ^(Chi/Alg)Si-SPs per sample). The amount of ¹²⁵I NT-3 released was quantified using a Perkin Elmer WIZARD automatic gamma counter with 1 min measurement time.

Auditory Neurons Cultures. SPs were prepared and loaded as described above. The concentration of the ¹²⁵I NT-3 released from the SPs was determined via gamma radiation levels. Samples of native NT3 maintained at 37 °C over the duration of the analysis and fresh NT3 prepared on the day of culture were used as controls. The concentrations of the SPs (Si-SPs and ^(Chi/Alg)Si-SPs)-released ¹²⁵I NT-3 and control samples were adjusted to 50ng/ml prior to application on the cultures.

Time-mated pregnant rat mums were obtained (Albino Wistar). Rat pups were used at postnatal day 5. The use and care of the experimental animals in this study were approved by the St Vincent's Hospital (Melbourne) Animal Ethics Committee (#18-393AB).

Cochleae were dissected in Hank's balanced salt solution and the modiolus isolated. The modiolus collected were treated with TrypLE for 20 min at 37 °C. Single cells obtained by mechanical trituration were filtered (40 μ m) and resuspended in Neurobasal media with NT-3 adjusted to concentrations of 50 ng/mL. The auditory neurons were plated on polyornithine/laminin coated 8-well culture slides and cultured for 3 days *in vitro*. Two wells were fixed 4 h post-plating as a control.

The cultures were fixed in 4% paraformaldehyde for 10 min at room temperature (~22 °C) and then washed 3 times in PBS. The cultures were blocked in 0.1% Triton

X-100 and 10% heat inactivated donkey serum in PBS for 1 hour. Primary antibodies Tuj1(1:500) and S100 (1:500) were diluted in blocking solution and applied overnight at 4 °C. The relevant secondary antibodies were diluted 1:500 in PBS. Nuclei were visualised using DAPI. All staining was visualised and imaged using the Zeiss Axiocam Microscope.

The cultures were analysed by counting the total number of neurons in each well. Neurite lengths were analysed using the ImageJ neurite tracer software.

Animal Surgery for Implantation of $^{(Chi/Alg)}$ Si-SPs. In this study, the use of guinea was approved by the St Vincent's Hospital (Melbourne) Animal Ethics Committee and 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).

Before the surgery, Si-SPs was pre-loaded with ^{125}I NT-3 followed by coating with two bilayers of 0.5 wt% chitosan and alginate. Then the $^{0.5(Chi/Alg)^2}$ Si-SPs were embedded in 10 μ L of clinically approved gelation system consisted of thrombin and fibronectin. The gel (called "fibrin sealant") can form a small pellet containing the loaded $^{0.5(Chi/Alg)^2}$ Si-SPs, which can be easily placed on the RWM to secure the fibrin sealant in place by a surgical applicator.

Prior to injection, cats were first anaesthetised with a 60 mg kg⁻¹ ketamine and 4 mg kg⁻¹ xylazine mixture via intramuscular injection. 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, five loaded $^{0.5(Chi/Alg)^2}$ Si-SPs were placed onto RWM, followed by sealing with superglue. For inner ear injection, a cochleostomy was applied to retract

the membrane on the top of the basal turn of the cochlea via a sharpened mental probe. A hole was drilled at the scala tympani of the cochlea and one $^{0.5(\text{Chi}/\text{Alg})_2}\text{Si-SP}$ embedded in fibrin sealant was injected into the basal turn of the cochlea, followed by sealing with superglue. At specific time points (1, 2 or 4 weeks), the cats were perfused by using 400 mL 0.9 % (w/v) saline (the saline contains 0.025 % (w/v) sodium nitrite and 0.1 % (w/v) heparin sodium). Then 400 mL neutral buffered formalin was administered for the intracardial perfusion followed by the dissection of bulla. The cochlea was dissected with semi-circular canals attached. The organs of cats were also separated for the measurement of radioactivity. Auditory brainstem responses (ABRs) were recorded prior to surgical implantation of the using techniques described previously.¹⁵ Auditory thresholds were measured for tone pip acoustic stimuli at a range of frequencies (1kHz to 24kHz).

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,²⁶ and include a companion checklist of these components in the Supporting Information.

6.4 RESULTS AND DISCUSSION

Synthesis and Characterisation of $^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$. Uncoated Si-SPs suffer from an initial burst release, where ~60% of a model protein drug (lysozyme) is typically released in the first 3 days. To control the release of loaded protein from supraparticles, Si-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.²⁷ The surface structure of SPs following LbL coating with chitosan and alginate was characterised by SEM. At low resolution, the morphology of Si-SP and ^(Chi/Alg)Si-SP appear similar (**Figure 1a, c**), but at higher magnification, distinct surface morphology differences can be observed (**Figure 1b, d**), which strongly suggests the successful coating of the Si-SPs with the chitosan/alginate film. FTIR spectroscopy of the Si-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 1e, black curve**). ^(Chi/Alg)Si-SPs displayed a comparable FTIR spectrum (**Figure 1e, red curve**). The 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. The dissipation (ΔD) values increased with time suggesting that the chitosan and alginate coating is soft, not rigid.²⁸ To quantify the mass of chitosan/alginate deposited, thermogravimetric analysis (TGA) was performed. Chitosan and alginate are two organic components that decompose at high temperatures. TGA showed that one bilayer coating of chitosan/alginate accounted for ~4% of the weight (~2 μ g considering the weight of each SP is ~0.05 mg) of one Si-SP (**Figure S1**). SEM-EDX elemental mapping

confirmed the presence of silicon (Si), oxygen (O), calcium (Ca) and nitrogen (N) uniformly distributed on the surface of $(\text{Chi}/\text{Alg})\text{Si-SPs}$ (**Figure 1g**). In addition, SEM images of $(\text{Chi}/\text{Alg})\text{Si-SPs}$ cross-sections (**Figure S2**) and confocal laser scanning microscopy images (**Figure S3**) of $^{0.5}(\text{Chi-FITC}/\text{Alg})^1\text{Si-SPs}$ also confirmed the presence of a chitosan/ alginate coating on the surface of Si-SPs.

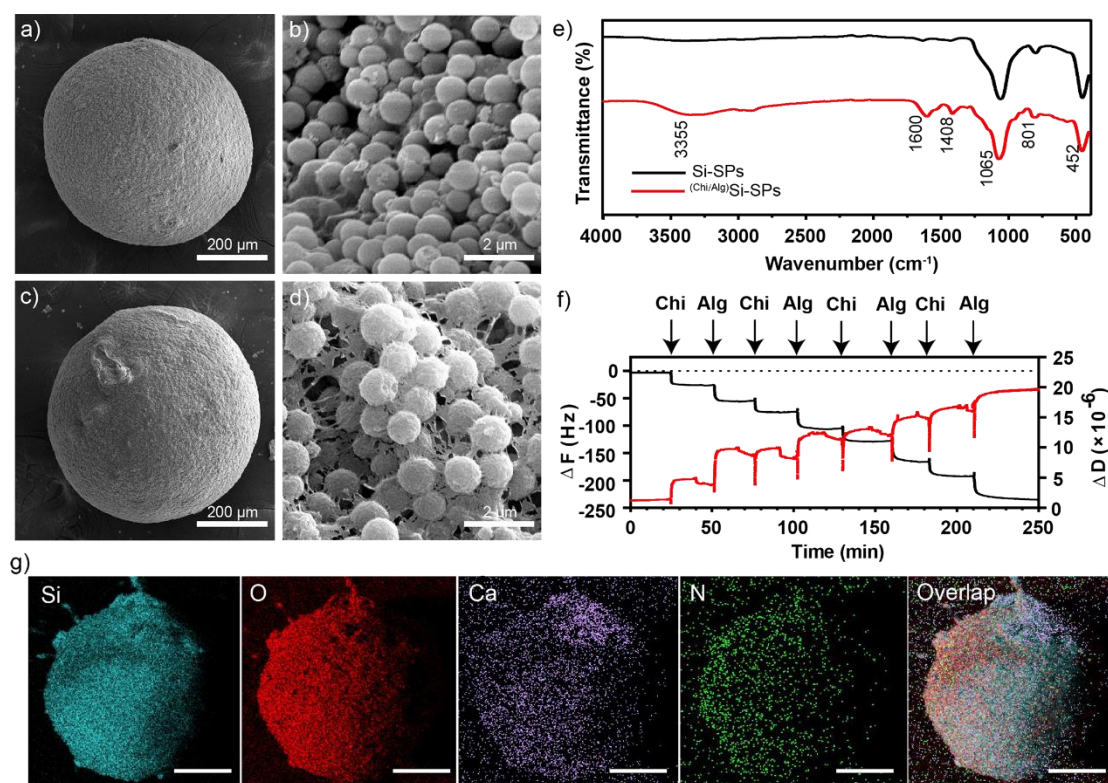


Figure 1. Characterisation of Si-SPs and $(\text{Chi}/\text{Alg})\text{Si-SPs}$. (a) SEM image of a Si-SP and (b) high-resolution SEM image displaying the surface of a Si-SP. (c) SEM image of a $(\text{Chi}/\text{Alg})\text{Si-SP}$ and (d) high-resolution SEM image displaying the surface of a $(\text{Chi}/\text{Alg})\text{Si-SP}$. (e) FTIR spectra of Si-SP and $(\text{Chi}/\text{Alg})\text{Si-SP}$. (f) Frequency and dissipation changes monitored by QCM-D for the layer by layer assembly of chitosan and alginate. (g) Elemental distribution of a $(\text{Chi}/\text{Alg})\text{Si-SPs}$ (scale bar is 200 μm) .

