

Bioreactivity of ZnO and Ag Nanostructures in the Lung

Ioannis G. Theodorou

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Abstract

One-dimensional nanomaterials (NMs) have become the focus of intensive research due to their unique physicochemical properties and their application in the fabrication of nanoscale devices. Silver (Ag) and zinc oxide (ZnO) nanowires (NWs) are considered as potential building blocks for the next generation of optical, electronic and sensing devices. However, there is currently limited information on human health effects from Ag and ZnO NW exposures, but concerns have been raised about a potentially greater toxicity compared to their non-fibrous counterparts. Inhalation of Ag and ZnO NWs represents one of the primary routes for human exposure. Due to their size, these NMs are expected to preferentially deposit in the alveolar region of the lungs, where any damage would not only impact pulmonary homeostasis but would also determine possible translocations to other organs. Therefore, the interactions of Ag and ZnO NWs with the pulmonary epithelium urgently need to be addressed, in order to predict their adverse effects and provide guidelines for their safe use.

Along with concerns about the toxic effects of Ag NMs, there is also potential for enormous benefit *via* highly efficacious antibacterial modality. Bacterial infections of the pulmonary system, such as tuberculosis, are increasing; especially infections caused by bacterial strains resistant to existing conventional antibiotics. AgNPs represent exciting candidates for the development of novel platforms for the treatment of pulmonary bacterial infections. Whilst the biological activity of silver has been well demonstrated, the mechanisms by which AgNPs degrade at the bio-nano interface and how these processes correlate to their bioreactivity are poorly understood.

The motivation of this thesis is to investigate the bioreactivity of Ag and ZnO nanostructures in the lungs. The main objective of the work presented here is to examine the bioreactivity of Ag and ZnO NWs with human lung epithelial cells from the perspective of their putative toxicity. For inhaled NMs that reach the alveoli, the lung lining fluid (LLF) that covers their surface represents a first line of defence of the lungs. Therefore, the emphasis of this work is placed on characterizing the impact of the LLF on the physicochemical properties of Ag and ZnO NWs. The formation of a phospholipid/protein corona is shown to modify the ion release kinetics and aggregation states of the NWs. These effects are correlated with the cellular uptake and toxicity of the NWs to human lung epithelial cells. Furthermore, the mechanisms by which Ag and ZnO NWs degrade within epithelial cells are investigated. In the case of AgNWs, intracellular dissolution precedes the precipitation of

highly insoluble silver sulfide, which could significantly limit their short-term toxicological effects. ZnO NWs, on the other hand, rapidly dissolve intracellularly and cell death correlates with the amount of free Zn^{2+} ions released.

Furthermore, the antibacterial effects of AgNPs against *Mycobacterium tuberculosis* (*M.tb*) are examined. A set of analytical transmission electron microscopy techniques are employed to investigate the ultrastructural damage in bacterial cells and the mechanisms by which AgNPs induce cell damage. The antimicrobial effects of AgNPs are also examined in *M.tb*-infected macrophages, to explore the potential use of AgNPs as therapeutic agents for pulmonary bacterial infections.

Author's Declaration

The present thesis is a record of the work carried out by me in the Department of Materials and the London Centre for Nanotechnology at Imperial College London, UK. The work described herein is my own, unless otherwise stated. Wherever contributions of others are involved, every effort is made to indicate this, with references to the literature and acknowledgements of collaborative research. Some of the work presented in this thesis has been published in the following journal articles:

Theodorou, I. G.; Botelho, D.; Schwander, S.; Zhang, J.; Chung, K. F.; Tetley, T. D.; Shaffer, M. S.; Gow, A.; Ryan, M. P.; Porter, A. E. Static and Dynamic Microscopy of the Chemical Stability and Aggregation State of Silver Nanowires in Components of Murine Pulmonary Surfactant. *Environmental Science & Technology* **2015**, 49, 8048-56.

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Theodorou, I. G.; Botelho, D.; Schwander, S.; Zhang, J.; Chung, K. F.; Tetley, T. D.; Shaffer, M. S.; Gow, A.; Ryan, M. P.; Porter, A. E. Static and Dynamic Microscopy of the Chemical Stability and Aggregation State of Silver Nanowires in Components of Murine Pulmonary Surfactant. *Environmental Science & Technology* **2015**, 49, 8048-56.

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List of Abbreviations

AgNP	Silver Nanoparticle
AgNW	Silver Nanowire
BAL	Bronchoalveolar Lavage
BFTEM	Bright Field Transmission Electron Microscopy
BSA	Bovine Serum Albumin
CNT	Carbon Nanotube
DI	Deionized
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DPPC	Dipalmitoylphosphatidylcholine
EDX	Energy Dispersive X-Ray Spectroscopy
ENM	Engineered Nanomaterial
FIB	Focused Ion Beam
HAADF	High Angle Annular Dark Field
HAS	Harvested Alveolar Surfactant
HRTEM	High Resolution Transmission Electron Microscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
LA	Large Aggregate (fraction of the bronchoalveolar lavage)
LLF	Lung Lining Fluid
LSCM	Laser Scanning Confocal Microscopy
MWCNT	Multi-Walled Carbon Nanotube
NCS	Newborn Calf Serum
NM	Nanomaterial
NP	Nanoparticle
NW	Nanowire
PBS	Phosphate Buffered Saline
PS	Pulmonary Surfactant
PVP	Polyvinylpyrrolidone
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
SA	Small Aggregate (fraction of the bronchoalveolar lavage)
SAED	Selected Area Electron Diffraction
SEM	Scanning Electron Microscopy
SP-A/B/C/D	Surfactant Protein A/B/C/D
STEM	Scanning Transmission Electron Microscopy
SWCNT	Single-Walled Carbon Nanotube
TEM	Transmission Electron Microscopy
UA	Uranyl Acetate
UV-Vis	Ultraviolet Visible Spectroscopy
ZnONP	Zinc Oxide Nanoparticle
ZnONW	Zinc Oxide Nanowire

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1 Introduction

The rapidly expanding and advancing field of nanotechnology is expected to revolutionize many aspects of human life, offering extraordinary opportunities in various technological fields. The unique physical and chemical properties of materials that emerge at the nanoscale could be harvested to enable a wide range of novel applications, such as in medicine, electronics, biomaterials, energy production, and consumer products. However, engineered nanomaterials (ENMs) have also raised concerns about their potential harmful effects on human health and the environment. At the cellular level, ENMs may resemble biomolecules or biomolecular assemblies in terms of their function. Studies suggest that ENMs are not inherently benign but may affect biological behaviours at the cellular, sub-cellular and protein levels. These concerns have led to an urgent need to establish accurate risk assessment procedures and a tighter regulation of ENMs, ensuring their safe manufacture and use.

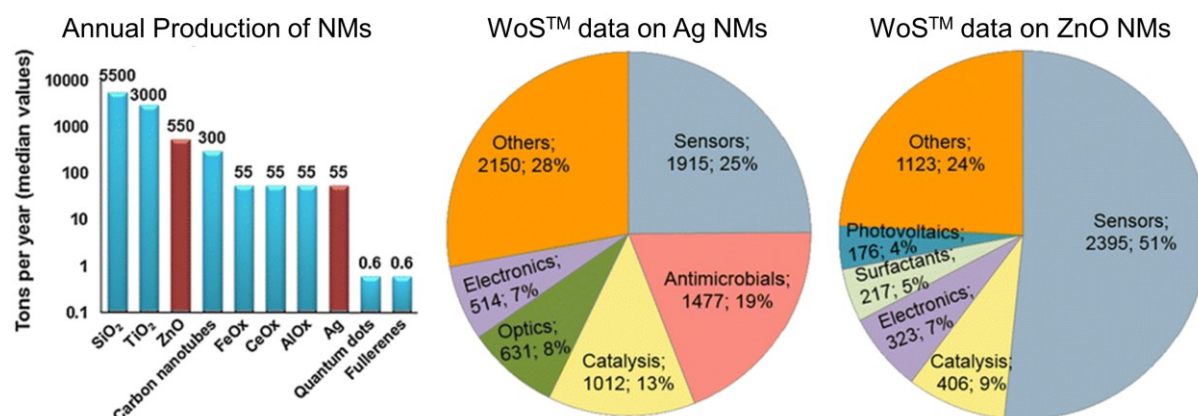


Figure 1.1 Annual production volumes of different classes of NMs and fields of application of Ag and ZnO NMs, based on the number of publications indexed by Thomson Reuters™ Web of Science™ (search performed in March 2013). Reprinted with permission from ¹. Copyright 2013 Bondarenko *et al.*

Silver (Ag) and zinc oxide (ZnO) nanomaterials (NMs) represent two of the most important classes of ENMs at the forefront of application-driven nanotechnology. The well-known antibacterial

properties and the unique optoelectronic properties of Ag NMs have led to a dramatic increase in their industrial production and implementation in consumer products, which range from electronic and photonic devices to textiles, food storage containers and antiseptic sprays (**Figure 1.1**). The median production volumes of Ag NMs are about 55 tons/year worldwide (**Figure 1.1**) and 5.5 tons/year in Europe.² Similarly, nanostructures of ZnO, which is a wide band gap semiconductor with a high exciton binding energy and piezoelectric properties, are being used for optoelectronic devices, energy harvesting devices, electronic devices, sensors and catalysts (**Figure 1.1**). The worldwide annual production of ZnO NMs is estimated to be around 550 tons/year (**Figure 1.1**), with 55 tons/year produced in Europe.² Despite the widespread use of Ag and ZnO NMs, their potential adverse effects to human health upon exposure are still unclear. Exposures are expected to be higher in occupational settings, during production and packaging of these NMs. However, their increasing application in consumer products may also increase general public exposures in the coming years.

Along with concerns about the toxic effects of Ag and ZnO NMs, there is also potential for enormous benefit *via* the development of novel strategies for biomedical applications. Both materials exhibit antibacterial activities, therefore they are considered as potential therapeutic agents that could have a significant impact on medical research, by offering multifunctional platforms that simultaneously diagnose and treat bacterial infections. Moreover, efforts are underway to exploit the properties of Ag and ZnO NMs for cancer detection and therapeutic intervention, through targeted drug delivery or selective destruction of tumour cells.

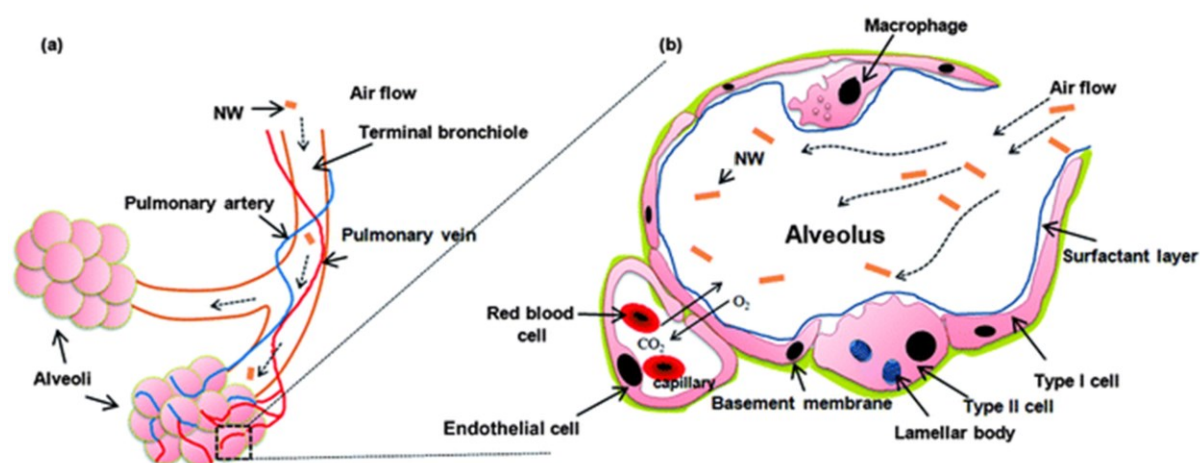


Figure 1.2 (a) A schematic diagram showing the deposition of inhaled particles into the pulmonary alveolar region, comprising the respiratory bronchioles, alveolar ducts and alveoli. Abundant pulmonary capillaries run through the alveoli to allow air-blood gas exchange. (b) A schematic diagram of the cross section of a single alveolus. The alveolar epithelium consists of type 1 and type 2 epithelial cells, and alveolar macrophages, which are covered with a surfactant layer produced by type 2 cells. Gas exchange takes place at the epithelial–endothelial gas–blood barrier, which consists of a mixed monolayer of type 1 and type 2 cells, which share a basement membrane with the underlying capillary endothelium. Reprinted with permission from ³. Copyright 2013 The Royal Society of Chemistry.

Inhalation of Ag and ZnO NMs represents a key route of human exposure, both from the perspective of intentional (diagnostic and therapeutic applications) and unintentional scenarios. Upon inhalation, the small size of NMs enables them to access the distal areas of the lungs, and deposit in the gas-exchange region of the alveoli (**Figure 1.2**). On the one hand, this ability of NMs to reach the alveoli could allow for more efficient delivery of therapeutic agents in the lungs. On the other hand, particle clearance may be insufficient in the alveoli, giving rise to a higher probability of adverse health effects due to continuous particle-tissue interactions. Once NMs reach the alveoli, further barriers to diffusion into the blood circulation are limited because the epithelial-endothelial gas-blood barrier is very thin. Consequently, any cellular or protein damage in this region could not only have an impact on pulmonary homeostasis but would also determine possible translocations of NMs to other organs, enabling them to elicit toxic effects at extrapulmonary sites. Therefore, it is paramount to understand the interactions of Ag and ZnO NMs with the alveolar epithelium, in order to anticipate their adverse effects and manage the risk of their toxicological potential.

Both Ag and ZnO NMs have been shown to cause inflammation in the lung *in vivo* following inhalation. In inhalation studies using rodents, Ag nanoparticles (NPs) induced pulmonary inflammation with mixed cellular infiltrates and decreased minute volumes, leading to the development of several types of pulmonary function abnormalities. Similar studies showed that ZnO NPs caused eosinophilia, proliferation of airway epithelial cells and pulmonary fibrosis. Prolonged inhalation exposures to ZnO NPs have also been linked with degeneration and necrosis of the myocardium. Meanwhile, the toxicity of Ag and ZnO NMs has been reported for various *in vitro* mammalian systems, including lung epithelial cells and alveolar macrophages. Toxic responses included the generation of reactive oxygen species (ROS) and oxidative stress, apoptosis or genotoxicity.