***In Vitro* FTIC-Lysozyme Release.** The *in vitro* drug release behaviour was first investigated using a model protein lysozyme (lysozyme $M_w = 14.3 \pm 0.5$ kDa,

isoelectric point = 11),^{29,30} which has a similar molecular weight and isoelectric point to the neurotrophin of interest, BDNF (M_w = 13 kDa, isoelectric point = 10).^{14,31} Fluorescein-labelled lysozyme (FITC-lysozyme) was first encapsulated into Si-SPs (typical loading of $\sim 7 \mu\text{g}/\text{Si-SP}$), followed by chitosan and alginate LbL coating. The loading percentage of FITC-lysozyme in each group of (Chi/Alg) Si-SPs studies were shown in **Figure S4**. The concentration of the chitosan/alginate solution was adjusted during LbL coating (0.1, 0.5, 1 and 2 wt% of both chitosan and alginate) to determine the influence of the coating on the release behaviour of FITC-lysozyme. Overall, the presence of a chitosan/alginate coating delayed the release of FITC-lysozyme. At day 12, for example, $\sim 75\%$ of FITC-lysozyme has been released from noncoated Si-SPs, whereas only $\sim 40\%$ of FITC-lysozyme was released from $(\text{Chi}/\text{Alg})^2$ Si-SPs prepared using 0.1, 0.5 or 1 wt% chitosan (i.e., $^{0.1}(\text{Chi}/\text{Alg})^2$ Si-SPs, $^{0.5}(\text{Chi}/\text{Alg})^2$ Si-SPs and $^1(\text{Chi}/\text{Alg})^2$ Si-SPs). An increase to a chitosan concentration of 2 wt% during coating $^2(\text{Chi}/\text{Alg})^2$ Si-SPs resulted in further delay of FITC-lysozyme release with $\sim 28\%$ released at day 12. By increasing the number of chitosan and alginate bilayers (from 2 to 6 bilayers), there were negligible differences in the release profile when the concentrations of chitosan and alginate were 0.1 and 0.5 wt%, respectively (**Figure 2a, b**). At these concentrations and bilayer numbers, it takes approximately 20 days to achieve $>75\%$ of FITC-lysozyme release, whereas it only takes ~ 6 days for non-coated Si-SPs to achieve the same extent of the release. When the chitosan concentration was increased to 1 and 2 wt%, the number of chitosan/alginate bilayers influenced the release—a slower release was observed with increasing layer numbers (**Figure 2c, d**). However, at these high chitosan concentrations (1 or 2 wt%), the resulting (Chi/Alg) Si-SPs are very sticky (Si-SPs was entrapped by a gel shell) and not easy to handle.

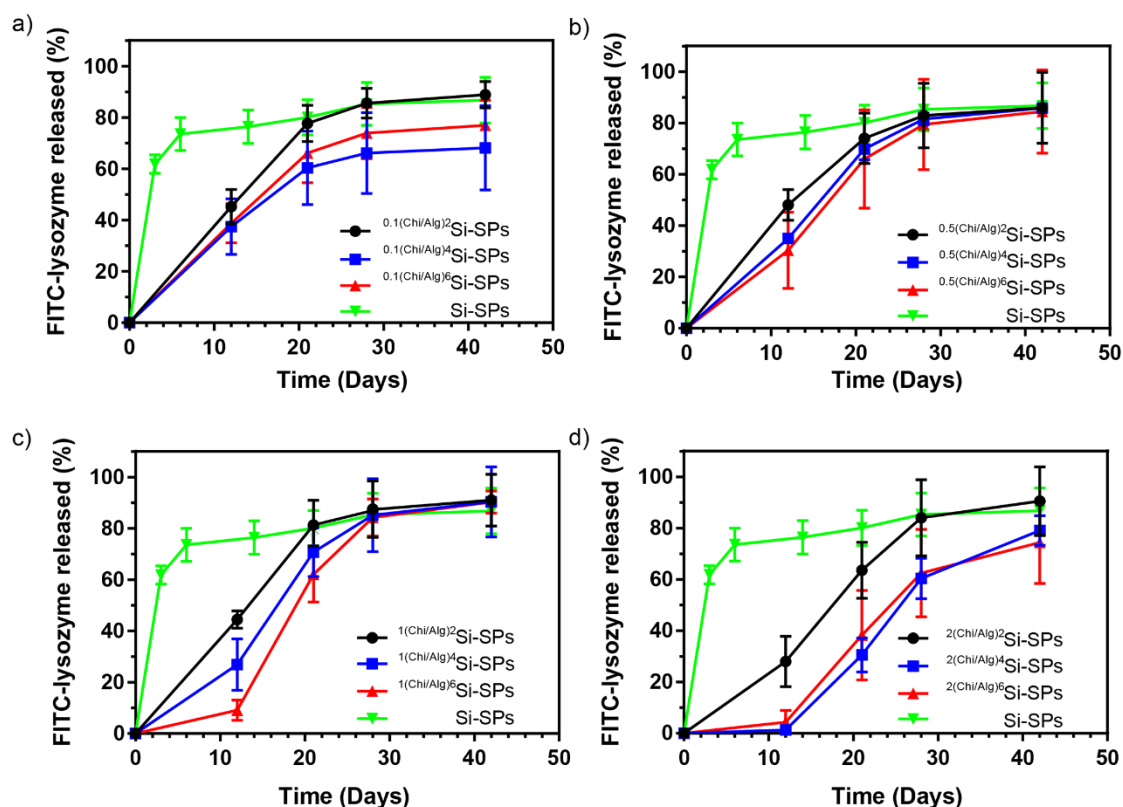


Figure 2. *In vitro* cumulative release profiles. FITC-lysozyme released from uncoated Si-SPs and (Chi/Alg) Si-SPs coated with chitosan and alginate coating at concentrations of (a) 0.1, (b) 0.5, (c) 1.0 and (d) 2.0 wt% and coated with 2, 4 and 6 bilayers). Data presented in (a–d) are averages of triplicates with error bars indicating standard deviations, each measuring release from 10 SPs.

***In Vitro* Neurotrophins Release and (Chi/Alg) Si-SPs Degradation.** To further evaluate the release profile of the target drug, neurotrophins, for the treatment of inner ear disease, (Chi/Alg) Si-SPs were pre-loaded with neurotrophins, followed by coating with 0.5 wt% of chitosan and alginate, with a total of 2 bilayers. **Figure 3a, b** show that the neurotrophins release from (Chi/Alg) Si-SPs extended to ~28 days when compared with the release observed from uncoated Si-SPs (3 days) in which ~60% of BDNF or NT-3 was released at ~28 days. ELISA showed that the average loading amount of BDNF and NT-3 were 6.75 and 7.39 μg per SP, respectively.

Previously researchers used radiolabelled NT-3 to characterise the pharmacokinetics distribution of NT-3 in the cochlea because it is a straightforward technique to quantify the amount of neurotrophins in different parts of tissue in the cochlea by measuring the radioactivity.^{25,32} This technique was used to characterise the *in vitro* NT-3 release profile shown in **Figure 3c**. The percentage of the radioactivity stemming from ¹²⁵I NT-3 in the washing solution, coating solution and the coating tube is very low, which suggests negligible drug loss during the post-coating steps (**Figure S5**). **Figure 3c** shows the cumulative release amount/percentage of ¹²⁵I NT-3 from uncoated Si-SPs, (Chi/Alg)Si-SPs and (Chi/Alg)Si-SPs embedded in fibrin sealant. It shows that coated (Chi/Alg)Si-SPs can slow the release, where ~60% of ¹²⁵I NT-3 was released by 14 days. (Chi/Alg)Si-SPs in fibrin sealant can further retard the release, where at day 14 only ~25% of ¹²⁵I NT-3 was released. There was no initial burst release observed from FITC-lysozyme loaded Si-SPs in fibrin sealant matrix, which further indicated that fibrin sealant can impede on protein release of SPs (**Figure S6**). In practice, the sealant is used for aiding the surgical implantation.

The drug release can be caused by both the concentration gradient between the loaded (Chi/Alg)Si-SPs and the degradation of (Chi/Alg)Si-SPs. ICP-OES was employed to quantify the amount of Si released from (Chi/Alg)Si-SPs at different time points (7, 14, 21, 28 and 42 days) (**Figure 3d**). In the first 7 days, ~96% of Si remained when compared with that of previously reported Si-SPs (~90% of Si remained).²² This demonstrates that the chitosan and alginate coating on the surface of Si-SPs impedes the degradation of Si-SPs, which further slows the drug release profile. SEM images reveal that the morphology of (Chi/Alg)Si-SPs significantly changed after the incubation for 42 days in PBS at 37 °C (**Figure 3e**). The primary silica particles on the surface of (Chi/Alg)Si-SPs were degraded into porous fragments, as shown in **Figure 3f**.

To confirm the stability of the *in vitro* released neurotrophins from $^{(Chi/Alg)}$ Si-SPs, SDS-PAGE and circular dichroism studies were performed. SDS-PAGE proved that the released neurotrophins were intact without any degradation where the *in vitro* released neurotrophins from $^{(Chi/Alg)}$ Si-SPs have the same molecular weight ($M_w = \sim 13$ kDa) compared to non-encapsulated neurotrophin (**Figure 3g, h**). Circular dichroism also demonstrated that the secondary structure of released neurotrophins or lysozyme is the same as that of native neurotrophins or lysozyme, in which the CD signals of neurotrophins and lysozyme overlap with that of native neurotrophins or lysozyme (**Figure S7**). The results confirm that the encapsulation and subsequent coating do not affect the overall structure of neurotrophins.

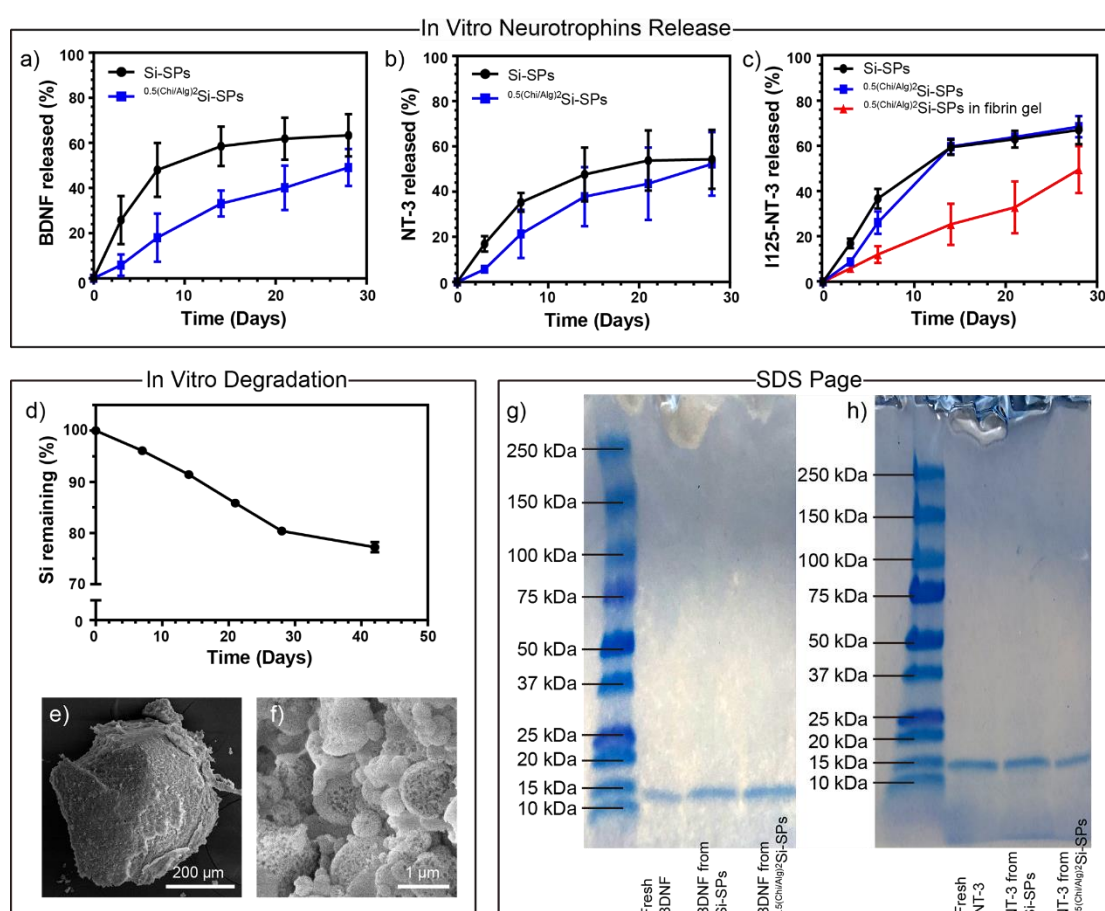


Figure 3. *In vitro* cumulative release profiles of (a) BDNF and (b) NT-3 from Si-SPs and $^{(Chi/Alg)}$ Si-SPs. (c) *In vitro* cumulative release profiles of 125 I NT-3 from Si-SPs, $^{(Chi/Alg)}$ Si-

SPs and $(\text{Chi}/\text{Alg})\text{Si-SPs}$ in fibrin sealant. Data presented in (a–c) are averages of triplicates with error bars indicating standard deviations, each measuring release using 1 SP except for the $(\text{Chi}/\text{Alg})\text{Si-SP}$ system in fibrin sealant where four ^{125}I NT-3-loaded SPs were used in each group. The loading amount of ^{125}I NT-3 in each group of SPs was shown in **Figure S8**. (d) ICP-OES analysis showing the Si amount remaining in $(\text{Chi}/\text{Alg})\text{Si-SPs}$. SEM images showing the surface of the $(\text{Chi}/\text{Alg})\text{Si-SPs}$ after 42 days incubation with PBS at (e) low magnification and (f) high magnification. Characterisation of the degradation of neurotrophins of *in vitro* released (g) BDNF and (f) NT-3 samples from Si-SPs and $(\text{Chi}/\text{Alg})\text{Si-SPs}$ using SDS-PAGE gels compared to the fresh BDNF and NT-3.

***In Vitro* SGNs Culture and Neurite Length Analysis.** Prior to the SGNs culture, the cytotoxicity of $(\text{Chi}/\text{Alg})\text{Si-SPs}$ was tested using U87MG human brain-derived glioblastoma cell line (**Figure S9**). Negligible cytotoxicity was observed even as the number of $(\text{Chi}/\text{Alg})\text{Si-SPs}$ increased to ten.

Auditory nerve culture experiments were carried out to test whether loading of ^{125}I NT-3 into the porous SP structure improved the bioactivity of ^{125}I NT-3 over time. *In vitro* SP (both Si-SPs and $(\text{Chi}/\text{Alg})\text{Si-SPs}$)-released ^{125}I NT-3 samples at 37 °C collected at multiple time points (3, 14 or 28 days) were compared to the control samples of native ^{125}I NT-3 and NT-3 maintained at 37 °C throughout the analysis and fresh NT-3 prepared on the day of culture. Compared to the fresh NT-3, the percentage of SGNs after treatment with NT-3 and ^{125}I NT-3 maintained at 37 °C was 11.1% at 3 (day *in vitro*) DIV and no survival was observed upon treatment with the sample collected at the later time points. In contrast, ~66% of SGNs was observed after treatment with ^{125}I NT-3 released from SPs at 3 DIV, a similar result was obtained from $(\text{Chi}/\text{Alg})\text{Si-SP}$ -released ^{125}I NT-3 and $(\text{Chi}/\text{Alg})\text{Si-SPs}$ incorporating fibrin glue (**Figure 4a**). Improved

nerve survival was observed after 14 and 28 DIV using SPs-released ^{125}I NT-3 compared to NT-3 and ^{125}I NT-3, although a decrease in the percentage of auditory neuron survival was observed at later time points.

The neurite lengths of the auditory neurons in the fresh and 3DIV NT-3 and SP-released ^{125}I NT-3 samples were comparable (**Figure 4b, c**). Similar results were observed to the nerve survival data, whereby there was no bioactivity of the NT-3 and ^{125}I NT-3 after 2 weeks (as assessed by neurite length). SP-released ^{125}I NT-3 maintained bioactivity over time, albeit with a decrease in neurite length.

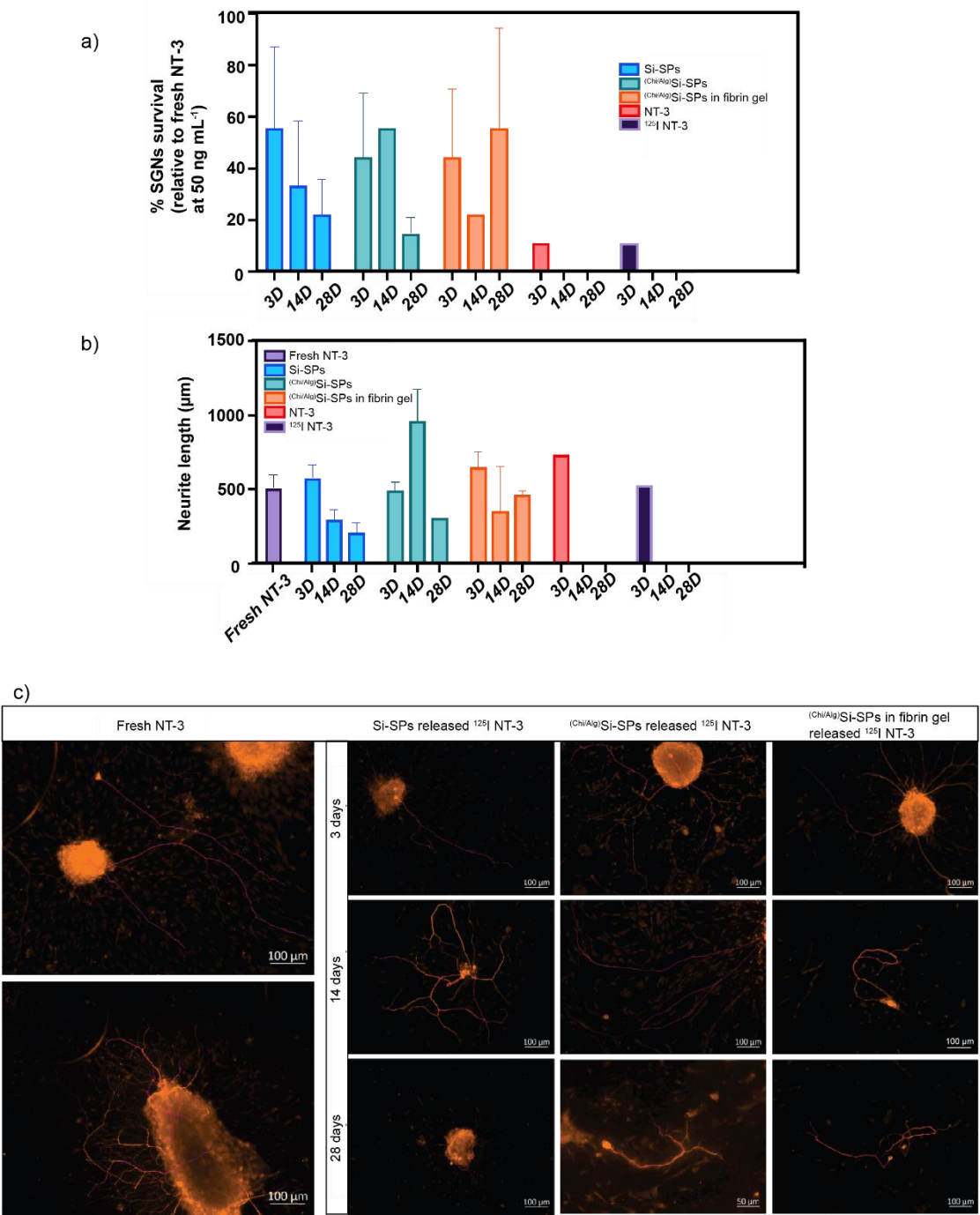


Figure 4. Bioactivity of the SP-released ¹²⁵I NT-3 over time. (a) SGNs of postnatal day 5 rats were cultured with SPs (both Si-SPs and (Chi/Alg)Si-SPs)-released ¹²⁵I NT-3 nor native INT-3 for 3 days *in vitro*. The eluates or native NT-3 samples were collected at 3, 14 and 28 days. The percentage of surviving neurons was calculated relative to the number of neurons present in the freshly prepared NT-3. (b) The neurite lengths of

the auditory neurons were measured using the neurite tracer ImageJ plugin. (c) The auditory neurons were immunolabelled with Neurofilament-H (orange). The neurites traced can be observed in purple. Scale bar= 100 μm .