Among the physicochemical properties of Ag and ZnO NMs, shape and size are important factors in determining their uptake mechanisms and the long term ramifications of NM exposures. One-dimensional (1D) NMs such as nanowires (NWs), rods and tubes have recently become the focus of intensive research, due to their unique applications in mesoscopic physics and the fabrication of nanoscale devices. However, there are currently few published studies on Ag or ZnO NW exposure-related health effects. Fibrous nanomaterials, such as carbon nanotubes (CNTs), have raised particular concerns due to their similarity with asbestos fibres in the lung and the induced mesothelioma. In contrast to CNTs, which are considered as a bio-persistent NM, the bioreactivity of Ag and ZnO NMs has been linked with their dissolution and release of Ag⁺ or Zn²⁺ ions. Some studies have already examined AgNWs with respect to their uptake mechanisms by alveolar macrophages, and the onset of frustrated macrophage phagocytosis has been stated as an important factor in the post-exposure inflammatory response. However, the mechanisms by which Ag or ZnO NWs interact with cells and how these NWs transform within cells over time is poorly understood. Moreover, the bioreactivity of

Ag or ZnO NWs with alveolar epithelial cells, which account for over 95% of the alveolar surface and are a major target of inhaled NMs, has hardly been investigated.

In order to draw accurate conclusions about the bioreactivity of Ag and ZnO NWs, further efforts are also required to understand the stability of these NMs and their dissolution kinetics in biological environments. Their properties can change drastically when incubated in biological media, leading for example to aggregation or the formation of biomolecule coronas. Additionally, several factors may affect ion release kinetics, such as particle size and shape, pH, complexing anions or organic components. Therefore, NM characterization at the local *point of exposure* in the cellular microenvironment is required. In the case of alveolar epithelial cells, the cells are covered by a thin liquid layer called the lung lining fluid (LLF), which is a complex mixture of phospholipids and proteins. Its main component is pulmonary surfactant (PS), which forms a monolayer at the air-liquid interface. Following deposition of inhaled Ag or ZnO NWs in the alveoli, the adsorption of LLF components on their surface may affect their physicochemical properties and their subsequent cellular effects. Therefore, the effects of the LLF on the physicochemistry of Ag and ZnO NWs need to be examined, because they will determine their interactions with proteins, cells and tissues in the lung.

The purpose of this thesis is to examine the *in vitro* bioreactivity of Ag and ZnO NWs with human alveolar epithelial cells. In previous studies in the literature, LLF components have not been commonly integrated in the *in vitro* testing of NMs. In the present work, LLF interactions are central in the experimental design, to provide an improved model of *in vivo* exposure scenarios. More specifically, the impact of individual LLF components on the dissolution kinetics and physicochemical properties of Ag and ZnO NWs is reported. These findings are correlated with the toxicity and cellular uptake and transformation of the NWs with human alveolar epithelial cells in the presence of LLF. In addition to these studies, the antibacterial activity of AgNPs against *Mycobacterium tuberculosis* (*M.tb*) is investigated, and the potential use of AgNPs as therapeutic agents in *M.tb*-infected alveolar macrophages is explored.

First, an overview of the literature regarding the bioreactivity of Ag and ZnO NMs is provided (Chapter 2). The focus of the literature review is placed on describing the mechanisms by which the dissolution kinetics and physicochemical properties (*e.g.* aggregation state, morphology and chemistry) of these NMs change in various biological milieu (*i.e.* relevant proteins, lipids and other molecules, and biofluids, such as the LLF) and how subsequent interactions with and within cells are affected. Furthermore, the antibacterial mechanisms of AgNPs are discussed in relation to the mechanistic information that a range of microscopy techniques (including transmission and scanning electron microscopy and 3D confocal microscopy) can provide about the bio-nano interface.

In Chapter 3, the principal experimental techniques employed to complete the work presented in this thesis are briefly described.

Next, the stability of AgNWs in the LLF and the subsequent effects on their bioreactivity with human alveolar epithelial cells are discussed in Chapter 4. The synthesis and characterization of AgNWs is reported and the effects of individual components of the LLF (phospholipids and proteins) on the physicochemical properties of AgNWs are presented. Moreover, the influence of the LLF on the cellular uptake and intracellular transformation of AgNWs within human alveolar epithelial cells is described.

Chapter 5 represents a first attempt to examine AgNPs as antibacterial agents against *M.tb*-infected alveolar macrophages. A range of analytical transmission electron microscopy (TEM) techniques are employed to provide information about bacterial infections and the mechanisms through which AgNPs induce bacterial cell damage.

In Chapter 6 the impact of PS on the stability of ZnO NWs and their toxicity to human alveolar epithelial cells is examined. The dissolution kinetics and physicochemical properties of commercially available ZnO NWs are characterized in the absence and presence of PS and serum. Moreover, the effects of pulmonary surfactant on the cellular uptake and intracellular dissolution of ZnO NWs, in relation to the viability of human alveolar epithelial cells, are discussed.

Chapter 7 reports the synthesis and characterization of ZnO NWs using two variations of electrochemical deposition, in an effort to obtain ZnO NWs with well-controlled physicochemical properties for further biological studies.

Finally, the key conclusions stemming from the present work are summarized in Chapter 8, along with suggestions for future studies.

2 Literature Review

2.1 Nanotechnology

Nanotechnology is the design, characterization, production and application of structures, devices and systems by controlling their shape and size at dimensions between 1 and 100 nm. At this size scale, unique physical and chemical properties emerge, different from the properties of the same material in bulk form, which can be exploited to enable a wide range of novel applications.⁴ Nanotechnology is expected to revolutionize many aspects of human life, offering extraordinary opportunities in various technological fields such as electronics, energy management and information technology, as well as the pharmaceutical and medical fields. Nanotechnology is a rapidly expanding and advancing field; according to a recent study, the annual worldwide market for products incorporating nanotechnology is expected to reach US \$3.3 trillion by 2018.⁵

2.2 Nanotoxicology

Natural nanoparticles (NPs) exist in all ecosystems and humans have been exposed to them throughout their evolutionary stages.⁶ With the advent of the industrial revolution, however, such exposures have increased dramatically due to anthropogenic sources, including internal combustion engines, power plants and other sources of thermo-degradation.⁷ The rapid development of nanotechnology is likely to become another source of human exposure to engineered NMs.⁷ **Table 2.1** summarizes some of the natural and anthropogenic sources of nanomaterials (NMs).⁷ In contrast to naturally occurring NPs that have a polydisperse nature, engineered NPs can be synthesized in highly homogeneous forms of desired sizes and shapes. They have unique physicochemical properties, attributable to their small size, chemical composition, surface chemistry, solubility and shape. Although these properties are what make NMs so interesting from the point of view of fundamental or applied research, they also raise concerns about their potential harmful effects on living organisms. At the cellular level, biological systems include structural arrangements that resemble NMs in terms of their function. Studies suggest that NMs are not inherently benign and that they affect biological

behaviours at the cellular, sub-cellular and protein levels.⁸ Therefore, NMs need to be evaluated in terms of their potential harmful effects on human health and the environment.

Natural	Anthropogenic	
	Unintentional	Intentional (NPs)
Gas-to-particle conversions	Internal combustion engines	Controlled size and shape, designed for functionality
Forest fires	Power plants	Metals, semiconductors, metal oxides, carbon, polymers
Volcanoes (hot lava)	Incinerators	Nanospheres, -wires, -needles, -tubes, -shells, -rings, -platelets
Viruses	Jet engines	
Biogenic magnetite: magnetotactic bacteria	Metal fumes (smelting, welding, <i>etc.</i>)	
protocists, mollusks, arthropods, fish, birds, human brain, meteorite (?)	Polymer fumes	Untreated, coated (nanotechnology applied to many products: cosmetics, medical, fabrics, electronics, optics, displays, <i>etc.</i>)
Ferritin (12.5 nm)	Other fumes	
Microparticles (< 100 nm; activated cells)	Heated surfaces	
	Frying, broiling, grilling	
	Electric motors	

Table 2.1 Natural and anthropogenic sources of exposure to ultrafine particles (UFPs) and engineered nanoparticles (NPs). Reproduced with permission from ⁷.

Nanotoxicology can be defined as “the science of engineered nanodevices and nanostructures that deals with their effects in living organisms”.⁷ Nanotoxicology emerged in the early 1990s, evolving from earlier studies on the pulmonary and other effects of ambient ultrafine particles.⁷ There was a lag of about 10 years before the number of nanotoxicological papers started to increase exponentially (**Figure 2.1**). Lam *et al.* and Warheit *et al.* reported the first peer-reviewed comparative toxicological assessments of nanotubes and quartz nanoparticles.^{9, 10} Lam *et al.* examined histopathological alterations in mice at 7 and 90 days following exposures to single-walled carbon nanotubes (SWCNTs) manufactured by different methods, demonstrating that all types of SWCNTs induced dose-dependent lung lesions.⁹ Warheit *et al.* found that SWCNTs could induce pulmonary inflammation and alter lung cellular proliferation and pathology.¹⁰ In contrast to quartz particles, SWCNTs induced multifocal granuloma formation, which occurred without evidence of ongoing pulmonary inflammation or cellular proliferation.¹⁰ Moreover, rodents developed asbestos-like effects following intracavitary injection of high doses of multi-walled carbon nanotubes (MWCNTs).¹¹ Dhawan and Sharma summarized the toxicological effects reported in around 100 *in vivo* and *in vitro* studies of the most common NMs, including SWCNTs and MWCNTs, fullerenes and Au, Ag and metal oxide nanoparticles.¹² The toxic effects reported ranged from induction of inflammatory responses and oxidative stress to DNA damage and genotoxicity.¹² In another review that evaluated the existing literature data on the toxicity of synthetic NMs, Ag and ZnO NMs were classified as

“extremely toxic”, fullerenes and CuO NMs as “very toxic”, SWCNTs and MWCNTs as “toxic” and TiO₂ NMs as “harmful”.⁶ Remarkably, none of the NMs tested was classified as “not harmful”.⁶

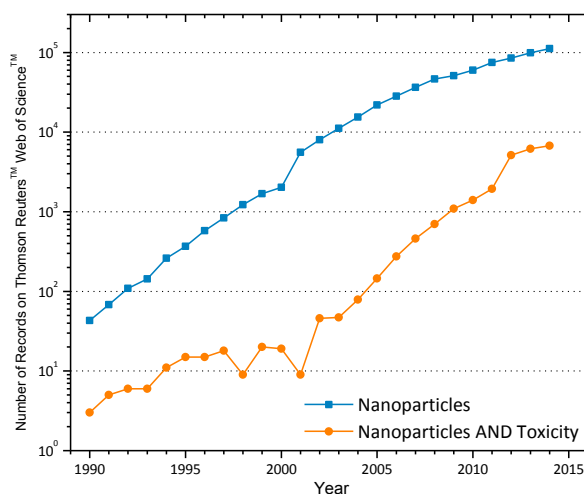


Figure 2.1 Number of records retrieved from Thomson ReutersTM Web of ScienceTM. The search was performed on April 8, 2015 using the keywords 'nanoparticles' and 'nanoparticles AND toxicity' (field: topic).

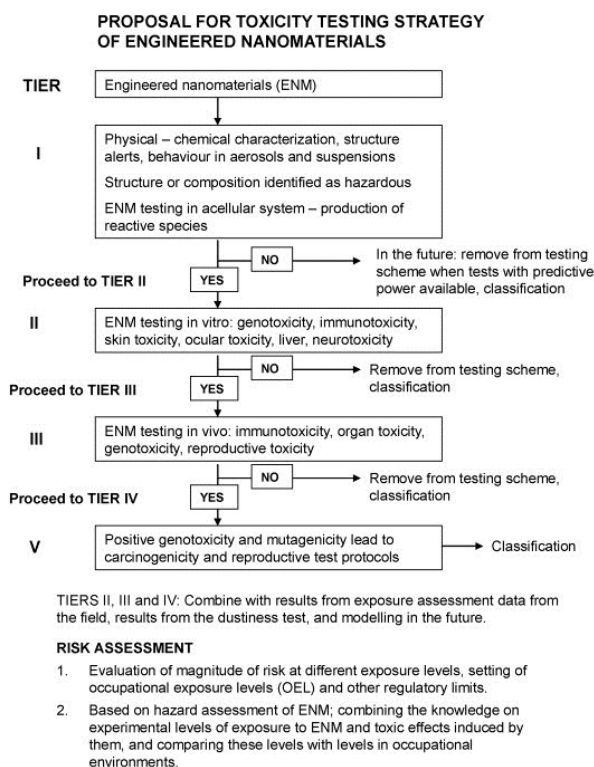


Figure 2.2 Schematic representation of a proposed tiered testing approach for engineered NMs. Reprinted with permission from ¹³. Copyright © 2010 Elsevier Ireland Ltd.

Consequently, there is an urgent need to establish the principles and test procedures that will ensure the safe manufacture and use of NMs. It is admittedly impossible to perform risk assessment without *in vivo* toxicological data. However, it is becoming increasingly clear that animal testing may not provide the best base test method when confronted with thousands of new NMs, from a cost, labour intensity or ethical point of view. A predictive *in vitro* toxicological screening approach that would rank NMs for priority of *in vivo* testing should be developed.⁸ Oberdörster *et al.* formulated three key elements that nanoparticle toxicity screening strategies should follow: (i) physicochemical characterization of the particles (size, surface area, shape, solubility and aggregation) and elucidation of their biological effects using (ii) *in vitro* and (iii) *in vivo* studies.⁷ Savolainen *et al.* proposed a detailed toxicity testing strategy of NMs based on a tiered approach (**Figure 2.2**).¹³

2.2.1 Physicochemical Properties of Nanomaterials that Determine their Biological Activity

Numerous physicochemical parameters are thought to determine the biological activity and biokinetics of NMs, such as size, shape, crystalline structure, chemical composition, surface properties (*i.e.* area, porosity, charge and surface modifications), agglomeration state, biopersistence and dose. However, toxicity cannot be wholly attributed to one single parameter. The cellular uptake, protein binding, translocation from portal of entry to target site and the possibility of causing tissue injury may be affected by all these factors. The most prominent property of NMs is their small size, which falls in the transitional zone between individual atoms or molecules and the corresponding bulk material. Their small size can facilitate their uptake and interaction with biological tissues, as size is a key factor that determines translocation across cellular barriers.¹⁴ A decrease in size also leads to an increase in surface area, and this allows for a larger proportion of atoms or molecules to be displayed on the surface rather than the bulk of the material. The number of atoms/molecules on the surface of a particle determines the material's reactivity; therefore smaller particles might have an increased chemical and biological reactivity.⁷ Data from several studies indicate that it may be possible to predict the acute *in vivo* inflammatory potential of NPs using *in vitro* cell-free and cellular assays based on NP surface area dose-response metrics.^{11, 15, 16}

A decrease in size may also create discontinuous crystal planes that increase the number of structural defects or disrupt the well-structured electronic configuration of the material, giving rise to altered electronic properties.^{7, 17} Surface groups can make NMs hydrophobic or hydrophilic, or catalytically active or passive. An example of how these surface properties could lead to toxicity is the interaction of electron donor or acceptor active sites with molecular oxygen (O₂) and the generation of reactive oxygen species (ROS). Single-component materials or the presence of transition metals on the material's surface can participate in the formation of such active sites.⁸ Several nanomaterial characteristics can culminate in ROS generation, which is currently the best-

developed paradigm for NP toxicity.¹⁸ Research has suggested that there is a direct relationship between the ROS-generating capability of NPs and their pro-inflammatory effects in the lung.⁸

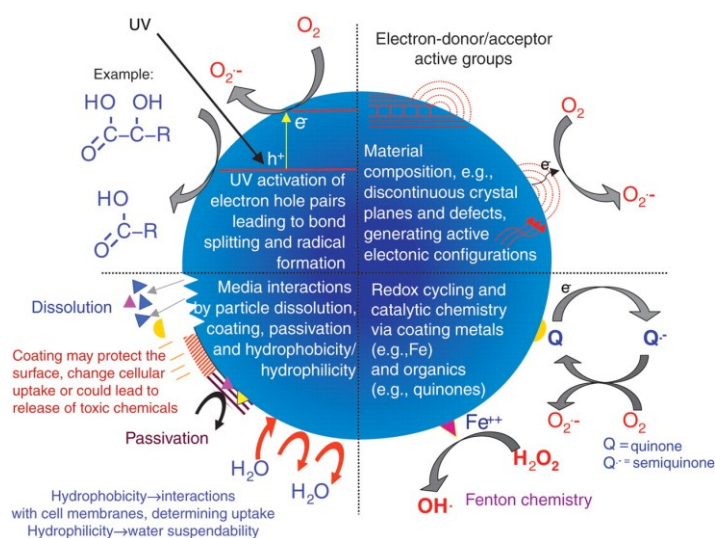


Figure 2.3 Possible mechanisms by which NMs may interact with biological tissue. Examples illustrate the importance of material composition, electronic structure, bonded surface species (*e.g.* metal-containing), surface coatings (active or passive), and solubility, including the contribution of surface species and coatings or interactions with other environmental factors (*e.g.* UV activation). Reprinted with permission from ⁸. Copyright 2006 American Association for the Advancement of Science.

In addition to the paradigm of oxidative stress and inflammation, other nanomaterial interactions may result in other forms of injury such as protein denaturation, membrane damage, immune reactivity and the formation of foreign body granulomas (**Figure 2.3**). Therefore, other NM properties such as shape, aggregation, surface coating and solubility may also affect their toxic potential, either diminishing or amplifying the size effects. For instance, shape is an important factor in determining the uptake mechanisms and the long-term ramifications of NM exposure. High aspect ratio or fibre-shaped NMs, such as nanotubes and nanowires have been studied with respect to their uptake mechanisms. The onset of frustrated macrophage phagocytosis has been stated as an important factor in the post exposure inflammatory response. CNTs are the best known and most widely studied of the fibrous NMs and emerging evidence supports that they may lead to similar pathologies (*e.g.* mesothelioma) as those induced by inhaled asbestos fibres.^{19,20} The increasing demand for other types of fibrous NMs, including AgNWs and ZnONWs, means that these materials should also receive similar toxicological scrutiny. In the case of Ag and ZnO NMs, solubility is another important factor to consider when investigating their bioreactivity. The observed toxicity of AgNPs has often been ascribed to the release of the Ag⁺ cation and its soluble complexes.²¹ Similarly, ZnONPs were more toxic than other non-soluble metal oxides and this was attributed to their dissolution in biologic environments and the release of toxic Zn²⁺ ions.^{22, 23} The current literature and the gaps or

discrepancies in published results regarding the bioreactivity of Ag and ZnO NMs are discussed in detail in Chapters 2.3 and 2.4.

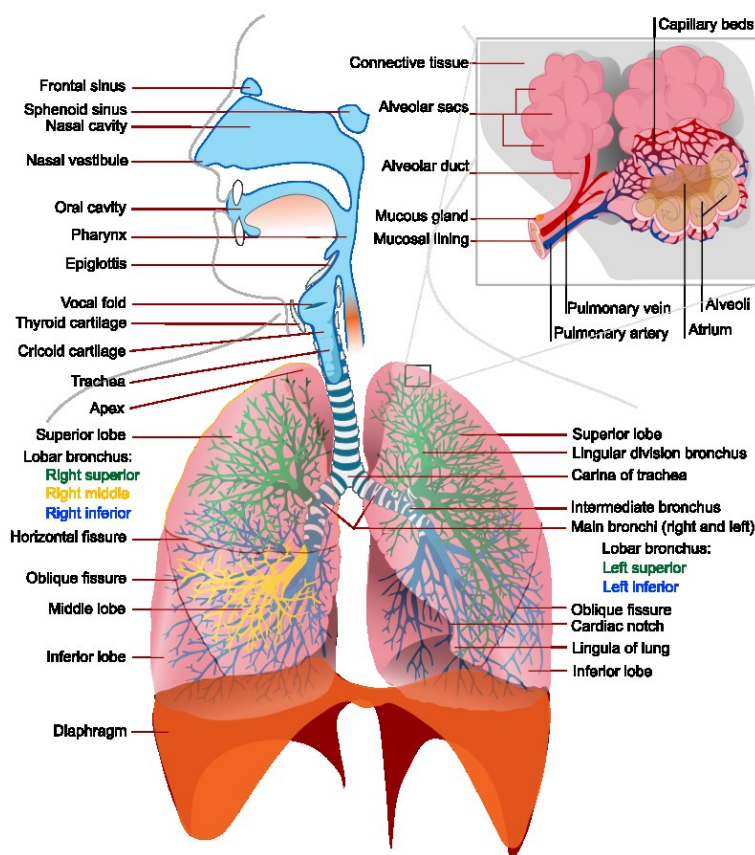


Figure 2.4 Schematic representation of the human respiratory system, which includes the airways, lungs, and the respiratory muscles that mediate the movement of air into and out of the body.

2.2.2 Pulmonary Exposure to Nanomaterials

Nanomaterials can enter the human body by different routes such as inhalation (respiratory tract), ingestion (gastrointestinal tract), through the skin or injection (blood circulation).⁷ They may then translocate to the blood circulation and reach secondary targets of interaction, causing adverse biological reactions in several organs.²⁴⁻²⁶ Nanomaterial interactions with cells, body fluids and proteins will determine their biological effects and their ability to distribute throughout the body. For example, NM binding to proteins may generate complexes that are more mobile in blood circulation and are able to enter tissue sites that would normally be inaccessible. Accelerated protein denaturation or degradation on the NP surface may lead to protein functional and structural changes, inducing an interference with enzyme function.²⁷ Throughout the human body, harmful materials encounter a number of defences during their uptake or transport, which can eliminate, sequester or dissolve them.

In addition, cells and tissues have effective antioxidant defences that deal with ROS generation.²⁸ However, these defences may not always be effective in the case of NMs.

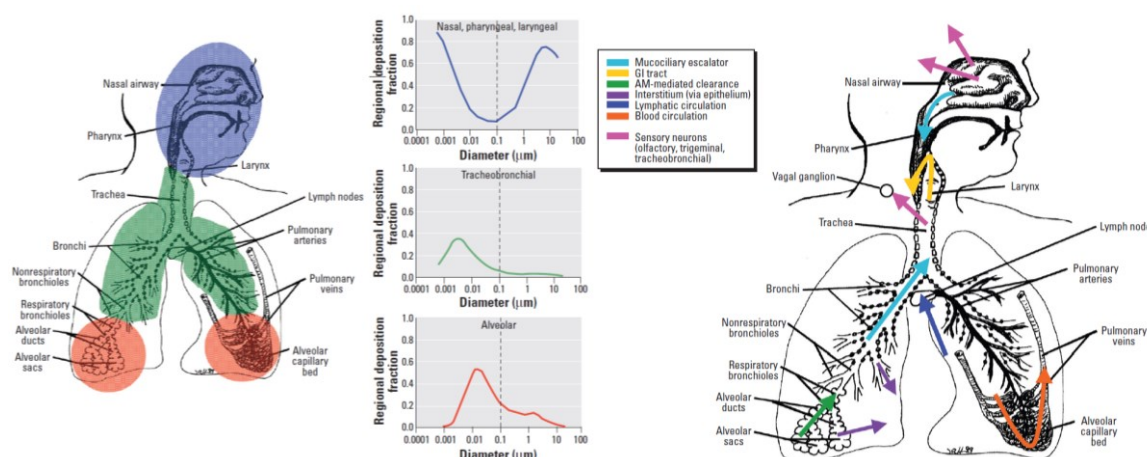


Figure 2.5 (a) Predicted fractional deposition of inhaled particles in the nasopharyngeal (blue), tracheobronchial (green) and alveolar region (red) of the human respiratory tract, under conditions of nose breathing during rest. Based on data from the International Commission on Radiological Protection (1994). (b) Pathways of particle clearance (disposition), in and out of the respiratory tract. Reprinted with permission from ⁷.

Since the pulmonary system is in continual contact with the environmental atmosphere and the pollutants present in it, one of the most important routes of exposure to NMs to consider is inhalation (**Figure 2.4**). Pulmonary exposure could be especially high during manufacture or handling of freely dispersible NPs. The human lungs consist of approximately 2300 km of airways and 300 million alveoli, giving rise to a surface area of about 150 m². This vast internal surface area facilitates a broad access of inhaled materials to the lung, which cannot always deal adequately with the wide range of airborne materials present in urban or occupational environments.²⁹ The most critical characteristic determining the deposition patterns of particulates in the respiratory tract is the particle size distribution (**Figure 2.5a**).²⁹ Larger particles (5–30 μm) are usually deposited in the nasopharyngeal region by the inertial impaction mechanism and smaller particles (1–5 μm) in the tracheobronchial region, mainly due to sedimentation. These particles are typically cleared by bodily responses, such as coughing, mucociliary transport and/or phagocytosis by alveolar macrophages (**Figure 2.5b**). The major deposition mechanism for particles smaller than 0.5 μm is diffusion; therefore NPs can penetrate deeply into the alveolar region, where clearance may be insufficient.^{29–31} The deeper the particle deposition, the longer it takes for pulmonary particle clearance; it is believed that this might give rise to a higher probability of adverse health effects due to continuous particle-tissue interactions.³² Additionally, phagocytosis by alveolar macrophages to remove inhaled NPs appears to be less efficient than for larger particles.^{33, 34}

For fibrous NMs, like Ag and ZnO NWs, their width or diameter is their key parameter that affects their deposition pattern in the lungs. This is due to the central role of fibre diameter in controlling the aerodynamic diameter (D_{ae}) and the dependence of pulmonary deposition on D_{ae} .³⁵ Length impacts little on D_{ae} for thin fibres;³⁶ the alignment of fibres along the stream lines of inhaled air may allow fibres of several μm in length to penetrate inside the alveoli. There are currently limited computational or experimental data on the fractions of inhaled NWs that may be deposited in the deep lung. According to one model for the deposition and clearance of rigid fibres in the human respiratory tract, fibres with diameters $< 100\text{ nm}$ - independent of their length and the breathing conditions - preferentially deposit in intermediately to terminally situated lung airways, with a peak alveolar deposition between 10% and 20%.³⁷ Similarly, a recent semi-analytical model describing the motion of nanofibres has shown that those with diameters in the range of 10-100 nm and lengths in the order of μm have the highest possibility to reach the gas-exchange area of the deep lung.³⁸

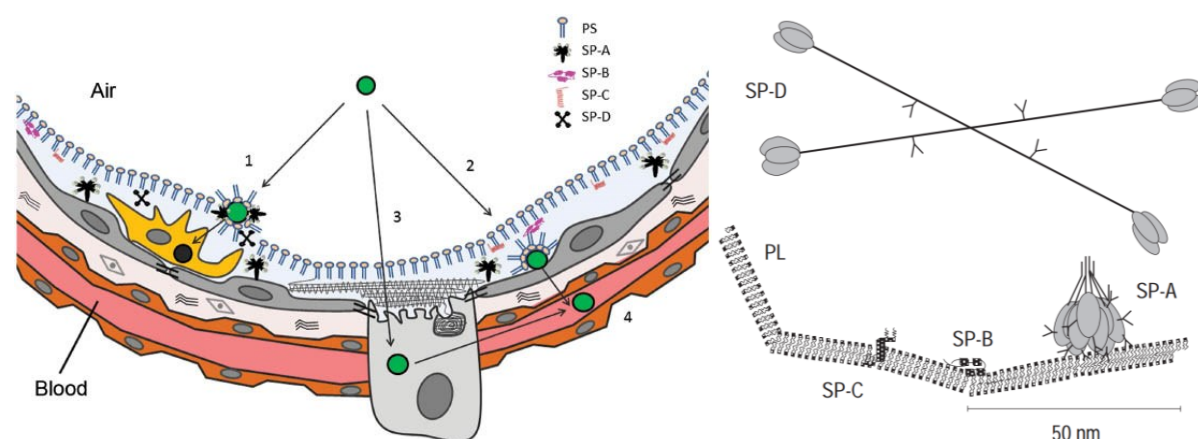


Figure 2.6 (a) Schematic diagram of the air-blood barrier at the alveolar region of the lungs. The air-blood barrier consists of a layer of pulmonary surfactant, composed of phospholipids (PL) and surfactant proteins (SP-A, B, C and D), followed by the tightly joined alveolar epithelium, which is built up by Type 1 (in grey) and the surfactant-producing Type 2 pneumocytes (in lighter grey). Underneath the alveolar epithelium, endothelial cells (in brown) represent the last cellular barrier before the bloodstream is reached. The fate of NPs may significantly be influenced after a first contact with the surfactant layer; PLs and SPs might adsorb to the surface of NPs, altering their clearance by alveolar macrophages (in yellow) (1). Alternatively, NPs may be immediately displaced to the hypophase, coming into close contact with the alveolar epithelium and internalized by Type 1 (2) or Type 2 pneumocytes (3). Eventually, NPs internalized by epithelial cells could penetrate into the capillaries, reaching the blood circulation (4). Reprinted with permission from ³⁹. (b) Schematic structure and relative size of the principal components of pulmonary surfactant. PLs are shown in the form of a bilayer, omitting the various other structural forms of organization, *e.g.* vesicles, sheets, tubular myelin, and lamellar bodies. SP-A is shown as an octadecamer with a bouquet-like appearance, and SP-D is depicted as a dodecamer with a cross-like structure. The carbohydrate recognition domains of these two proteins are shown as globular formations. SP-B is shown as a monomer, although a significant amount of dimers are present in the airspaces. SP-C is depicted as an integral membrane monomer. Reprinted with permission from ⁴⁰. Copyright 1999 ERS Journals Ltd.