Effects on Hearing Thresholds Following Implantation. To establish the safety of the surgical approach to deliver $^{(\text{Chi/Alg})}\text{Si-SPs}$ in fibrin sealant to the RWM and to the scala tympani, hearing thresholds were measured before and after treatment. ABRs were recorded prior to surgical implantation of the supraparticles using techniques described previously.¹⁵ Auditory thresholds were measured for tone pip acoustic stimuli at a range of frequencies.

$^{(\text{Chi/Alg})}\text{Si-SPs}$ containing ^{125}I NT-3 were delivered to the RWM ($n = 30$ SPs in each cat cochlea). At 6 weeks of the treatment period, the ABRs was remeasured. **Figure 5a** shows the thresholds for measured prior and after treatment for the round window delivery route. The result shows RWM delivery had minimal impact on normal functional hearing, and it is a safe administration route to deliver neurotrophins. **Figure 5b** shows the thresholds for measured prior and after treatment for the intracochlear delivery ($n = 6$ SPs in each cat cochlea) route. The Post treatment analysis indicated intracochlear delivery results in a high frequency hearing loss in normal hearing animals, which would not be applied to people with normal hearing.

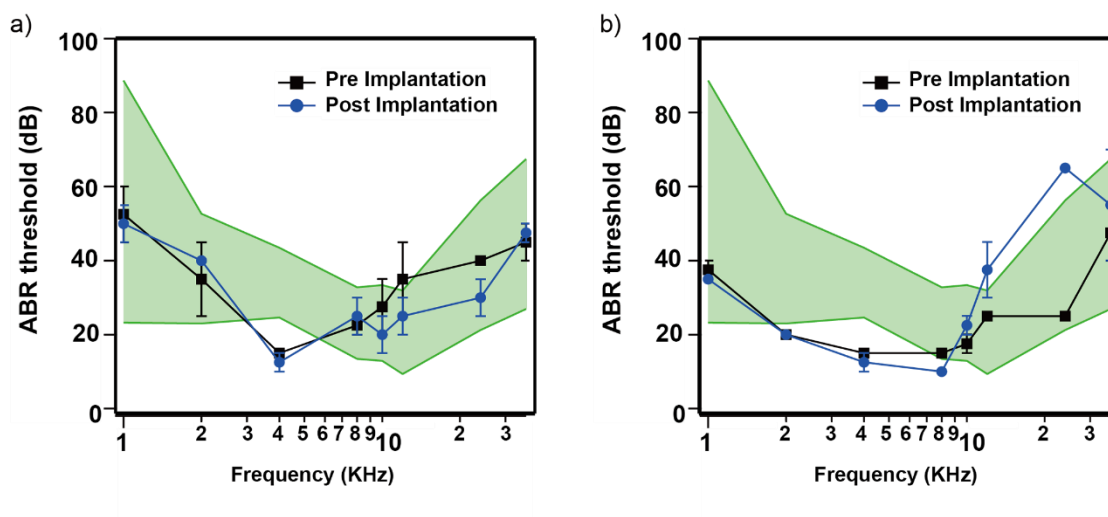


Figure 5. Auditory brainstem response (ABR) thresholds for tone pip acoustic stimuli before and after the delivery of ^{125}I NT-3 loaded supraparticles ($n = 6$ SPs to the (a) RWM) and ($n=30$ SPs to the (b) cochlea) after 6 weeks treatment. Graphs depict the pre and post ABR thresholds. The green region is from historical data on the 95% confidence range for normal hearing animals (i.e. normal hearing region)

After the implantation of $(\text{Chi/Alg})\text{Si-SPs}$ for 8 weeks, the cats were perfused and the $(\text{Chi/Alg})\text{Si-SPs}$ were collected. SEM images show the morphology of $(\text{Chi/Alg})\text{Si-SPs}$ after the *in vivo* implantation of RWM (**Figure 6**). The size of the $(\text{Chi/Alg})\text{Si-SPs}$ was decreased from $\sim 550 \mu\text{m}$ to $\sim 250 \mu\text{m}$ and the degree of sphericity of $(\text{Chi/Alg})\text{Si-SPs}$ decreased. The structure of the primary silica particles on the surface of $(\text{Chi/Alg})\text{Si-SPs}$ was compromised, as indicated by the presence of hollow primary particles. These results indicate the good biodegradability of $(\text{Chi/Alg})\text{Si-SPs}$, which is an important property for the safety consideration of nanoengineered particles.

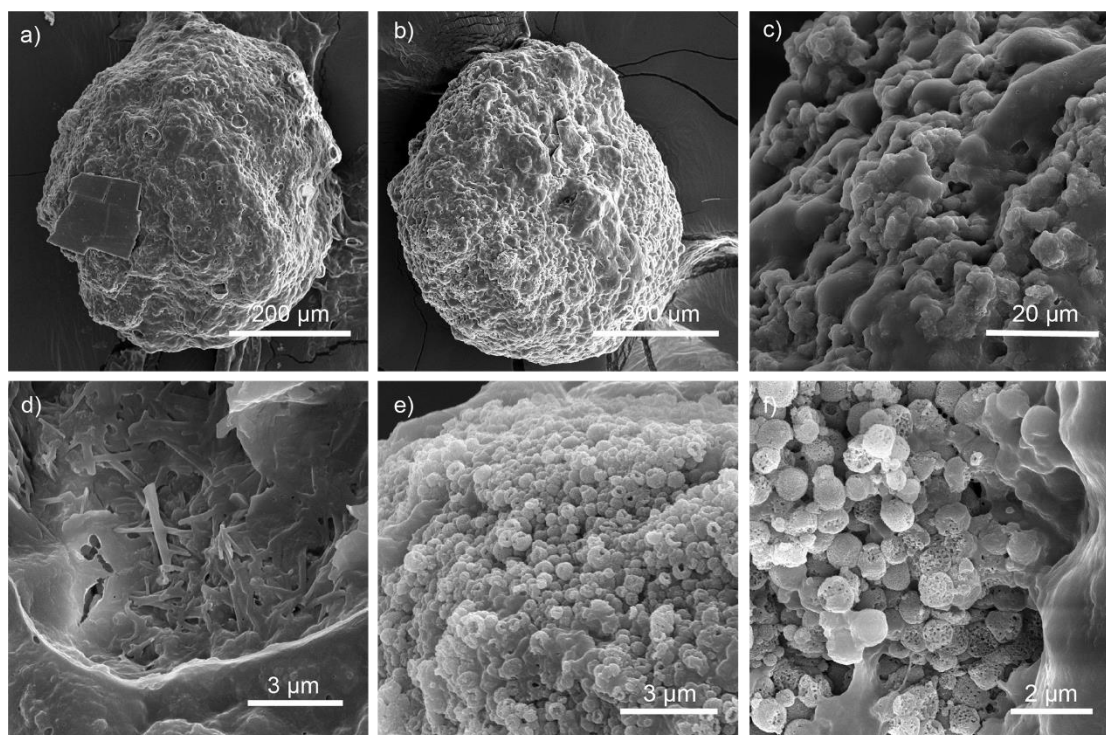


Figure 6. SEM images of the $(\text{Chi/Alg})\text{Si-SPs}$ after 8 weeks of implantation of the RWM of cats at (a, b) low magnification and (c–f) high magnification.

6.5 CONCLUSIONS

A $(\text{Chi/Alg})\text{Si-SPs}$ drug delivery system with a controlled drug release profile and excellent degradability both *in vitro* and *in vivo* was presented. The deposition of chitosan and alginate on the surface of Si-SPs slowed the initial burst release of neurotrophins, thus minimising potential risks of toxicity to the inner ear. ^{125}I NT-3 released from $(\text{Chi/Alg})\text{Si-SPs}$ showed higher bioactivity, as noted from the higher percentage of SGNs and longer neurite length, than the native NT-3 and free ^{125}I NT-3 (not loaded into SPs) over the duration of the analysis. The ABR measurements showed that $(\text{Chi/Alg})\text{Si-SPs}$ injected via intratympanic administration route ($(\text{Chi/Alg})\text{Si-SPs}$ embedded in fibrin sealant matrix for easy surgical handling of $(\text{Chi/Alg})\text{Si-SPs}$) is a safe surgical delivery route, whereas $(\text{Chi/Alg})\text{Si-SPs}$ injected via intracochlear administration

route resulted in a significant loss of high frequency. Therefore, $(\text{Chi/Alg})\text{Si-SPs}$ in fibrin sealant via RWM delivery route can serve as a versatile platform for the controlled release of neurotrophins for the treatment of inner ear diseases.

6.6 SUPPORTING INFORMATION

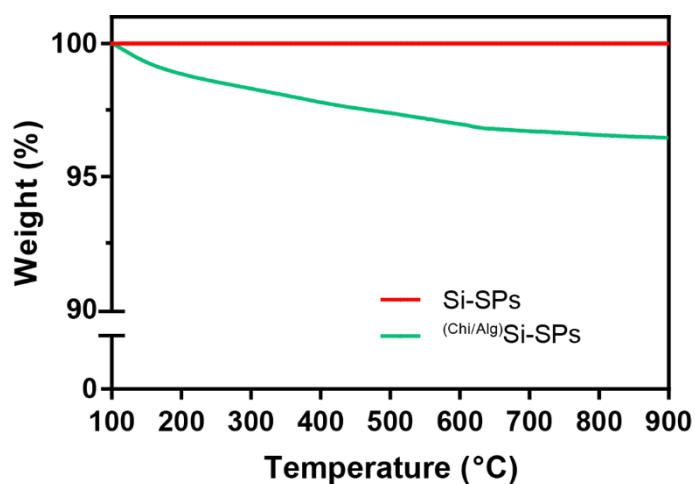


Figure S1. Thermogravimetric analysis (TGA) of Si-SPs and $(\text{Chi/Alg})\text{Si-SPs}$ showing the weight percentage of chitosan/alginate bi-layer coating.