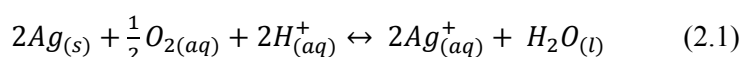
Nanoparticles and NWs that effectively access the alveolar region of the lungs will come into contact with the alveolar epithelium, where they could be taken up by epithelial cells and fibroblasts. Once NMs reach the alveoli, further barriers to diffusion into the blood circulation are limited. This is because the epithelium that separates the inhaled air from the blood capillaries is very thin ($<0.5\ \mu\text{m}$), consisting of a monolayer of type 1 and type 2 epithelial cells.⁴¹ Type 1 (squamous alveolar) cells form the structure of the alveolar wall, whereas type 2 (cuboidal alveolar) cells continuously release pulmonary surfactant by exocytosis. Any cellular or protein damage in this region could not only have an impact on pulmonary homeostasis but would also determine possible translocations of NMs to other organs and allow them to elicit toxic effects at extrapulmonary sites.

The epithelial cells in the alveolar region of the lungs are covered and protected by a thin liquid layer composed of various neutralizing agents, called the lung lining fluid (LLF) (**Figure 2.6a**).^{40, 42} Its main component is surfactant, which forms a monolayer at the liquid-air interface.⁴³ This monolayer reduces the surface tension of the lung on expiration, stabilizing the alveoli against lung collapse. By modulating the surface tension, it also minimizes the work of expanding the alveoli during inhalation.^{44, 45} Moreover, it can displace particulate matter and facilitate mucociliary clearance.⁴⁶ Human surfactant consists of about 80% phospholipids, 8% neutral lipids (cholesterol, triacylglycerol and free fatty acids) and 10 % surfactant-specific proteins. The most abundant component of surfactant is phosphatidylcholine (PC), which accounts for 70-80% of the total amount of lipids. Approximately 50-70% of PC is saturated, especially in the dipalmitoylated form (DPPC).⁴² There are four lamellar phases recognized in saturated phosphatidylcholines (*e.g.* DPPC) but at 37 °C, DPPC monolayers are rigid enough to be compressed to high surface pressure ($\pi > 50\ \text{mN}\cdot\text{m}^{-1}$) or low surface tension ($\gamma = 0\ \text{mN}\cdot\text{m}^{-1}$) and can sustain this low surface tension for a considerable amount of time without collapsing.^{47, 48} Furthermore, four surfactant-associated proteins have been described: the hydrophilic SP-A and SP-D and the hydrophobic SP-B and SP-C (**Figure 2.6b**).⁴⁹ SP-A and SP-D play a fundamental role in host defence, by facilitating phagocytosis of various bacterial and viral pathogens. SP-A may also have a regulatory function in the formation of the monolayer that lowers the lung surface tension. On the other hand, SP-B and SP-C promote the rapid adsorption of the surfactant phospholipids at the air-liquid interface and maintain the stability of the lung system by influencing the molecular ordering of the phospholipid layer.^{42, 44} Inhaled NMs that enter and deposit in the alveoli may adsorb LLF components, affecting their subsequent cellular effects.^{50, 51} Studies indicate that the LLF layer may promote interaction of inhaled particles with the underlying epithelium, through wetting forces that draw the particles into the surfactant towards the alveolar wall.^{52, 53} The effects of the LLF on the physicochemistry of NMs will determine their interaction with proteins, cells and tissues in the lung. An altered aggregation state could modify particle transport, the amount of NMs internalized by cells and subsequent interactions within cells.⁵⁴ Moreover, NMs could alter the physiological surface tension of the lung surfactant and affect immune responses by sequestering lipids or proteins.⁵⁵

2.3 The Bioreactivity of Silver Nanomaterials in the Lungs

The well-known antibacterial properties of AgNPs,⁵⁶⁻⁵⁹ as well as the unique optoelectronic properties of this material,^{60,61} has led to a dramatic increase in research and industrial production of silver NMs. Among over 1800 commercially available products, identified as containing NMs according to manufacturers' reports, about 25% contain silver NMs.⁶² The available products range from electronic and photonic devices (such as solar panels) to textiles, food storage containers, antiseptic and antibacterial sprays and more.

The increase in the number of products containing AgNMs has led to growing concerns about the potential adverse effects on human health upon exposure to AgNMs. As a result, nanosilver has been the subject of intensive investigation during the last decades. Elemental silver was considered to be of low toxicity to humans, although several cases have been reported of argyria (irreversible pigmentation of the skin) or argyrosis (irreversible pigmentation of the eyes) after chronic ingestion of colloidal silver.^{63,64} The toxicity of AgNPs has been demonstrated for several species of vertebrates, invertebrates, and prokaryotic and eukaryotic microorganisms, as well as mammalian cell lines.^{57,65} The toxicological outcomes upon exposure to AgNMs include oxidative stress, lipid peroxidation, inhibition of mitochondrial activity, damage of DNA and cell apoptosis.⁶⁶⁻⁷⁴ AgNPs are manufactured in several different formats, which may lead to varied toxicological potentials. Several physicochemical properties of the AgNPs have been shown to play a role in their bioreactivity (*e.g.* shape,^{71,75} size⁶⁷ and coating agent⁶⁹) but the exact mechanisms remain elusive. Several studies have linked the toxicity of AgNPs to their dissolution and the release of free Ag⁺ ions.²¹ Silver is an oxidation catalyst and undergoes slow oxidative dissolution as Ag⁺ so the chemical nature of the AgNPs in the environment changes with time (depending on the environment), therefore both size and oxidation state will critically determine any cytotoxicity. Liu and Hurt have shown that the dissolution of AgNPs is a cooperative oxidation that involves both protons (H⁺) and dissolved oxygen (O₂).⁷⁶ They suggested that in simple aqueous solutions containing no other oxidants or reductants, the global reaction stoichiometry is:



Zhang *et al.* developed a kinetic model to describe Ag⁺ release, based on the hard sphere theory.⁷⁷ They proposed that the oxidation reaction can be expressed by first-order reaction kinetics and therefore, the Ag⁺ release rate can be described by the Arrhenius equation:

$$\left(\frac{dm}{dt}\right)_{Ag} = k[AgNP][O_2]^{1/2}[H^{+}]^2 \quad (2.2)$$

$$k = A \exp\left(\frac{-E_a}{RT}\right) \quad (2.3)$$

$$\left(\frac{dm}{dt}\right)_{Ag} = A \exp\left(\frac{-E_a}{RT}\right) [AgNP][O_2]^{1/2}[H^+]^2 \quad (2.4)$$

where $\left(\frac{dm}{dt}\right)_{Ag}$ is the Ag^+ release rate (mol/L·h), k is the reaction rate constant (mol/h), $[AgNP]$, $[O_2]$ and $[H^+]$ are the molar concentrations (mol/L) of AgNPs, dissolved oxygen and protons respectively, E_a is the activation energy (J), T is the temperature (298 K) and A is the frequency factor. Replacing the molar concentration of AgNPs ($[AgNP]$) by the mass-based concentration ($[Ag]$):

$$[AgNP] = \frac{[Ag]}{N_A \rho \frac{4}{3} \pi r^3} \quad (2.5)$$

where ρ is the density of Ag (~ 10.5 g/cm³), r is the AgNP radius (nm) and N_A is the Avogadro constant ($\sim 6.023 \times 10^{23}$ mol⁻¹), yields:

$$\left(\frac{dm}{dt}\right)_{Ag} = A \exp\left(\frac{-E_a}{RT}\right) \frac{[Ag]}{N_A \rho \frac{4}{3} \pi r^3} [O_2]^{1/2}[H^+]^2 \quad (2.6)$$

Therefore, release of silver ions will rely on the presence of dissolved molecular oxygen,⁷⁸ and, given the presence of molecular oxygen, the amount and rate of silver ions released will increase as the pH becomes more acidic.⁷⁹ The kinetics of silver ion release will also depend on the size⁷⁷ and surface functionalization of the NPs,⁸⁰ temperature^{77, 81} and composition of the surrounding media.⁸²⁻⁸⁴ Once released, the Ag^+ ions will react with other species in their environment and may precipitate as insoluble silver compounds or undergo complexation with proteins.

There has been much discussion in the literature as to whether the reactivity of AgNPs arises due to an ionic^{82, 85} or particulate effect,⁸⁶⁻⁸⁹ or both. Attempts have been made to resolve this question by incubating cells with $AgNO_3$ or by culturing cells in anaerobic conditions.⁸⁵ However, several discrepancies still exist between the published results. This is most likely caused by the lack of sufficient controls over the particles used and the cellular systems investigated, making it hard to compare between experiments performed by different groups. On the one hand, further efforts are required to understand the stability of AgNPs and the kinetics of Ag^+ ion release in biological environments. AgNPs are highly dynamic and their properties can change drastically when incubated in biological media, leading for example to aggregation or the formation of biomolecule coronas. As a result, characterization of as-synthesized AgNPs alone is not enough to predict their biological activity but appropriate characterization should also take place at conditions that realistically simulate

in vivo exposure scenarios. When combined with molecular biological studies, this information can provide greater insight into the unique ENM behaviour and a better appreciation of potential effects on human health and the environment. Since experimental techniques commonly used in the past for AgNP characterization, such as atomic absorption spectroscopy or dynamic light scattering, may possess limitations in detecting transformations of the physicochemical properties of AgNPs in different environments (*e.g.* pulmonary surfactant), several complementary techniques need to be applied. On the other hand, it is becoming increasingly clear that the extracellular release of Ag^+ ions by AgNPs cannot wholly account for the observed toxicity. Additional effects at the particle-cell membrane interface and inside cells seem to play a role in the biological action of AgNPs.⁹⁰ Therefore, a synergistic effect between AgNPs and Ag^+ ions must be considered in order to obtain accurate conclusions about the mechanisms of toxicity. The focus should be placed on developing new metrology methods that will be able to link the existing discrepancies between the effects of AgNPs and Ag^+ ions. New approaches based on the correlative application of high spatial and energy resolution analytical microscopy techniques, combined with dynamic optical microscopy, may offer an improved understanding of the mechanisms by which AgNPs interact with cells, and can guide the selection of the most relevant toxicological assays to test. Imaging and analysis could also help to determine whether the toxicological findings relate *directly* to the localization of AgNPs inside cells, or whether they are more general. Finally, the development of new methods for the quantification of Ag^+ ions released intracellularly will prove invaluable in discriminating between the effects of AgNPs and Ag^+ ions.

2.3.1 Pulmonary Exposure to Silver Nanomaterials

An increase in the number and production volume of products containing AgNPs will lead to a larger release into the environment during manufacture,⁹¹ use, washing or disposal of the products. There is currently very little data on the magnitude of release of AgNPs but efforts have begun to provide quantitative estimations of the environmental concentrations of engineered NMs,^{92, 93}. During manufacturing, AgNPs can be present in either powder or liquid formats and potentially present a health risk to workers. In an industrial manufacturing facility, significant release of AgNPs was observed during processing as soon as the reactor, dryer and grinder were opened, leading to a possible occupational exposure even for wet production processes.⁹⁴ In a laboratory setting, handling of silver nanomaterial powders inside a fumehood, led to an *increase* in the number concentration of particles in the breathing zone of a worker.⁹⁵ Moreover, few data exist on the effects of exposure of consumers to NPs in realistic application scenarios relating to the use of nanotechnology-based consumer products. As two studies have shown, the use of sprays containing AgNPs can lead to the generation of nanosized aerosols and the release of NPs near the human breathing zone.^{96, 97} Ag is also

imbedded in textiles in a variety of different forms, such as ionic Ag, AgCl or metallic AgNPs, to provide them with an enhanced antimicrobial activity. These silver-treated textiles can be a source of AgNPs in the washing solution when laundering fabrics, regardless of the initial form of treatment.⁹⁸ Consequently, AgNPs are a potential occupational, environmental and public hazard and their effects must be assessed before the production and use of these NMs are spread more widely. Regulatory frameworks to protect both public health and the environment are still under development. Therefore, scientific evidence is needed in order to inform these decisions and allow for the establishment of risk assessment processes.

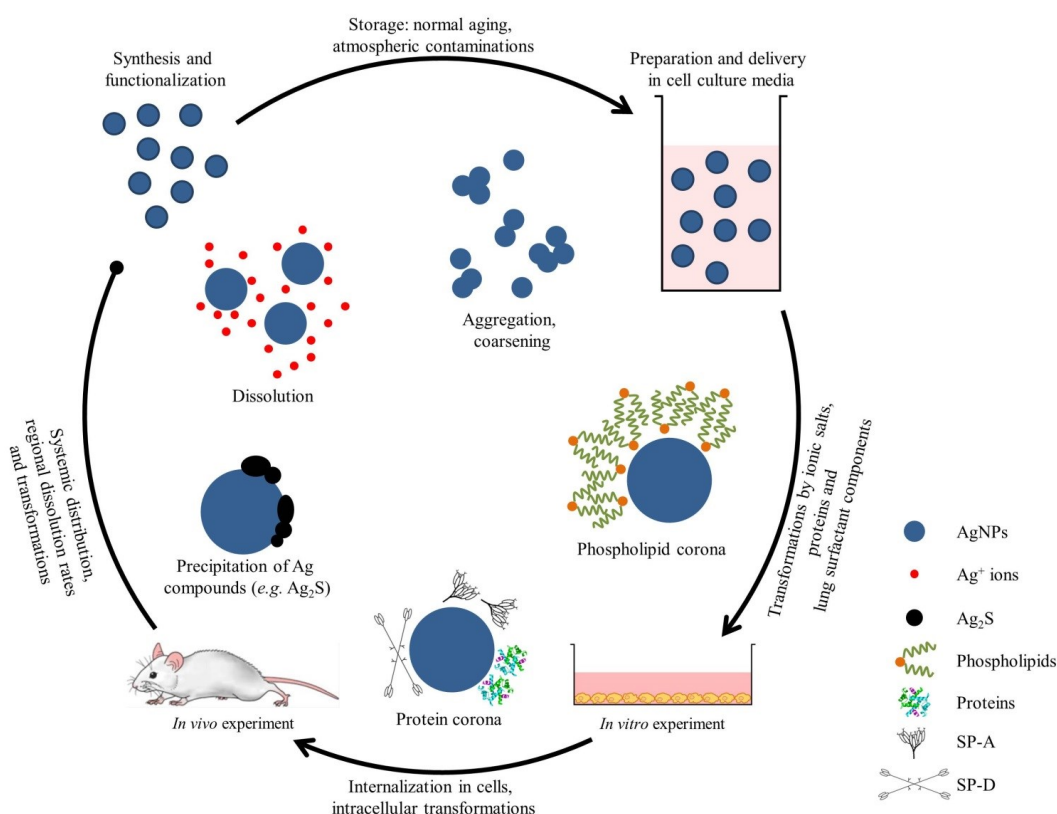


Figure 2.7 Examples of transformations to the physicochemical properties of AgNPs. To elucidate the mechanisms of biological action of AgNPs, these transformations must be carefully considered and comprehensive characterization should take place at each stage of the *in vitro* and *in vivo* testing.

Human exposure to AgNPs may take place through various routes, including the respiratory tract, the skin, the gastrointestinal tract, the reproductive system or the circulatory system (**Table 2.2**).^{7, 56, 59} For instance, the use of AgNPs in wound dressings, antibacterial textiles or cosmetic products could increase human skin exposure while AgNPs used in food packaging and kitchen utensils could lead to ingestion of AgNPs. Moreover, exposure of the reproductive system could occur through the use of contraceptive devices or hygiene products containing AgNPs. Another important

uptake route is the circulatory system, following intravenous injection of AgNP-based drugs or drug delivery/diagnostic systems. Although all these scenarios need to be assessed as potential portals of entry, inhalation of AgNPs is considered as one of the most important routes of exposure, since the respiratory system serves as a major portal for ambient particulate materials. Pathologies resulting from airborne particle materials at the micron level, such as quartz, asbestos and carbon, have been topics thoroughly researched in occupational and environmental medicine and associations between ultrafine particle inhalation and increased cardiovascular and pulmonary morbidity and mortality have been made.^{29, 99} Several healthcare, hygiene and antibacterial spray products containing AgNPs have now entered daily use. Therefore, AgNP aerosols directly applied into the nasal or oral cavity (e.g. nasal drops for rhinitis treatment) are of great concern, as concentrated NPs can be channelled into the lungs. The increasing use of sprays containing AgNPs, such as deodorants, shoe sprays or disinfectant cleaning products could also lead to accidental AgNP inhalation. Finally, as described above, AgNPs pose a risk of occupational respiratory exposure in manufacturing and research facilities.

Route of Exposure	Sources of Exposure
Respiratory System	Workers in manufacturing or research facilities working with AgNPs Aerosols directly applied in the nasal or oral cavities Sprays (<i>e.g.</i> deodorants, shoe sprays, cleaning products) Air filters, breathing masks Ambient airborne AgNPs
Skin	Wound dressings Antibacterial textiles (<i>e.g.</i> sheet, towels, socks, underwear, fitness wear) Antibacterial surfaces, paints Cosmetic products (<i>e.g.</i> lotions, roll-on deodorants, hair products) Computer hardware and mobile devices
Gastrointestinal Tract	Food packaging, cooking utensils and coatings Water filters Health supplements Oral hygiene products (<i>e.g.</i> toothpastes, toothbrushes)
Reproductive System	Contraceptive devices Feminine hygiene products
Circulatory System	Intravenous injection of AgNP-enabled drugs or drug delivery/diagnostic systems Implants, medical catheters

Table 2.2 Possible routes of human exposure to silver nanoparticles

2.3.2 The Bioreactivity of Silver Nanomaterials *in Vivo*

Although AgNPs have been identified as a possible exposure hazard, there is very limited information about how this class of nanomaterial interacts with the lung *in vivo*. Nanotoxicology assessment studies performed *in vivo* using the appropriate dosing amounts and routes of exposure can provide useful information because of the diversity of systemic phenotypic response and the anatomic influence, which can be directly translatable from animal models to human exposures. Particle pharmacokinetics combined with studies of regional dissolution rates can provide insights into the impact of dosing on systemic physiology and positional anatomy.¹⁰⁰ For instance, inhalation and instillation of 15 nm AgNPs in rats showed that lung particle content rapidly decreased following inhalation. The AgNPs were subsequently detected in the blood and other organs, such as the liver, kidney and brain.¹⁰¹ In several cases, a gender-dependent difference for AgNP accumulation in kidneys has been reported, with females exhibiting a higher concentration than males.¹⁰²⁻¹⁰⁴ Another study on the distribution and accumulation of AgNPs in rats following subcutaneous injection also revealed that the particles had translocated to the blood circulation and distributed throughout the main organs, especially in the kidney, liver, spleen, brain and lung.¹⁰⁵ Ultrastructurally, AgNPs that had accumulated in organs were observed within different types of cells, such as renal tubular epithelial cells and hepatic cells. Moreover, AgNPs induced blood-brain barrier destruction and astrocyte swelling, and caused neuronal degeneration.¹⁰⁵ In most studies that report on the anatomic distribution of silver following inhalation of AgNPs *in vivo*, silver is quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) due to its high sensitivity.^{101, 103, 105} However, this technique requires the digestion of tissue samples by strong acids and therefore ICP-MS detects the total elemental silver content of samples. Consequently, it is difficult to draw conclusions about the form of silver that has reached each organ, *i.e.* distinguish between metallic AgNPs, ionic Ag⁺ or Ag compounds. Other groups have employed the silver-enhancement method to track Ag in tissues,^{104, 106} which is based on the autometallography (AMG) technique.¹⁰⁷ This method is based on the principle that small-sized clusters of silver enhance the reduction of silver ions that are added on their surface, resulting in an increase in the size of the clusters. These can become large enough to be viewed under a light microscope, thus confirming the presence of Ag in tissue, but without being able to characterize the morphology or chemistry of the original AgNPs. Moreover, this method presents several drawbacks that could lead to artefacts. For example, autonucleation of the enhancement solution may lead to the formation of nanoparticles that are subsequently catalytic for further growth.

A 28-day inhalation toxicity study of AgNPs on rats by Ji *et al.* showed no significant changes in the haematology and blood biochemistry in either the male or female rats.¹⁰⁸ To further evaluate these findings, a sub-chronic 90-day inhalation study was performed by the same group.¹⁰² Among the lung function test measurements, the tidal volume and minute volume showed a statistically significant decrease during the 90 days of AgNP exposure. Moreover, histopathological

examinations indicated dose-dependent increases in lesions related to AgNP exposure, such as mixed cell infiltrate and chronic alveolar inflammation, including thickened alveolar walls and small granulomatous lesions. Their findings indicated that prolonged exposure to AgNPs can induce lung function changes, along with inflammation, at much lower mass dose concentrations, when compared to Ag microparticles. In contrast, in a 28-day inhalation exposure performed by Hyun *et al.*, histopathological examination showed that the nasal cavity and lungs from the AgNP-exposed groups exhibited no “remarkable” changes compared to the control group.¹⁰⁹

In contrast, Lee *et al.* found that a 14-day inhalation exposure of mice to AgNPs led to alterations in brain gene expression.¹¹⁰ A total of 468 genes in the cerebrum and 952 genes in the cerebellum were identified as AgNP-responsive. The largest groups of gene products affected by AgNP exposure included 73 genes in the cerebrum and 144 genes in the cerebellum. AgNP exposure modulated the expression of several genes associated with motor neuron disorders, neurodegenerative disease, and immune cell function, indicating potential neurotoxicity and immunotoxicity associated with AgNP exposure.¹¹⁰ In a recent 90-day inhalation study it was demonstrated that a decrease in lung function and pulmonary inflammation could persist even after termination of the exposure in male rats, while in females there was no effect on lung function and a gradual improvement in lung inflammation following cessation of exposure.¹¹¹

While the results of the above studies clearly demonstrate a pro-inflammatory potential of AgNPs in the lung, the mechanism through which lung injury occurs and the characteristics of AgNPs that contribute to lung injury have not been assessed systematically. Recently, Wang *et al.* compared the effects of 20 and 110 nm AgNPs with two different surface coatings, citrate and polyvinylpyrrolidone (PVP), on mice.¹⁰⁶ Both size and surface coating were found to affect the cellular toxicity of AgNPs as well as their acute *versus* sub-chronic lung injury potential. Smaller particles induced more cellular toxicity and oxidative stress than the larger particles, possibly due to a higher rate of dissolution and Ag⁺ ion bioavailability. Moreover, there was a higher propensity for citrate-20 nm AgNPs (C20) to generate acute neutrophilic inflammation in the lung and to produce chemokines compared to citrate-110 nm AgNPs (C110). In contrast to the more intense acute pulmonary effects of C20, C110 induced mild pulmonary fibrosis at day 21, likely as a result of slow but persistent Ag⁺ release, leading to a sub-chronic injury response.¹⁰⁶ Further investigation is therefore necessary in order to elucidate the mechanisms through which the physicochemical properties of inhaled AgNPs affect their interactions with the lung. Additionally, understanding how the impact of AgNPs at the cellular level *in vitro* translates to their *in vivo* toxicological potential will be essential for the development of a predictive toxicological paradigm for AgNP toxicity, the design of accurate screening assays and ultimately the development of safer products.

Species	Size (nm)	Dose	Exposure Method	Exposure Time	Reference
C57Bl/6 Mice, 8 weeks old	20, 110	0.1, 0.5 and 1.0 mg/kg	Oropharyngeal aspiration	Single injection	106
Sprague-Dawley rats, 6 weeks old	15	0.66×10 ⁶ particles/cm ³ (49 µg/m ³) 1.41×10 ⁶ particles/cm ³ (117 µg/m ³) 3.24×10 ⁶ particles/cm ³ (381 µg/m ³)	Inhalation	6 h/day, 5 days/week, for 12 weeks	111
Sprague-Dawley rats, 8 weeks old	18	0.7×10 ⁶ , 1.4×10 ⁶ and 2.9×10 ⁶ particles/cm ³	Inhalation	6 h/day, 90 days	112
Wistar rats, 8–10 weeks old	15-40	4, 10, 20 and 40 mg/kg	Intravenous injection	32 days (injected at 5-day intervals)	113
C57BL/6 mice	20	1.91×10 ⁷ particles/cm ³	Inhalation	6 h/day, 5 days/week, for 2 weeks	110
Wistar rats, female	50-100	62.8 mg/kg	Subcutaneous injection	Single injection	105
C57Bl/6N mice, adult male	30	100, 500 and 1000 mg/kg	Intraperitoneal injection	24 h	114
Sprague-Dawley rats, 6 weeks old	55	0.7×10 ⁶ , 1.4×10 ⁶ and 2.9×10 ⁶ particles/cm ³	Inhalation	6 h/day for 90 days	102
Sprague-Dawley rats, 8 weeks old	13-15	1.73×10 ⁴ particles/cm ³ (0.5 µg/m ³) 1.27×10 ⁵ particles/cm ³ (3.5 µg/m ³) 1.32×10 ⁶ particles/cm ³ (61 µg/m ³)	Inhalation	6 h/day, 5 days/week, for 4 weeks	109
Sprague-Dawley rats, 8 weeks old	15	1.73×10 ⁴ , 1.27×10 ⁵ and 1.32×10 ⁶ particles/cm ³	Inhalation	6 h/day, 5 days/week, for 4 weeks	108
Fischer 344 rats, female	15	3×10 ⁶ particles/cm ³ (133 µg/m ³)	Inhalation	6 h	101

Table 2.3 Exposure doses used in *in vivo* studies of silver nanoparticles

Although the importance of dosimetry in biological studies is well-known, the relevance of AgNP doses used in animal inhalation studies to realistic exposure scenarios has rarely been assessed. This is in part due to the fact that very few data on AgNPs are currently available to evaluate occupational and consumer exposure. The recommended threshold limit value (TLV) set by the American Conference of Industrial Hygienists (ACGIH) for AgNP inhalation is 100 µg/m³,^{115, 116} while the recommended exposure level (REL) for nano-Ag by the US National Institute for Occupational Safety and Health (NIOSH) is 10 µg/m³.¹¹⁷ A study on airborne NP exposures

associated with the manual handling of nanosilver in fume hoods showed a peak airborne concentration of 7000 particles/cm³.¹¹⁸ Another assessment reported a similar particle number concentration in the workplace air, while mass concentrations were between 0.02 and 1.02 µg/m³.¹¹⁹ A recent study estimated the potential exposure of workers in nanosilver manufacturing facilities by performing personal sampling, area monitoring and real-time monitoring over 3 days.¹²⁰ The highest AgNP concentrations were obtained from area sampling in the injection room, ranging from 5.01 to 288.73 µg/m³. These values were used in the study by Wang *et al.* to calculate the relevant doses to administer in their animal experiments.¹⁰⁶ Assuming a monthly deposition in human lungs, the equivalent dose per mouse was estimated at 0.33 mg/kg, therefore 0.1, 0.5 and 1.0 mg/kg were selected as the dose range to perform bolus instillation studies. Based on their findings, they predicted that a human lung burden equivalent to a bolus dose of 0.1–1.0 mg/kg in a mouse could be associated with incremental acute pulmonary inflammation, whereas a lung burden equivalent to 1.0 mg/kg in a mouse may lead to sub-chronic pulmonary effects in a human.¹⁰⁶ For comparison, the doses used in other *in vivo* studies are summarized in **Table 2.3**.

2.3.3 The Bioreactivity of Silver Nanomaterials *in Vitro*

In vitro studies offer a relatively straightforward method to determine which features of a nanosubstance could predict an increased cellular reactivity. *In vitro* testing is one of the major options suggested in recent proposals on strategic approaches to discover any undesirable effects of NPs or to study NP cell targeting. However, the lack of standardized procedures for the evaluation of NP toxicity makes it difficult to compare between studies performed by different laboratories. One of the factors that can lead to inconsistencies between different studies is the use of different cell types, because the toxic effects of NPs are highly dependent on the type of cell encountered.¹²¹ This is due to the variation in cell physiology (*e.g.* epithelial or macrophage cells), proliferation state (tumoral or resting cells), membrane characteristics and phagocyte characteristics among different cell types.¹²² For example, cancer cell lines can be more resilient towards NP toxicity than normal cells due to an increased rate of proliferation and metabolic activity.^{123, 124} Also, since membrane transport depends on the composition of cellular membranes, cell type can greatly influence the amount of uptake of NPs into cells as well as their fate in the intracellular environment. Therefore, in order to identify the exact effect of NPs on the organs or cells of interest, the *in vitro* study should include cells that represent the relevant exposure scenario. Since NPs will encounter different cell types depending on the exposure route, different levels of toxicity can be expected with different exposure routes. Consequently, numerous cell types ranging from endothelium, blood, spleen, liver, nervous system, heart and kidney are all of interest in toxicity studies of NPs in general and AgNPs in particular.