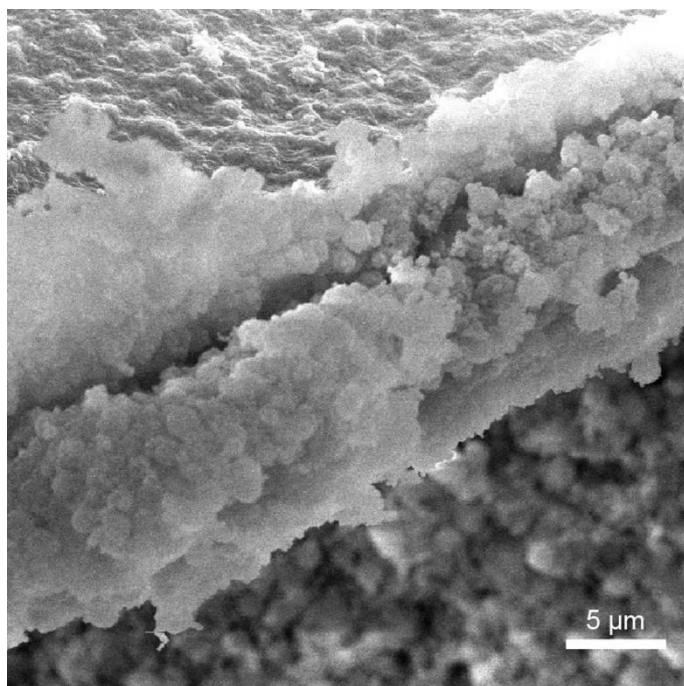


Figure S2. SEM image of the cross-section of chitosan/alginate coating on the surface of (Chi/Alg)Si-SPs.

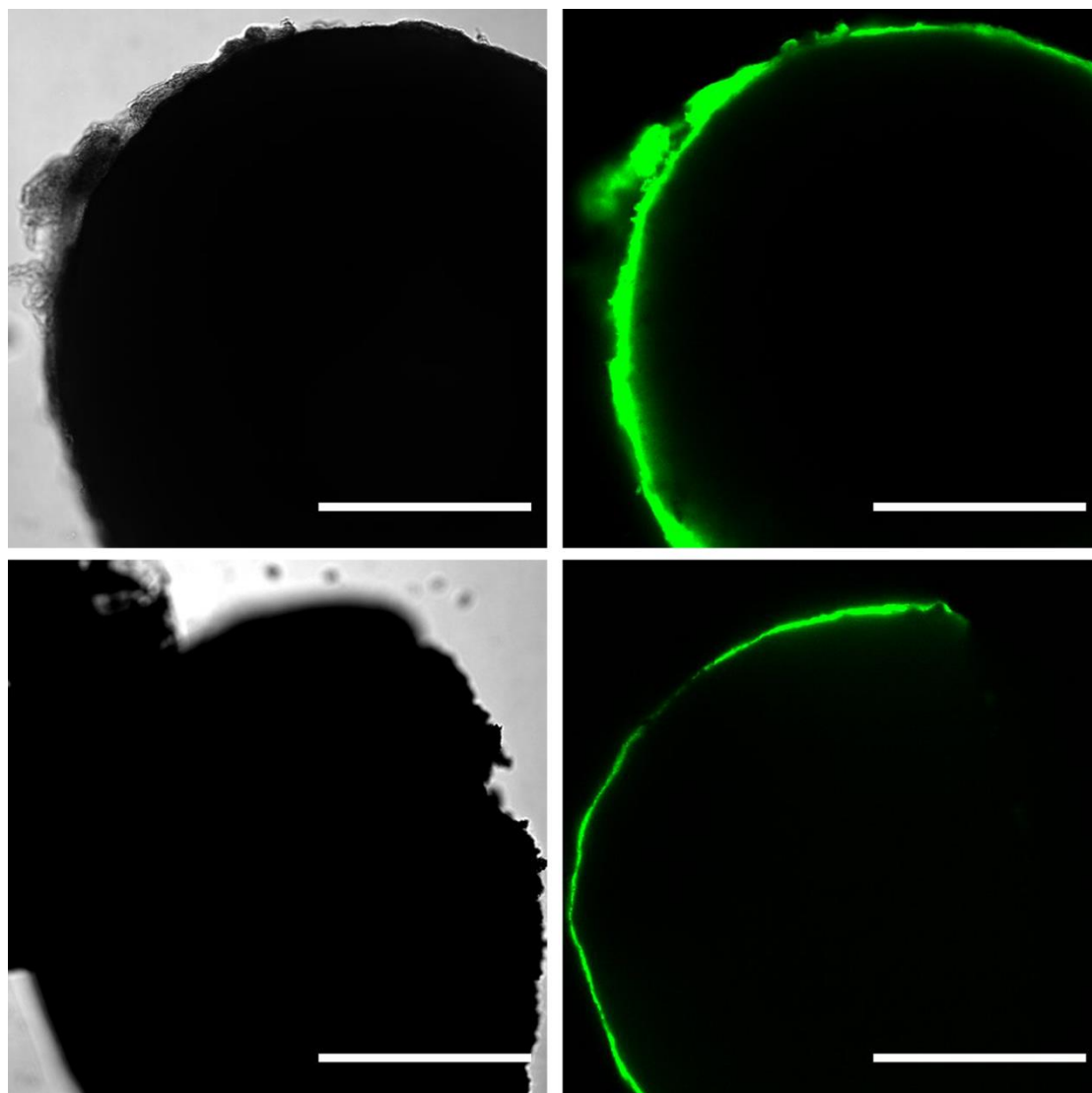


Figure S3. Optical microscopy images of (a) intact and (b) fragmented chitosan-FITC and alginate coated Si-SPs. Confocal laser scanning microscopy images of (c) intact and (d) fragmented chitosan-FITC and alginate coated Si-SPs (green). Scale bars are 200 μm .

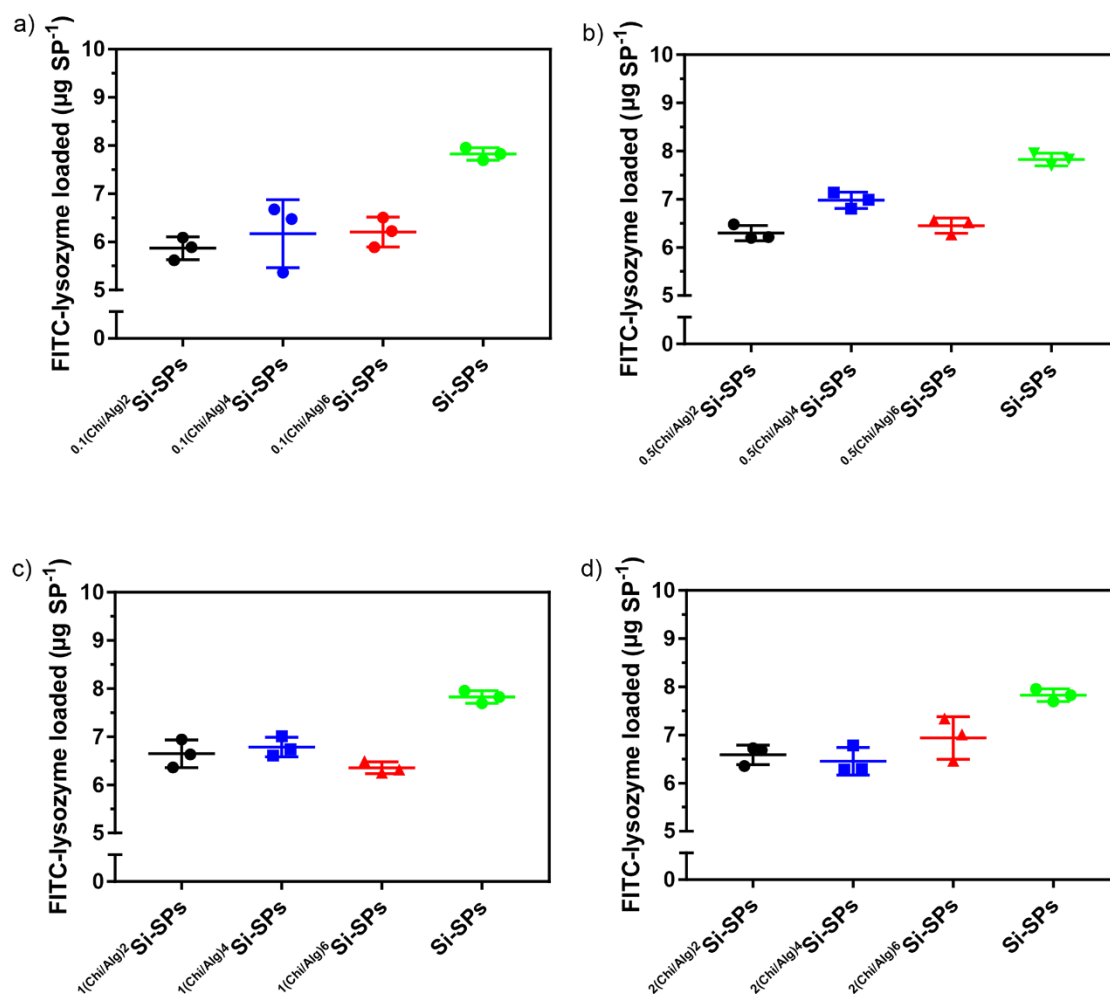


Figure S4. Amount of FITC-lysozyme loaded in individual Si-SPs before coating with chitosan/alginate. The concentration of chitosan/alginate varied from (a) 0.1, (b) 0.5, (c) 1.0 to (d) 2.0 wt% of samples and coated with 2 cycles, 4 cycles and 6 cycles.

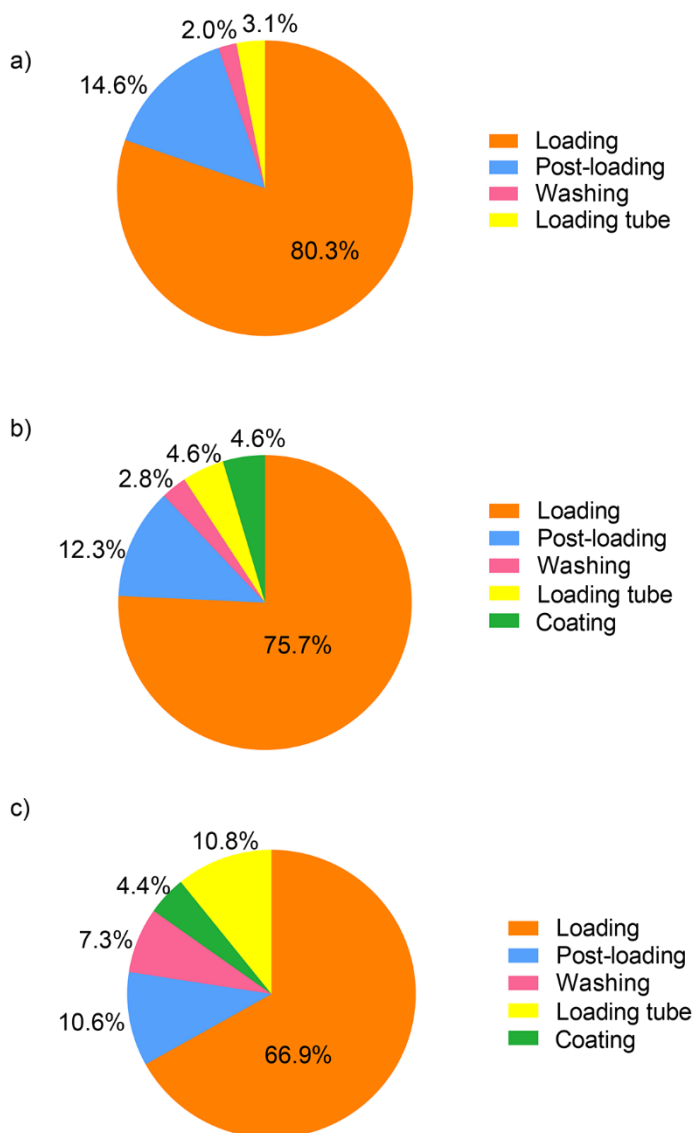


Figure S5. Percentage of ^{125}I NT-3 in each part of *in vitro* loading steps. ^{125}I NT-3 stock solution (1 mg mL^{-1}) was incubated with SPs. After 3 days of incubation, the supernatant was aspirated denoted as “post loading”. Then the SPs were washed with Milli-Q water denoted as “washing”. The tube was used for loading, denoted as “loading tube”. Finally, SPs were coated with chitosan/alginate and the supernatant of the coating solution and the washing buffer during the coating steps were collected, denoted as “coating”. Then the SPs (a) without coating and (b, c) with chitosan/alginate coating were denoted as “loading”.

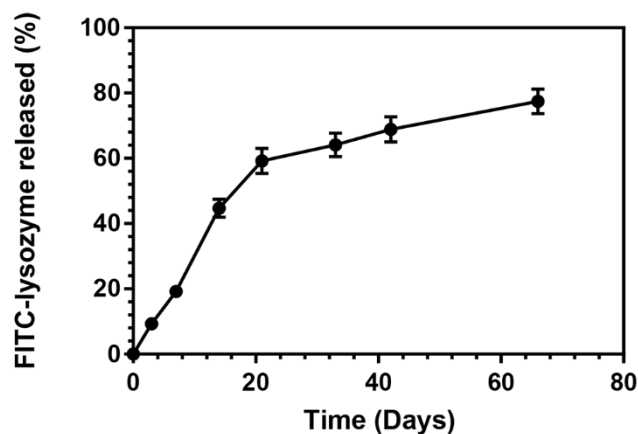


Figure S6. *In vitro* cumulative FITC-lysozyme release profiles of Si-SPs in fibrin sealant.

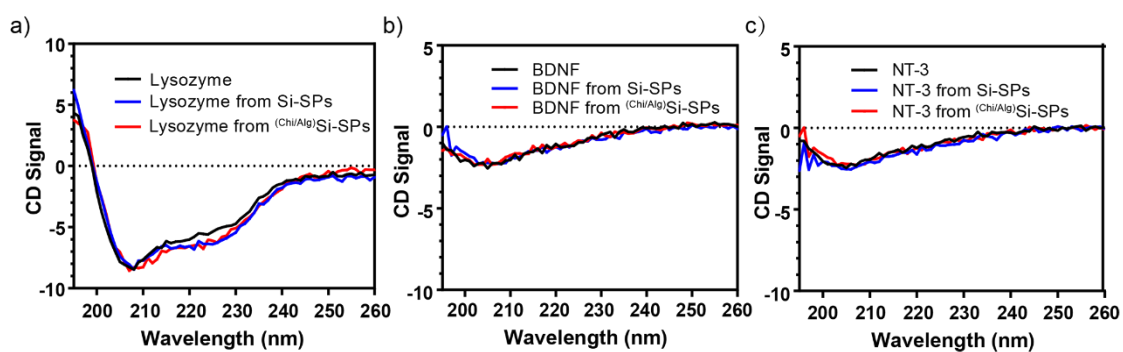


Figure S7. Circular dichroism spectra of (a) lysozyme, (b) BDNF and (c) NT-3 released from Si-SPs and $(\text{Chi}/\text{Alg})\text{Si-SPs}$ in PBS (pH 7.4). The standard samples are fresh lysozyme, BDNF and NT-3 which is the black line in the above figure.

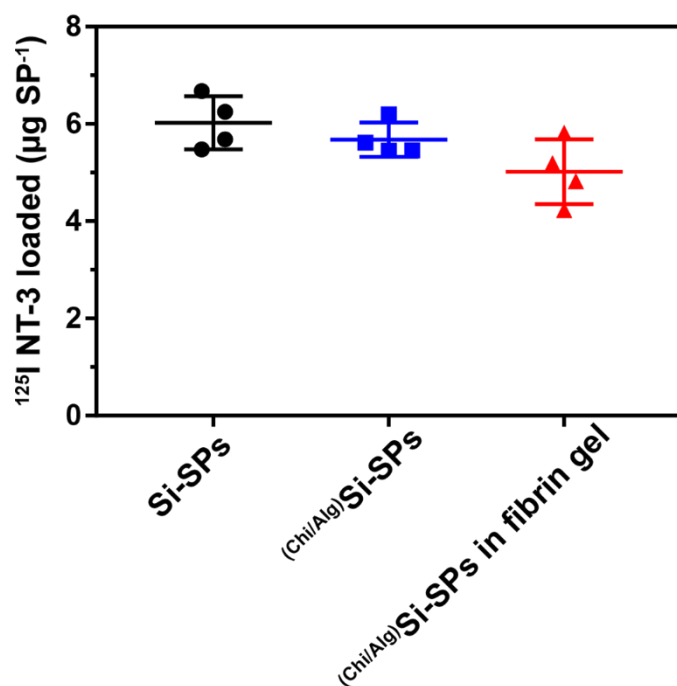


Figure S8. Amount of ^{125}I NT-3 loaded in individual Si-SPs before coating with chitosan/alginate for Figure 3c.

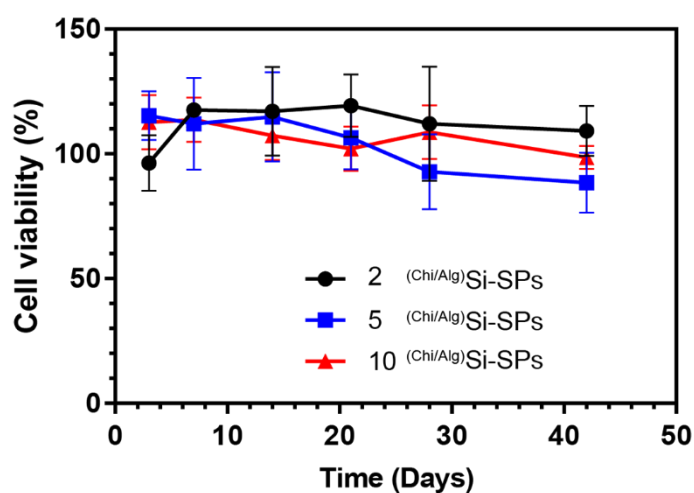


Figure S9. Cytotoxicity of U87MG human brain glioblastoma cells exposed to *in vitro* cell culture media from 2, 5, or 10 $(\text{Chi/Alg})\text{Si-SPs}$. The $(\text{Chi/Alg})\text{Si-SPs}$ were incubated in cell media at 37 °C. At different time intervals, the cell media was aspirated and kept in the freezer. Each data point represents the average of triplicates. Untreated cells were used to express 100% viability.

Table S1. Particle systems used in this study (see Scheme 1 for a graphical overview of the preparation procedure)

Name	Description
SP	supraparticle, assembly of primary silica particles
Si-SPs	silica supraparticle
(Chi/Alg) Si-SPs	Chitosan-alginate-coated silica supraparticles
$x(\text{Chi/Alg})_y\text{Si-SPs}$	Chitosan-alginate-coated silica supraparticles prepared with x wt% of chitosan/alginate and y represents the bi-cycles of chitosan/alginate layers

Checklist

Minimum Information Reporting in Bio–Nano Experimental Literature

The MIRIBEL guidelines were introduced here: <https://doi.org/10.1038/s41565-018-0246-4>

The development of these guidelines was led by the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology: <https://www.cbns.org.au/>. Any updates or revisions to this document will be made available here: <http://doi.org/10.17605/OSF.IO/SMVTF>. This document is made available under a CC-BY 4.0 license: <https://creativecommons.org/licenses/by/4.0/>.