In summary, a variety of oxidative stress-related changes have been observed in various mammalian cell types after exposure to AgNPs.^{125, 126} For example, the generation of reactive oxygen species (ROS), is induced after the treatment of cells with AgNPs at concentrations as low as 0.2 µg/ml in human glioblastoma cells (U251),²¹ human Jurkat T cells,¹²⁷ human colon cancer cells (HCT116 and HT29),^{128, 129} human hepatoma cells (HepG2),^{130, 131} human Chang liver cells,⁷⁴ mouse germline stem cells (C18-4),^{132, 133} mouse fibroblasts (NIH3T3),¹²⁸ mouse neuroblastoma cells (N2A),¹³⁴ rat liver-derived cell line (BRL 3A),¹³⁵ and rat pheochromocytoma cells (PC12).¹³⁶ The level of lipid peroxidation is also increased by AgNP treatment in human skin carcinoma and fibrosarcoma cells,⁷² as well as in Chang liver cells.⁷⁴ Moreover, biochemical and molecular changes related to genotoxicity have been found following AgNP exposure in a variety of cells,^{21, 74, 127, 130} including human mesenchymal stem cells,¹³⁷ human hepatocyte cell line (L02)¹³⁸ and mouse embryonic stem cells and embryonic fibroblasts.¹³⁹ Furthermore, AgNP-induced apoptosis has been demonstrated in human-derived cells such as colon cancer^{129, 140} and hepatoma cells¹³¹ as well as in Jurkat T¹²⁷ and HeLa cells.¹⁴¹ Evidence of apoptosis has also been provided in some animal-derived cells, including mouse blastocysts,¹⁴² NIH3T3 and L929 fibroblasts,^{128, 143} embryonic fibroblasts and stem cells¹³⁹ and baby hamster kidney cells.¹⁴⁰

As far as the lungs are concerned, various cells have been used to model the epithelial barrier. Primary cells directly isolated from tissue are not preferable due to their limited life span and variations in quality (donor variations and quality of preparation).¹⁴⁴ Immortalized cell lines, although not as well differentiated as primary cells, provide high reproducibility and are most often used for the assessment of cellular toxicity and permeation, with human lung adenocarcinoma-derived A549 cells being the most popular.¹⁴⁴ Although very useful for toxicity testing, A549 cells are less suitable to assess permeation as they do not form tight intercellular junctions. To assess the bronchial barrier, Calu-3, 16HBE14o-, and BEAS-2B cells are usually used. The HPV-E6/E7 and hTERT immortalized bronchial epithelial cell line NuLi-1 has recently been employed as a model for the bronchial epithelial barrier.¹⁴⁵ Finally, models for the alveolar barrier use either primary or immortalized AT-II cells or NCI-H441 cells. Despite the fact that numerous studies have been published, investigating a wide range of cells that represent different exposure routes, including inhalation, determining a trend for AgNP toxicity can still be considered complex. In many of these studies, independent experimental methods have been employed, especially in regards to the physicochemical properties of the AgNPs tested. Consequently, it is not completely feasible to compare data from different studies, especially quantitative data such as half-maximal inhibitory concentration (IC₅₀) values.

Moreover, the selection of appropriate cytotoxicity assays is vital to the accurate assessment of NP toxicity, especially since some may interfere with the actual toxic effect produced by the NP.¹⁴⁶⁻¹⁴⁹ For instance, single walled carbon nanotubes (SWCNTs) have been shown to interact with MTT-formazan crystals formed after the reduction of MTT.¹⁴⁶ This led to a false cytotoxic effect of SWCNTs on A549 human alveolar epithelial cells within the MTT assay. Cytotoxic assays can

provide a large amount of information about toxic endpoints, such as membrane integrity (lactate dehydrogenase -LDH), cellular metabolic activity (MTT), oxidative stress (ROS) and apoptosis (fluorescent Annexin V or caspase substrates). These assays are generally employed to measure acute toxic effects of cultured cells and, although they can be used to investigate cell viability, the results from one assay are difficult to directly compare with another as they measure different parameters.¹⁵⁰ AgNPs have been shown to cause various effects and interact with biological components in numerous ways, making it nearly impossible to cover the whole scale of cell-particle interactions in a single study. Correlating the information provided by toxicity assays with imaging techniques could provide very valuable information on local alterations in cell morphology, the localization of AgNPs and their transformation within cells and tissues. This would improve our understanding of the cytotoxicity mechanisms induced by AgNPs, depending on their concentration, size, shape, surface modification but also on the target cell type.

Several studies have reported morphological changes in the cell membrane and cell shape after exposure to AgNPs. Using phase contrast microscopy, Lee *et al.* examined live cells from the human alveolar cell line A549 exposed to 10, 50 and 200 µg/ml AgNPs for 24 hours. Significant morphological changes characteristic of cell death, including cell shrinkage, few cellular extensions, restricted spreading pattern, and increased floating of cells from the substrate were observed in a dose-dependent manner.¹⁵¹ A rat alveolar macrophage cell line incubated with hydrocarbon-coated AgNPs of different sizes (15 nm, 30 nm and 55 nm) presented size-dependent changes in cellular morphology.¹⁵² The most pronounced changes were observed for cells treated with 15 nm AgNPs, with cell shrinkage and cellular debris floating in the medium. Cells exposed to 30 nm AgNPs presented abnormal sizes, with agglomerated AgNPs inside and outside the cells, whereas 55 nm AgNPs-treated cells had minor morphological changes. Nguyen *et al.* compared the toxicity of PVP-coated AgNPs and uncoated AgNPs in a mouse macrophage cell line (J774A.1). Both types of AgNPs induced cell damage, including shrinkage, deformation and enlargement of mitochondria. However, uncoated AgNPs resulted in cell shrinkage, whereas coated Ag-NPs induced cell elongation and enlargement. These different responses to AgNPs suggest different mechanisms of cell damage depending on their surface coating.¹⁵³ A systematic investigation on the cytotoxic effects, cellular response and membrane damage caused by four types of AgNPs with different surface charge was carried out on mouse macrophage (RAW-264.7) and lung epithelial (C-10) cell lines. Cytotoxicity was found to be strongly dependent on the surface charge of the AgNPs, with the positively charged AgNPs being the most toxic. The same study concluded that the response to AgNPs is dependent on the cell type, as lung epithelial cells were found to be more resistant to the AgNPs than macrophages, regardless of the surface coating.⁶⁹ This was attributed to the fact that the aggregate size of AgNPs in the cell culture medium was more than 100 nm, allowing them to be taken up more efficiently by macrophages than epithelial cells.¹⁵⁴

Moreover, the exposure of cells to AgNPs has been proven to induce stress responses, such as production of reactive nitrogen species (RNS) and reactive oxygen species (ROS). AgNPs have been shown to act on various cellular targets resulting in the induction of apoptosis, the stimulation of inflammatory signalling pathways or the production of free radicals, culminating in cell death.^{21, 155} For instance, enlargement of the mitochondria has been observed in human colonic epithelial (HT-29), mouse macrophage (J774A.1) and human macrophage (U937) cell lines, in a dose-dependent manner, after exposure to citrate or PVP coated AgNPs.^{153, 156} According to Asharani *et al.*, AgNPs reduced the ATP production of human lung fibroblast cells (IMR-90), causing damage resulting in altered mitochondrial respiratory chain activity, dissipation of mitochondrial membrane potential and stimulation of apoptotic pathways.²¹ Structural damage of the mitochondria may in turn lead to a disruption of cellular ROS balance. Evidence in the literature suggests that AgNPs can bind to thiol containing proteins or peptides, such as Glutathione (GSH), restricting their availability for ROS neutralization and resulting in an oxidant-mediated response to AgNPs.^{157, 158} Lim *et al.* showed that exposure of a human macrophage cell line (U937) to 5 nm PVP-coated AgNPs resulted in swelling of the mitochondria and formation of double-layered membrane structures. The authors suggested that mitochondrial swelling was due to necrotic changes in the cell and that the presence of double-layered membrane structures was related to autophagosome formation.¹⁵⁶ Therefore, mechanisms other than apoptosis may also be involved in the initiation of cell death. Similarly, Gliga *et al.* reported that 10 nm polymer-grafted AgNPs led to morphological changes suggestive of autophagy in a normal human bronchial epithelial cell line (BEAS-2B).¹⁵⁹ The induction of autophagy, *i.e.* the degradation of unnecessary or dysfunctional cellular components through the action of lysosomes, has been reported for several types of NPs, including AgNPs and AgNWs, and may represent a common cellular response to NPs.^{160, 161}

In some studies, evidence of genotoxicity upon exposure to AgNPs has also been obtained. Nanoparticles may interact with DNA chains through nuclear penetration and consequently induce DNA damage.^{162, 163} Occasionally, AgNPs have been found inside nuclei.^{70, 164} Cronholm *et al.*¹⁶⁵ detected agglomerates of AgNPs (from 0.5 to 1 μm) inside the nuclei of A549 type 2 alveolar epithelial cell line. Due to the large size of the aggregates, compared to the size of nuclear pores, it is possible that AgNPs enter *via* vesicles lodged to the nucleus,²¹ individual particles are internalized *via* the nuclear pores and agglomerate inside the nucleus or AgNPs enter during mitosis.^{166, 167} Furthermore, a mouse macrophage cell line (RAW264.7) exposed to chitosan-coated AgNPs showed dose-dependent nuclear shrinkage and chromosome abnormalities, such as double-strand breaks.¹⁶⁸

The results of *in vitro* studies on the toxic responses to AgNPs have been mixed. There is an on-going debate about the properties of AgNPs that determine their interactions with cells and the extent to which their bioreactivity depends on the release of free Ag^+ ions. Exposure of mouse macrophages (J774A.1) to citrate and PVP-coated AgNPs resulted to an increase in ROS generation after 24 hours for both types of particles, suggesting that surface modification did not have a great

effect on cytotoxicity.¹⁵³ Similarly, Gliga *et al.* compared uncoated and citrate- and PVP-coated AgNPs of different primary sizes (10 nm up to 75 nm). They showed that only 10 nm AgNPs, regardless of surface coating, were cytotoxic on human bronchial epithelial cells (BEAS-2B). Their findings suggested that particle size was the key property determining their cytotoxicity.¹⁵⁹ A size-dependent toxicity of AgNPs on a rat alveolar macrophage cell line was also demonstrated by Carlson *et al.*, who found the predominant mechanism of toxicity to be mediated through oxidative stress.¹⁵² More recently, however, Wang *et al.* argued that both size and surface coating can affect the cellular toxicity of AgNPs, as well as their acute *versus* sub-chronic lung injury potential.¹⁰⁶ In their work, human bronchial epithelial (BEAS-2B) and mouse macrophage cell (RAW 264.7) lines were exposed to 20 nm and 100 nm PVP- and citrate-coated Ag-AuNPs (AgNPs with a gold core). The 20 nm Ag-AuNPs induced significant toxicity, in the dose range from 6.25-50 µg/mL for 24 hours. Significant ROS generation, intracellular calcium flux and decline of the mitochondrial membrane potential were also measured in both cell types.¹⁰⁶ The higher toxicity of the smaller particles was attributed to their higher rate of dissolution and Ag⁺ bioavailability. In comparison, Herzog *et al.* applied nebulized AgNPs to a triple cell co-culture system composed of A549 epithelial cells combined with monocyte-derived macrophages (MDM) and dendritic cells (MDDC) cultured at an air-liquid interface. In this work, however, AgNPs did not significantly increase the LDH after a 24 hour exposure and the secretion of pro-inflammatory markers did not alter.¹⁶⁹ In a recent study, Ag nanowires (AgNWs) were applied to a human alveolar type 1-like epithelial cell line for 24 hours, at a dose of 25 µg/ml. Cell viability studies showed no evidence of cytotoxicity or release of reactive oxygen species.³ Analytical transmission electron microscopy revealed the precipitation of Ag₂S within the cell, which acts as a “sink” for free Ag⁺, significantly limiting short-term toxicological effects. The differences in toxic outcomes between these studies reflect the different chemistries and formats of the particles applied to the cells, the cell culture model and differences in cell types. An improved understanding of how the AgNPs transform within each component of the cell culture model, and whether this is representative of the lung *in vivo*, may provide improved insights into the putative pulmonary bioreactivity of AgNPs.

2.3.4 Evaluation of the *in Vitro* Testing of Silver Nanomaterials

One of the challenges of evaluating the toxicity of engineered NMs in the coming years is the development of viable alternatives to *in vivo* testing. Although several *in vitro* systems exist, providing some mechanistic understanding of AgNP toxicity, these systems will have to be optimized in the future to simulate better the *in vivo* situation and provide a holistic understanding of the “bionano” systems. Valid *in vitro* systems will lead to decreased costs compared to expensive *in vivo* studies, faster results and improved animal welfare.¹⁷⁰ One of the limitations of most *in vitro* studies

conducted in the past is the use of single cell types, which is not physiologically relevant. Not only is the pulmonary system diverse in its cellular makeup, but also different cell types often participate in coordinated responses.¹⁷¹ Rothen-Rutishauser's group has established and evaluated an *in vitro* model of the human epithelial airway barrier composed of epithelial cells and two of the most important immune cells of the lung (macrophages and dendritic cells; derived from blood monocytes), to study NP lung-cell interactions and their possible responses.¹⁷² Their model can be used at the air-liquid interface, allowing the direct exposure of cells to an aerosol,¹⁷³ therefore representing a realistic situation following NP inhalation. Using this model, they have demonstrated that cultured epithelial cells, macrophages, and dendritic cells can cooperate in NP trafficking. Nanoparticle uptake into the cells was also enhanced in co-culture compared with monocultures.^{174, 175} Additionally, inhaled NPs reaching the alveoli will first come into contact with pulmonary surfactant, therefore any interactions between particles and the surfactant could have an impact on their subsequent cellular effects. However, pulmonary surfactant components are not commonly integrated in *in vitro* testing of NMs. In the future, surfactant interactions should be integrated into the experimental design, to better represent the *in vivo* situation.

Moreover, in order to understand the discrepancies between the results of previous studies and elucidate the mechanisms of biological action of AgNPs, any transformations of the particles must be carefully considered at each stage of the *in vitro* experiment. More specifically, there are three steps to consider: (i) preparation of the AgNPs and delivery to the biological medium, (ii) transformations in the cell culture medium, either due to ionic salts or proteins, as well as the lung surfactant, prior to interaction with the cells and (iii) transformations of the AgNPs following internalization into the cell. This will require meticulous particle characterisation and sophisticated imaging techniques under difficult experimental conditions.

2.3.4.1 Preparation and Delivery to Biological Media

The last decade has witnessed the successful synthesis of silver nanocrystals in a variety of shapes (*e.g.* sphere, cube, plate, rod¹⁷⁶), using a wide range of experimental setups. These Ag nanocrystals can be described by a set of physical parameters that may include their size, shape, composition, structure, coating and surface charge. In principle, the properties of the nanocrystal can be tailored and fine-tuned by controlling any one of these parameters.¹⁷⁶ Therefore, adequate physicochemical characterization of AgNPs prior to undertaking experiments for *in vitro* toxicity assessments is paramount in order to correlate biological responses with NP properties.¹⁷⁷ Some published nanotoxicity studies report characteristics of the particles using only manufacturer's data, which provides incomplete information about the particle being used. There is a wide range of methods that can be used for NP characterization. Due to its simplicity and rapidity of analysis,

dynamic light scattering (DLS) is increasingly being used in many fields of science and industry for the characterization of NPs, including AgNPs.¹⁷⁸ However, this technique could be problematic when measuring samples with wide size distributions, multimodal distributions or containing NP aggregates. This is because, in the presence of even a low percentage of larger particles, these will dominate the light scattering signal and mask the presence of smaller particles.^{179, 180} Moreover, DLS may be incompatible with biological media because of the presence of various light scattering components.¹²¹ Therefore, precise characterization of AgNPs requires the use of a combination of techniques. The key strength of electron microscopy (EM) techniques, combined with elemental analysis, is their ability to provide spatially resolved information about the morphology, size and chemistry of NPs at the same time. Sample preparation for EM may lead to drying-induced artefacts, such as NP aggregation or sample fractionation, but these limitations may be overcome by the use of cryogenic temperature EM (cryo-EM). Cryo-fixation can be applied to rapidly freeze samples, allowing cryo-EM to acquire high-resolution images of NPs, as well as cells, in their native aqueous state.¹⁸¹

Even though silver is considered as a noble metal, it is far from being chemically inert. Therefore, transformation of AgNPs caused by poor experimental control will likely interfere with the prediction of silver toxicity results. Moreover, information of AgNP behaviour during their full life cycle is limited. Normal aging of the NPs (*e.g.* Ostwald ripening, corrosion, aggregation, surface-state modification) may already affect their properties. The atmospheric corrosion of silver is an example of transformation that could take place over poorly controlled storage conditions. Due to existing gaseous hydrogen sulfide (H₂S), carbonyl sulfide (OCS) and carbon disulfide (CS₂) in the atmosphere, silver sulfidises upon exposure to the atmosphere.¹⁸² Additionally, it is well known that AgNPs can be oxidized and shed Ag⁺ ions in aqueous media, by reacting with dissolved O₂.⁷⁸ Storage of AgNPs in dispersion for several weeks was shown to considerably increase the toxicity of the AgNP dispersion toward human mesenchymal stem cells, compared to freshly synthesized dispersions of AgNPs, due to an increased concentration of Ag⁺ ions. Finally, the toxicity of AgNPs can be affected by sonication dispersion protocols.¹⁸³ During sonication a large amount of energy is applied to the AgNP dispersion to enable the rupture of larger AgNP aggregates. However, the majority of this energy is converted into thermal energy, raising the temperature and potentially promoting the dissolution of AgNPs.⁷⁸ Higher concentrations of Ag⁺ ions in AgNP dispersions prepared without cooling the samples over ice during sonication were measured by atomic adsorption spectroscopy (AAS). Sonicated AgNP dispersions were more toxic to A549 epithelial cells than when prepared with cooling.¹⁸⁴

2.3.4.2 Transformation in Cell Culture Media

To allow interaction with cells in culture, tissue or organisms, as-synthesized AgNPs are usually suspended in biocompatible aqueous tissue culture medium. Depending on the composition and exposure time to the biological environment, agglomeration/aggregation of the particles could take place or there may be alteration of their dissolution profile. Several factors such as ionic strength and composition of the dispersion medium,^{82-84, 185, 186} pH,^{78, 187} dissolved organic matter,¹⁸⁸ dissolved oxygen concentration⁶⁹ and temperature⁸¹ have been shown to affect the stability of AgNPs and are consequently expected to have an impact on their bioreactivity. Therefore an assessment of the properties of AgNPs in the extracellular medium, before or during cell exposure tests, is also crucial. This has recently been highlighted by Chen *et al.*, who reported cell culture media-induced changes to the chemistry of silver nanowires (AgNWs) using high resolution analytical electron microscopy (HRTEM) (**Figure 2.8**).¹⁸⁹ It was demonstrated that silver sulfide (Ag₂S) crystals formed on the surface of AgNWs following incubation in DCCM-1 cell culture medium. Ionic silver released from the surface of AgNWs will transform to Ag₂S due to its extremely low water solubility ($K_{sp} = 5.92 \times 10^{-51}$).¹⁹⁰ This silver to silver sulphide transformation will substantially reduce the Ag⁺ ion release rate; therefore reduced AgNP toxicity could be expected. Indeed, some studies have shown low toxicity of Ag₂S NPs and reduced antibacterial activity of AgNPs because of sulfidation.^{191, 192}

Moreover, studies to determine the amount of Ag⁺ ions dissolved in media, which are known as one of the major factors affecting Ag toxicity, and the impact of this ionic species on cells have produced inconsistent results.^{82, 83} Inductively coupled plasma sources (ICP-OES and ICP-MS) and atomic absorption spectroscopy (AAS) techniques are commonly used to correlate the dissolution rate of AgNPs with their toxicity profiles. **Table 2.4** shows that a wide variety of experimental setups and analytical methodologies are employed in *in vitro* studies to quantify the amount of Ag⁺ ions released from AgNPs. However, there are discrepancies in the data provided by different authors, probably due to the use of inconsistent methodologies (*e.g.* experimental setup, separation method) and incomplete NP characterization^{77, 106, 159, 193}. This might explain, to some extent, why inconsistent dissolution rates are observed even when the same types of AgNPs are used. For instance, in the case of 10 nm citrate-AgNPs, different dissolution percentages were measured by UV-Vis absorption spectroscopy and AAS, which required a previous centrifugation step.¹⁹⁴ Due to the small size of AgNPs, they could not be effectively separated from solution by centrifugation and the values obtained by AAS were anomalously high.

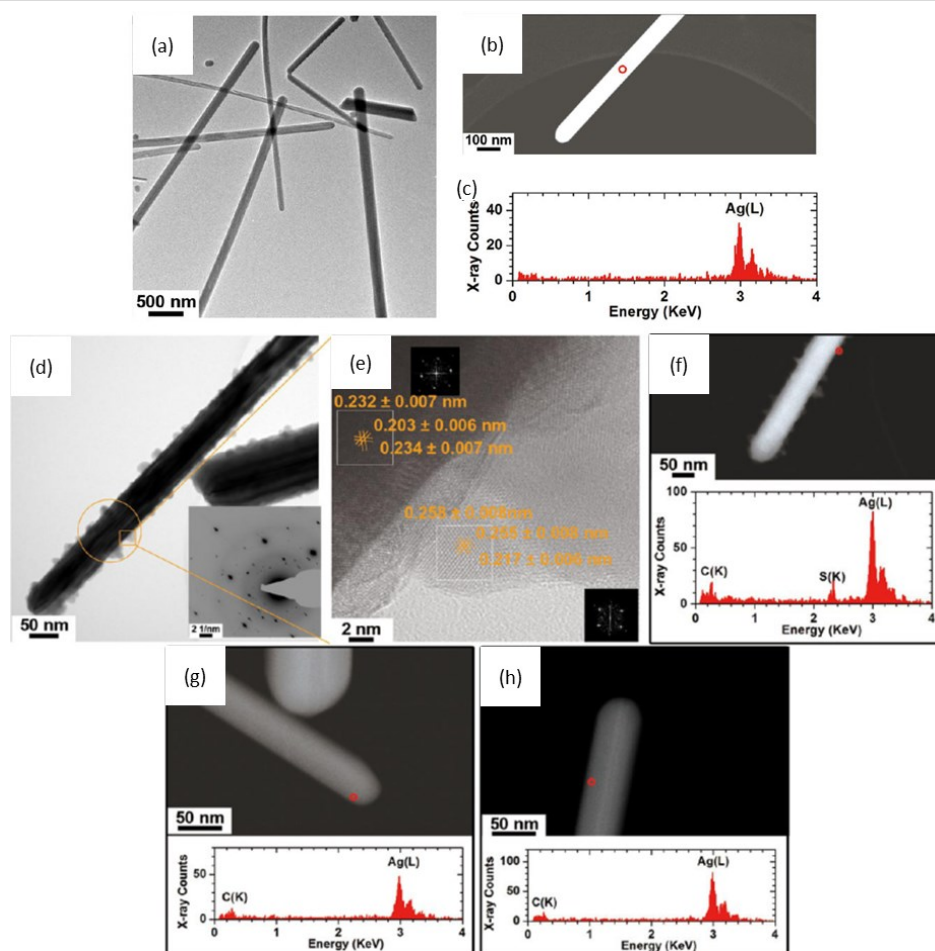


Figure 2.8 (a-b) Low-resolution bright field transmission electron microscopy (BF-TEM) image (a) and high angle annular dark field scanning transmission electron microscopy (HAADF-STEM) image (b) of as-synthesized AgNWs. (c) The corresponding energy dispersive X-Ray (EDX) spectrum collected from the area circled in (b). The two peaks at 2.98 and 3.15 keV correspond to the $\text{Ag(L}_\alpha)$ and $\text{Ag(L}_\beta)$ peaks, respectively. (d-h) Physicochemical characterization of AgNWs incubated in various cell culture media for 1 hour at 37 °C: (d-f) DCCM-1, (g) DMEM, and (h) RPMI-1640. (d) Representative BF-TEM image of AgNWs incubated in DCCM-1 medium, showing the formation of crystallites on the surface of the AgNWs. The inset is a SAED pattern taken from the circled area (aperture size ~ 130 nm). (e) HRTEM image collected from the boxed area in (d) reveals that the crystallites have a different crystal structure than the original AgNWs. The lattice spacings of the crystals formed at the surface of the AgNWs (~ 0.26 and 0.22 nm) correspond to the (112) and (031) lattice spacings of Ag_2S , respectively. The lattice spacings of the core of the nanowires (~ 0.23 and 0.20 nm) correspond to the interplanar spacings of metallic Ag. Insets are FFT patterns taken from the two boxed areas. (f) HAADF-STEM image (top) taken from the same area as (d) and STEM-EDX spectra collected from the circled area (bottom), confirming the formation of Ag_2S (g-h) HAADF-STEM images (top) and EDX spectra (bottom) of AgNWs incubated in DMEM (g) and RPMI-1640 (h) cell media, indicating that AgNWs do not sulfidise in DMEM or RPMI-1640. Reprinted with permission from ¹⁸⁹. Copyright 2013 American Chemical Society.

Type of AgNPs	Experimental Setup	Analytical Methodology and Analysis Technique	Released Ag ⁺ ions	Ref.
10.5 ± 4.3 nm PVP-AgNPs	10 µg/ml of AgNPs incubated in test media (half-strength NaCl-free lysogeny broth (LB)) for 4 h at 30°C	Ultracentrifugation (1 h at 390,000 g) AAS	4.4 %	195
14 ± 1.6 nm Citrate-AgNPs	25 µg/ml of AgNPs incubated in non-interacting perchlorate (ClO ₄) buffer solutions at pH 5 for 336 h	Ultrafiltration through 2 kDa filter membranes (13,000 rpm) ICP-OES	~2 %	79
20 nm Citrate-AgNPs	300 µg/ml of AgNPs incubated in quarter-strength Hoagland media for 24 h and 336 h	Ultrafiltration through 3 kDa filter membranes (40 min at 5000 g) ICP-MS	17 % (24 h) 40% (336 h)	196
20 nm Citrate-AgNPs	12.5 µg/ml of AgNPs incubated in BEGM cell media for 24 h at 37°C	Centrifugation (1 h at 15,000 rpm) ICP-OES	~4.3 %	106
40 nm Citrate-AgNPs	10 µg/ml of AgNPs incubated in BEGM cell media at pH 4.5 for 24 h at 37°C	Centrifugation (1 h at 15,000 rpm) AAS	~7 %	159
PVP-AgNWs Diameter: 129±74 nm Bimodal length: 2.8±2.4 µm and 7.0±2.0 µm	10 µg/ml of AgNPs incubated in DMEM, RPMI1640 and DCCM-1 cell media for 168 h at 37°C	Ultrafiltration through 2 KDa filter membranes (13,000 rpm) ICP-OES	n.q.	189
10 nm Citrate-AgNPs	12.5 µg/ml of AgNPs incubated in DI water or bacterial growth media (pH 7) for 24 h at 37°C	UV-Vis Centrifugation (1 h at 21,000 g) AAS	3.5% (DI) 7% (bacterial growth media) 16.1% (DI) 26% (bacterial growth media)	194
n.q.: not quantified ICP-MS: Inductively coupled plasma mass spectrometry ICP-OES: Inductively coupled plasma optical emission spectroscopy AAS: Atomic absorption spectroscopy				

Table 2.4 Summary of Ag⁺ ion release studies reported in the literature

Silver nitrate (AgNO₃) is frequently used as a control to study the effects of free Ag⁺ ions but the fact that addition of AgNO₃ to cell culture media rapidly leads to the precipitation of insoluble Ag compounds has not been addressed. Using analytical TEM techniques, precipitates from AgNO₃ in

RPMI-1640 medium have been shown to consist of small particles, with sizes from 20 to 200 nm, that were likely a mixture of silver oxide ($K_{sp}=4 \times 10^{-11}$) and silver chloride ($K_{sp}=1.77 \times 10^{-10}$) (**Figure 2.9**). The amount of free Ag^+ measured by ICP-OES was less than 5 % of the total Ag^+ added.¹⁸⁹ Therefore, a combination of characterization techniques is necessary in order to understand which particulate species actually interact with the cells.

On the other hand, the addition of proteins in the cell culture medium could alter what the cell “sees”.¹⁹⁷ It is known that NPs are covered with serum proteins after their dispersion in serum-containing physiological media,¹⁹⁸ leading to a so-called protein corona.¹⁹⁹ These proteins may be responsible for modifying the aggregation state of AgNPs and may even directly affect the degree of Ag^+ ions released from the NP surface.²⁰⁰ For instance, Kittler *et al.* reported that 50 nm PVP-capped AgNPs suspended in RPMI medium containing Bovine Serum Albumin (BSA) agglomerated, but the same particles remained dispersed if the BSA was replaced by foetal calf serum (FCS).²⁰¹ The authors suggested that the albumin lipoprotein, glyco-protein and globulin content of FCS may have been responsible for a steric stabilization of the AgNPs. Yen *et al.* linked variations in serum protein attachment to the surface of AgNPs and AuNPs to differences in their uptake mechanism and toxicity to macrophages. They suggested that negatively charged AuNPs (zeta potential from -56.64 mV to -78.81 mV) were able to adsorb serum proteins and enter cells *via* the phagocytic as well as the pinocytic pathway. However positively charged AgNPs (zeta potential from 5.35 mV to 15.43 mV) could not adsorb serum proteins, which reduced uptake and exerted a lower cytotoxicity than AuNPs.²⁰² In another published study, BSA was found to interact strongly only with uncapped AgNPs compared to PVP- and citrate-capped AgNPs of similar sizes (surface charge not reported). The presence of serum albumin led to the aggregation of uncapped AgNPs and consequently lowered their antibacterial activity compared to the capped AgNPs.²⁰³

There are also conflicting data in the literature about whether sulfur containing proteins such as albumin are able to alter the chemistry of AgNPs. Previous work has shown that PVP-capped AgNWs do not sulfidise in phosphate buffered saline (PBS) containing 5% cysteine or 5% BSA, but do sulfidise in the inorganic fraction of a serum-free cell culture medium (DCCM-1).¹⁸⁹ Other reports have shown that Ag^+ ions can bind strongly to both inorganic sulfur groups as well as organosulfur compounds, with the highest affinity for thiols, such as cysteine.²⁰⁴ Ag^+ ions may have a high affinity for thiols and cysteine, changing their biological activity; however, Ag^+ ions may not be able to remove sulfur from biological molecules to form an inorganic sulfide without the existence of other oxidizing species.