The MIRIBEL guidelines were developed to facilitate reporting and dissemination of research in bio–nano science. Their development was inspired by various similar efforts:

- MIAME (microarray experiments): *Nat. Genet.* **29** (2001), 365; <http://doi.org/10.1038/ng1201-365>
- MIRIAM (biochemical models): *Nat. Biotechnol.* **23** (2005) 1509; <http://doi.org/10.1038/nbt1156>
- MIBBI (biology/biomedicine): *Nat. Biotechnol.* **26** (2008) 889; <http://doi.org/10.1038/nbt.1411>
- MIGS (genome sequencing): *Nat. Biotechnol.* **26** (2008) 541; <http://doi.org/10.1038/nbt1360>
- MIQE (quantitative PCR): *Clin. Chem.* **55** (2009) 611; <http://doi.org/10.1373/clinchem.2008.112797>
- ARRIVE (animal research): *PLOS Biol.* **8** (2010) e1000412; <http://doi.org/10.1371/journal.pbio.1000412>
- Nature’s reporting standards:
 - Life science: <https://www.nature.com/authors/policies/reporting.pdf>; e.g., *Nat. Nanotechnol.* **9** (2014) 949; <http://doi.org/10.1038/nnano.2014.287>
 - Solar cells: <https://www.nature.com/authors/policies/solarchecklist.pdf>; e.g., *Nat. Photonics* **9** (2015) 703; <http://doi.org/10.1038/nphoton.2015.233>
 - Lasers: <https://www.nature.com/authors/policies/laserchecklist.pdf>; e.g., *Nat. Photonics* **11** (2017) 139; <http://doi.org/10.1038/nphoton.2017.28>
- The “TOP guidelines”: e.g., *Science* **352** (2016) 1147; <http://doi.org/10.1126/science.aag2359>

Similar to many of the efforts listed above, the parameters included in this checklist are **not** intended to be definitive requirements; instead they are intended as ‘points to be considered’, with authors themselves deciding which parameters are—and which are not—appropriate for their specific study.

This document is intended to be a living document, which we propose is revisited and amended annually by interested members of the community, who are encouraged to contact the authors of this document. Parts of this document were developed at the annual International Nanomedicine Conference in Sydney, Australia: <http://www.oznanomed.org/>, which will continue to act as a venue for their review and development, and interested members of the community are encouraged to attend.

After filling out the following pages, this checklist document can be attached as a “Supporting Information” document during submission of a manuscript to inform Editors and Reviewers (and eventually readers) that all points of MIRIBEL have been considered.

Supplementary Table 1. Material characterization*

Question	Yes	No
1.1 Are “ best reporting practices ” available for the nanomaterial used? For examples, see <i>Chem. Mater.</i> 28 (2016) 3535; http://doi.org/10.1021/acs.chemmater.6b01854 and <i>Chem. Mater.</i> 29 (2017) 1; http://doi.org/10.1021/acs.chemmater.6b05235		X
1.2 If they are available, are they used ? If not available, ignore this question and proceed to the next one.		
1.3 Are extensive and clear instructions reported detailing all steps of synthesis and the resulting composition of the nanomaterial? For examples, see <i>Chem. Mater.</i> 26 (2014) 1765; http://doi.org/10.1021/cm500632c , and <i>Chem. Mater.</i> 26 (2014) 2211; http://doi.org/10.1021/cm5010449 . Extensive use of photos, images, and videos are strongly encouraged. For example, see <i>Chem. Mater.</i> 28 (2016) 8441; http://doi.org/10.1021/acs.chemmater.6b04639	X	
1.4 Is the size (or dimensions , if non-spherical) and shape of the nanomaterial reported?	X	
1.5 Is the size dispersity or aggregation of the nanomaterial reported?	X	
1.6 Is the zeta potential of the nanomaterial reported?		Reported in original study (see Ma et al. <i>ACS Appl. Mater. Interfaces</i> 2018 , <i>10</i> , 31019)
1.7 Is the density (mass/volume) of the nanomaterial reported?		Reported in previous study (see Ma et al.

		<i>Biomacromolecules</i> 2019 , 20, 3425)
1.8 Is the amount of any drug loaded reported? ‘Drug’ here broadly refers to functional cargos (e.g., proteins, small molecules, nucleic acids).	X	
1.9 Is the targeting performance of the nanomaterial reported, including amount of ligand bound to the nanomaterial if the material has been functionalised through addition of targeting ligands?		not applicable
1.10 Is the label signal per nanomaterial/particle reported? For example, fluorescence signal per particle for fluorescently labelled nanomaterials.		not applicable
1.11 If a material property not listed here is varied, has it been quantified ?		not applicable
1.12 Were characterizations performed in a fluid mimicking biological conditions ?	X (PBS and cell culture media)	
1.13 Are details of how these parameters were measured/estimated provided?	X	
Explanation for No (if needed):		

*Ideally, material characterization should be performed in the same biological environment as that in which the study will be conducted. For example, for cell culture studies with nanoparticles, characterization steps would ideally be performed on nanoparticles dispersed in cell culture media. If this is not possible, then characteristics of the dispersant used (e.g., pH, ionic strength) should mimic as much as possible the biological environment being studied.

Supplementary Table 2. Biological characterization*

Question	Yes	No
2.1 Are cell seeding details , including number of cells plated , confluency at start of experiment , and time between seeding and experiment reported?	X	
2.2 If a standardised cell line is used, are the designation and source provided?	X	
2.3 Is the passage number (total number of times a cell culture has been subcultured) known and reported?		X
2.4 Is the last instance of verification of cell line reported? If no verification has been performed, is the time passed and passage number since acquisition from trusted source (e.g., ATCC or ECACC) reported? For information, see <i>Science</i> 347 (2015) 938; http://doi.org/10.1126/science.347.6225.938		X
2.5 Are the results from mycoplasma testing of cell cultures reported?	X	
2.6 Is the background signal of cells/tissue reported? (E.g., the fluorescence signal of cells without particles in the case of a flow cytometry experiment.)		not applicable
2.7 Are toxicity studies provided to demonstrate that the material has the expected toxicity, and that the experimental protocol followed does not?	X	
2.8 Are details of media preparation (type of media , serum , any added antibiotics) provided?	X	
2.9 Is a justification of the biological model used provided? For examples for cancer models, see <i>Cancer Res.</i> 75 (2015) 4016; http://doi.org/10.1158/0008-5472.CAN-15-1558 , and <i>Mol. Ther.</i> 20 (2012) 882; http://doi.org/10.1038/mt.2012.73 , and <i>ACS Nano</i> 11 (2017) 9594; http://doi.org/10.1021/acsnano.7b04855	X	
2.10 Is characterization of the biological fluid (<i>ex vivo/in vitro</i>) reported? For example, when investigating protein adsorption onto nanoparticles dispersed in blood serum, pertinent aspects of the blood serum should be characterised (e.g., protein concentrations and differences between donors used in study).		not applicable

2.11 For animal experiments , are the ARRIVE guidelines followed? For details, see <i>PLOS Biol.</i> 8 (2010) e1000412; http://doi.org/10.1371/journal.pbio.1000412		not applicable
Explanation for No (if needed):		

*For *in vitro* experiments (e.g., cell culture), *ex vivo* experiments (e.g., in blood samples), and *in vivo* experiments (e.g., animal models). The questions above that are appropriate depend on the type of experiment conducted.

Supplementary Table 3. Experimental details*

Question	Yes	No
3.1 For cell culture experiments: are cell culture dimensions including type of well, volume of added media , reported? Are cell types (i.e.; adherent vs suspension) and orientation (if non-standard) reported?	X	
3.2 Is the dose of material administered reported? This is typically provided in nanomaterial mass, volume, number, or surface area added. Is sufficient information reported so that regardless of which one is provided, the other dosage metrics can be calculated (i.e. using the dimensions and density of the nanomaterial)?	X	
3.3 For each type of imaging performed, are details of how imaging was performed provided, including details of shielding, non-uniform image processing , and any contrast agents added?	X	
3.4 Are details of how the dose was administered provided, including method of administration, injection location, rate of administration , and details of multiple injections ?	X	
3.5 Is the methodology used to equalise dosage provided?		not applicable
3.6 Is the delivered dose to tissues and/or organs (in vivo) reported, as % injected dose per gram of tissue (%ID g ⁻¹)?		not applicable
3.7 Is mass of each organ/tissue measured and mass of material reported?		not applicable
3.8 Are the signals of cells/tissues with nanomaterials reported? For instance, for fluorescently labelled nanoparticles, the total number of particles per cell or the fluorescence intensity of particles + cells, at each assessed timepoint.		not applicable
3.9 Are data analysis details , including code used for analysis provided?		Data analysis details provided, but not code.

3.10 Is the raw data or distribution of values underlying the reported results provided? For examples, see <i>R. Soc. Open Sci.</i> 3 (2016) 150547; http://doi.org/10.1098/rsos.150547 , https://opennessinitiative.org/making-your-data-public/ , http://journals.plos.org/plosone/s/data-availability , and https://www.nature.com/sdata/policies/repositories		X
Explanation for No (if needed):		

* The use of protocol repositories (e.g., *Protocol Exchange* <http://www.nature.com/protocolexchange/>) and published standard methods and protocols (e.g., *Chem. Mater.* **29** (2017) 1; <http://doi.org/10.1021/acs.chemmater.6b05235>, and *Chem. Mater.* **29** (2017) 475; <http://doi.org/10.1021/acs.chemmater.6b05481>) are encouraged.

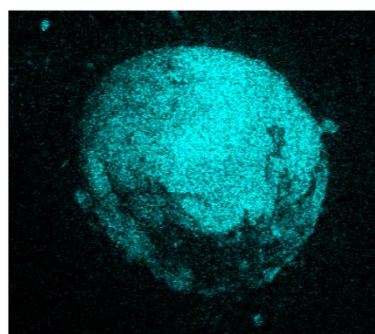
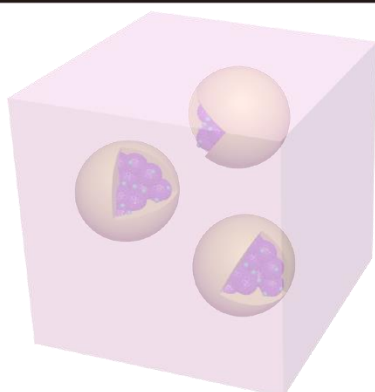
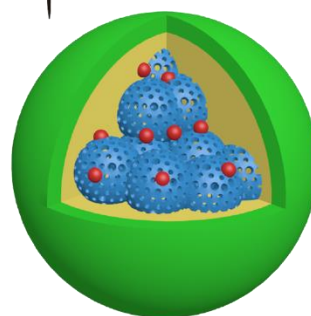
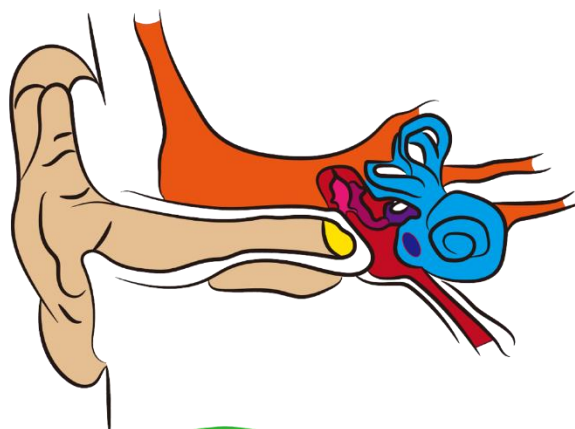
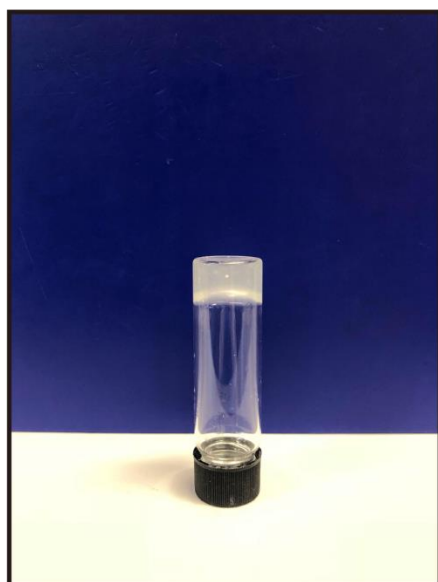
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Chapter 7

Conclusions and Outlook



Over the last few decades, nanoengineered particle-based systems have been applied in various biomedical applications, including drug delivery, imaging and diagnostics. Particularly, nanoengineered particles are a promising candidate for treating sensorineural hearing loss (SNHL) as they can achieve safe drug delivery or specific cell targeting in the cochlea by modification of the particle surface to protect or repair auditory neurons or their synapses. However, translating particle systems from the research laboratory setting to the clinical context for widespread application is challenging because of complex synthesis protocols and specialised skills required to fabricate particles. In this thesis, silica supraparticles (SPs) were synthesised, developed and characterised to address the key challenges in treating SNHL. In Chapter 1, a literature review was presented on pre-clinical strategies and current challenges for treating SNHL. In Chapter 2, the instruments and methods used to fabricate and characterise the SPs were described.

In Chapter 3, a rapid and automatable gel-mediated electrospray approach to fabricate SPs was presented. Primary silica particles were embedded into alginate bead matrices and electrosprayed for the high-throughput synthesis of SPs. The size and shape of the SPs were adjusted reproducibly by changing the parameters of the instrument set-up and sample composition. Primary silica particles without pores, as well as with small pores (2–3 nm) and large bimodal pores (2–3 nm and 15–64 nm) were investigated as building blocks to synthesise SPs. Among the particle systems synthesised, the SPs assembled from the large porous primary silica particles demonstrated the largest loading capacity (10 $\mu\text{g SP}^{-1}$ with a loading concentration of 5.0 mg mL^{-1} FITC-lysozyme and 7.17 $\mu\text{g SP}^{-1}$ with a loading concentration of 1.0 mg mL^{-1} BDNF). The *in vitro* release studies showed that the large porous SPs showed sustained release profiles of FITC-lysozyme for more than 150 days and BDNF for over 40 days. These

findings together with the SPs synthesis technique that is rapid and automatable (important properties for clinical translation and commercialisation) demonstrate that the large porous SPs can serve as promising candidates for long-term and sustained drug delivery, for instance, neurotrophins delivery for the treatment of inner ear diseases.

In Chapter 4, the pharmacokinetics of BDNF released from SPs in the cochlea by either direct implantation at the inner ear or placing the SPs on the round window membrane (RWM) were investigated. The BDNF distribution was investigated over 3 and 7 days. Although the SPs were implanted at the basal turn of the cochlea, the highest concentration of BDNF was achieved at the apex and the distribution of BDNF was bell-shaped. Moreover, this study demonstrated that the BDNF delivery efficiency from SPs was higher than delivery using an osmotic pump via intracochlear implantation. The SPs collected 7 days after RWM implantation displayed some structural damage to the surface of the SPs. However, negligible Si accumulation was observed in different organs, including the heart, liver, intestines, kidney, ventricles, cochlea nucleus, inferior colliculus, of the guinea pig subjects, further indicating the biocompatibility and biodegradability of the SPs. The findings provide a general understanding of the pharmacokinetics of BDNF released from SPs in the cochlea.

In Chapter 5, biocoatings on the SPs were investigated to address the undesirable initial burst release of protein drugs observed from the uncoated SPs. Fibrin-coated SPs showed high biocompatibility and improved drug release behaviour both for FITC-lysozyme and neurotrophins, thus demonstrating the potential of such coating strategy in circumventing fast initial burst release of drugs and potential side effects associated with initial high doses of drugs. Specifically, the fibrin-coated SPs improved the drug release efficiency by more than 6 times compared with the uncoated SPs, where 60%

of protein cargo was released from the fibrin-coated SPs over 20 days and the same amount of protein cargo released from the uncoated SPs in only 3 days. This release behaviour was further adjusted by incorporating the SPs within an alginate CaCO_3 hydrogel (alg- CaCO_3 hydrogel), which also facilitates SP localisation and retention for clinical use. The results demonstrate the versatility of the present SP drug delivery system with biocoatings or hydrogel platforms with tailorable drug release behaviours, thereby facilitating their future clinical application for long-term protein delivery to treat specific disease such as SNHL.

In Chapter 6, another type of film coating on SPs was presented. The layer-by-layer assembly technique was used to deposit biocompatible polysaccharides (i.e. chitosan and alginate) on the surface of the SPs. By adjusting the thickness of the coatings, different *in vitro* release behaviours of chitosan-alginate-coated silica supraparticles ($^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$) were obtained. The released neurotrophins retained their activity, leading to the survival of spiral ganglion neurons (SGNs) and increased neurite length compared to the native control neurotrophins, thus confirming that $^{(\text{Chi}/\text{Alg})}\text{Si-SPs}$ can extend the half-life of neurotrophins. The findings also showed that intracochlear SP delivery results in a significant loss of high frequency hearing in normal hearing, while the hearing thresholds were maintained via RWM SP delivery strategy.

In summary, this body of work demonstrates the application and potential of SPs synthesised via a gel-mediated electrospray technique with tailored properties (high loading capacity, long-term and sustained drug release) for the treatment of SNHL. The SPs are fabricated by embedding primary silica particles into alginate beads followed by calcination to remove organic materials. This electrospray technology can scale up the yield of SPs where thousands of SPs can be fabricated in 30 min (important for clinical translation and commercialisation). The generated SPs are robust and spherical

shape that enables application in long-term and sustained therapeutic delivery. The pharmacokinetics of BDNF released from SPs provide insight into the concentration, therapeutic level and BDNF clearance in the cochlea. Moreover, SPs incorporating a surface biocoating can slow the initial burst release that can overcome problems associated with (i) rapid initial depletion of the drug reservoir and (ii) potential side effects of high initial dosing.

A previous surgical approach has involved the delivery of SPs to the scala tympani or the RWM via the use of a cannula. Although successful for intracochlear delivery, such an approach presented challenges when delivering the SPs to the RWM. Specifically, it is difficult to contain individual particles to the RWM once delivered in saline. To overcome this problem, a new technique was developed using a clinically approved gelation system comprising thrombin and fibronectin. The approach involves the application of the gelation system in combination with the loaded SPs, thus forming a small pellet of gel containing the SPs. The gel pellet can then be easily handled during surgery and lock the SPs in place on the RWM. Once the SP/gel pellet is placed on the RWM, a surgical applicator for the gelation system is used to deliver additional gel to the RW area to secure the pellet in place. This system was successfully applied in trials using guinea pig and cat cochlea.

Despite the tremendous research outcomes generated annually towards the treatment of SNHL, research translation to clinical application remains slow. For instance, inner ear drug delivery via osmotic pumps can potentially introduce infections and toxicity by delivering a high drug concentration in the cochlea. The use of hydrogels alone can lead to burst release of drug because the drug is normally loaded in the hydrogel via entrapment or adsorption in which there is weak binding between drug and the hydrogel matrix. Furthermore, nanoparticles have limitations including the potential to migrate

from the cochlea to the brain via the cochlear aqueduct because of their small size. Moreover, some of the synthesis protocols for nanoparticles are complex and have low yields, which makes it difficult to translate to clinical trials. Therefore, collaborations and interactions are still required among researchers in diverse areas such as mathematics, physics, engineering, chemistry and biology to unlock the promising potential of applying nanoengineered particles to treat SNHL and improve the lives of hearing-impaired patients worldwide.

The SP system can be applied for treating other diseases requiring long-term drug delivery and the size of SPs can be adjusted by changing the parameters of electrospray. This includes applications in fertility or treating ovarian diseases. For example, SPs can be made a similar size as the ovum and loading with drugs such as metformin, spironolactone, eflornithine is possible for achieving sustained and long-term delivery in the female reproductive system to address fertility issues. Moreover, lysozyme and neurotrophins are positively charged proteins, which were loaded into SPs via electrostatic interactions. Other building blocks (including modified cationic silica nanoparticles) can be applied to synthesize SPs to achieve negatively charged protein or nucleic acid loading. The effect of loading and release of other small molecules (besides protein) with hydrophilic or hydrophobic properties should be tested. In addition, pre-loading of drugs into nanoparticles, followed by synthesis of SPs can also be tested and compared with the loading and release behaviour of post-loaded drugs to further understand and exploit the SPs system.