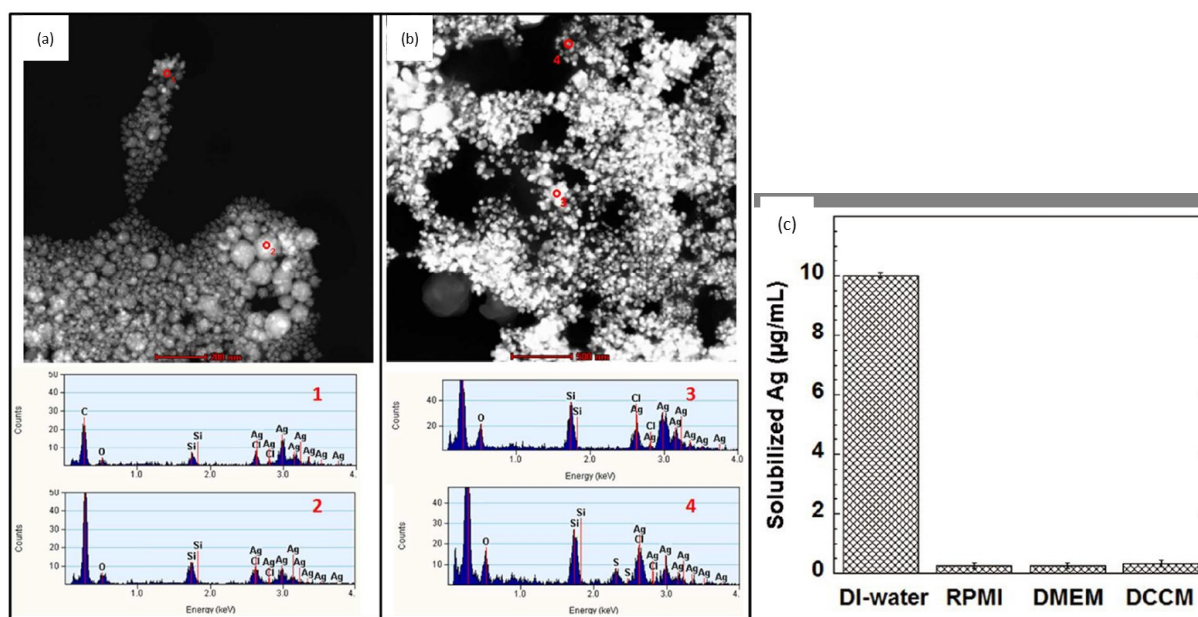


Figure 2.9 (a-b) HAADF-STEM-EDX characterization of the precipitates formed after the incubation of 17.0 $\mu\text{g/mL}$ AgNO_3 (equal to an Ag concentration of 10 $\mu\text{g/mL}$) in (a) RPMI-1640 and (b) DCCM-1 cell culture medium, at 37 $^\circ\text{C}$ for 0.5 hours. The corresponding STEM-EDX spectra 1-4 were collected from the areas 1-4 marked in (a-b). The precipitates were collected by filtering the solution through 2 kDa filter membrane and were washed 3 times with DI-water. In both cell culture media, the particles have sizes that range from ~ 20 to ~ 200 nm. STEM-EDX analysis reveals that the precipitates in RPMI-1640 are probably a mixture of silver oxide and silver chloride. The insoluble compounds formed in DCCM-1 likely also contain silver sulfide. (c) ICP-OES analysis of solubilized silver concentrations of 17.0 $\mu\text{g/mL}$ AgNO_3 (equal to an Ag concentration of 10 $\mu\text{g/mL}$) in DI-water, RPMI-1640, DMEM, and DCCM-1 solutions, incubated at 37 $^\circ\text{C}$ for 0.5 hours ($n = 3$). Although 100% of the free Ag^+ ions were detected in the AgNO_3 DI-water solution, the amount of solubilized silver in cell culture media was less than 0.5 $\mu\text{g/mL}$ after incubation. The dissolved Ag^+ ions are probably sequestered as insoluble precipitates in the cell media, indicating the limitation of using ICP-OES to study the kinetics of Ag^+ dissolution in cell culture media. Reprinted with permission from ¹⁸⁹. Copyright 2013 American Chemical Society.

2.3.4.3 Transformation in Pulmonary Surfactant

Pulmonary surfactant covers the entire alveolar region, decreasing the surface tension in the alveoli to prevent alveolar collapse. So far, only a few studies have investigated the stability of AgNPs in environments fully representative of the lung. For example, Stebounova *et al.* studied the stability of two types of AgNPs, PVP-coated and polymer-coated, in artificial interstitial and lysosomal fluids.⁸³ Their experimental results and the extended DLVO model calculations showed that PVP-capped AgNPs precipitated but polymer-coated AgNPs, with a higher negative surface charge, were more stable in the simulated fluids. However, these simulated fluids were lacking any surfactant components. A few other studies have examined the colloidal stabilization of other types of NPs in rat bronchoalveolar lavage (BAL),²⁰⁵ phosphate buffered saline (PBS) containing bovine serum albumin (BSA) and DPPC^{206, 207} or semisynthetic lung fluids consisting of DPPC, palmitoyl-

oleoyl-phosphatidylglycerol and SP-B (70:30:1 wt. %).²⁰⁸ These studies found that NPs were well-dispersed in the presence of DPPC and proteins and that synthetic lung fluid was as effective as BAL in dispersing the NPs. Recently, the stability of citrate-capped AgNPs in DPPC was investigated as a function of pH (**Figure 2.10**).⁷⁹ A decrease in pH was found to accelerate the kinetics of Ag^+ ion release but also promoted particle aggregation and coarsening. DPPC however, delayed the release of Ag^+ ions, without significantly altering the total amount of Ag^+ released after two weeks. By coating the AgNPs, DPPC improved their dispersion and inhibited aggregation and coarsening. This may allow AgNPs to enter cells more easily, leading to a greater ROS formation within cells, reduced cell viability and increased DNA damage.

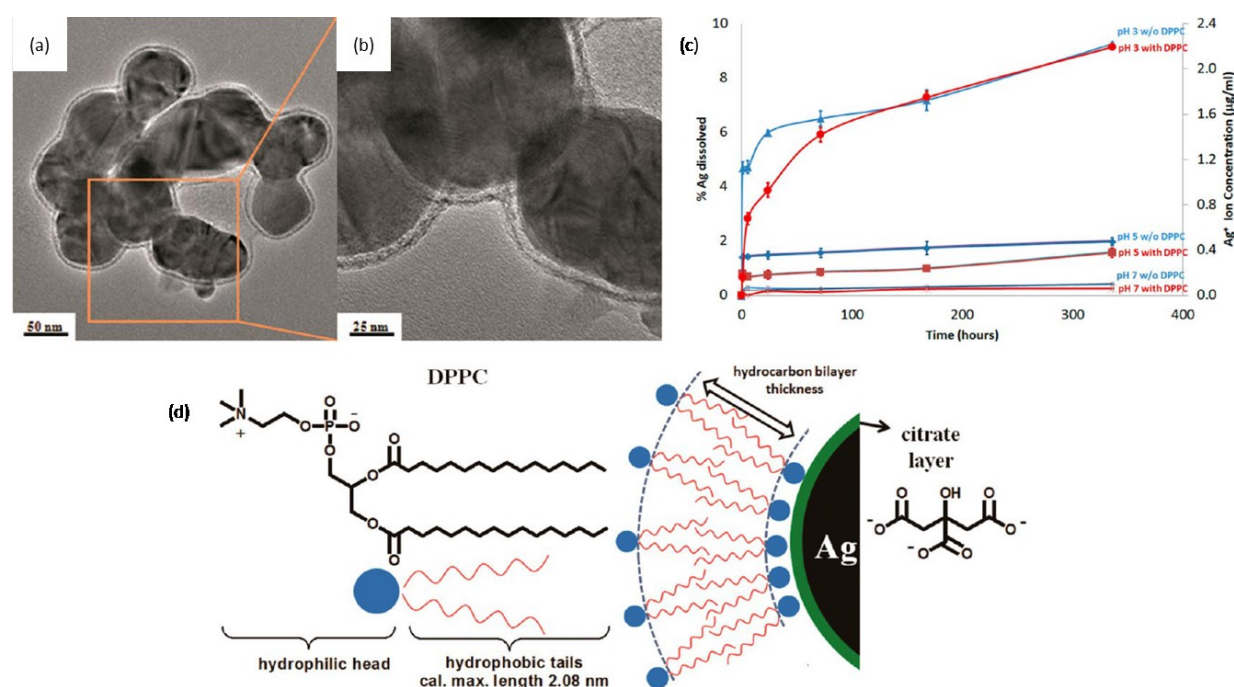


Figure 2.10 (a-b) BF-TEM images of 20 nm citrate-coated AgNPs incubated in a perchlorate pH 3 solution, in the presence of DPPC, for 1 day. Incubation of AgNPs in solutions containing DPPC resulted to a significant increase of the amorphous layer thickness around the particles, which implies the formation of DPPC layer(s) on the surface of AgNPs. The samples were negatively stained with uranyl acetate to enhance the contrast of lipid coating. The high affinity of electron dense uranyl ions to the carboxyl groups of citrate and the phosphate groups of DPPC results in dark contrast. Therefore, the outside layer of AgNPs, showing dark contrast, is likely to be the polar groups of DPPC facing the aqueous environment, whereas the hydrophilic heads of the inner layer of DPPC likely interact with the citrate layer. (c) Ag^+ ion release from AgNPs incubated in perchlorate solutions at pH 3, 5, or 7, in the presence and absence of DPPC. The DPPC coating of AgNPs may serve as a semipermeable layer, delaying the release of Ag^+ ions but without significantly altering the total amount of Ag^+ released after two weeks. (d) A schematic illustration of a model of DPPC bilayer structure on the surface of AgNPs. The DPPC surfactant molecule consists of a trimethyl ammonium bounded to an acidic phosphate, providing a hydrophilic zwitterionic headgroup and two hydrophobic fatty acid tails comprised by 16 hydrocarbons. There is likely a formation of a lipid bilayer structure on the surface of citrate coated AgNPs, with hydrophobic tails associating with each other, whereas the hydrophilic groups are oriented towards the aqueous environment and the citrate layer, respectively. Reprinted with permission from ⁷⁹. Copyright 2013 American Chemical Society.

The coating of AgNPs by phospholipids could have serious implications on pulmonary homeostasis by interfering with biophysical surfactant function. One study, for example, investigated the effect of gold nanoparticles (AuNPs) as a model metal air pollutant on the surfactant function of a semisynthetic surfactant, composed of DPPC, palmitoyl-oleoyl-phosphatidylglycerol (POPG), and SP-B (70:30:1 wt.%)²⁰⁸. AuNPs markedly inhibited the adsorption of pulmonary surfactant at the air-liquid interface, induced a dysfunction during film compression and inhibited the re-spreading during film expansion. Since AuNPs were shown to be coated by phospholipids, the authors speculated that these coated particles could adsorb at the air-liquid interface and thereby inhibit the adsorption of free phospholipids.

Furthermore, how the binding of surfactant components on AgNPs may affect their toxicity is not known. Research on other types of NMs has shown, for instance, that a bovine surfactant preparation (Survanta) and SP-A increased the uptake of TiO₂ particles into primary rat alveolar macrophages.²⁰⁹ Konduru *et al.* showed that single walled carbon nanotubes (SWCNTs) could bind phosphatidylserine (PS), which facilitated their recognition and internalization by several types of phagocytic cells, such as mouse RAW264.7 macrophages, primary monocyte-derived human macrophages and dendritic cells and primary rat brain microglia.²¹⁰ Bound surfactant components could also affect the intracellular response to AgNPs. Although there are currently no data on AgNPs, studies on other systems have emphasised the importance of the NP-surfactant interactions. For example, recent findings demonstrated that pre-coating multi-walled carbon nanotubes MWCNTs with Curosurf[®] evoked an increase in ROS, inflammatory chemokine release and apoptosis in human monocyte derived macrophages.²¹¹ An increased ROS production by normal human primary bronchial epithelial cells and A549 type 2 alveolar epithelial cells was also reported for single-walled carbon nanotubes (SWCNTs) dispersed in DPPC.²¹² Moreover, coating various types of NPs with the surfactant-associated proteins SP-A and SP-D, led to the agglomeration of the particles.^{150, 213} The selective binding of SP-A and SP-D to carbon nanotubes,²¹⁴ the adsorption of SP-A on metal oxide NPs¹⁵⁰ and the adsorption of SP-D on AuNPs²¹³ has already been shown. Recently, Ruge *et al.* revealed a pronounced binding of SP-A to hydrophobic magnetite nanoparticles (mNPs) whereas SP-D preferentially adsorbed on hydrophilic mNPs.²¹⁵ These findings stress the need to further understand possible interactions between AgNPs and components of the lung surfactant and how these could affect cellular uptake, clearance, translocation and toxic effects in the lung.

2.3.4.4 Internalization of Silver Nanomaterials by Cells and Transformation Inside the Cell

Uptake, internalization and transformation of AgNPs inside cells can be used to predict their potential bioreactivity. The pathways by which NPs enter cells can vary based on the cell type, chemical composition of the NP surface,²⁰² particle size and agglomeration state.¹⁶⁴ Endocytosis is a

form of active transport in which cells take up objects by enclosing them in vesicles or vacuoles pinched off from their cytoplasmic membrane.²¹⁶ The endocytotic processes that enclose NPs in membrane vesicles include phagocytosis, pinocytosis, and caveolae-dependent or clathrin-mediated endocytosis.²¹⁷⁻²²⁰ Phagocytosis is typically restricted to specialized mammalian cells, like macrophages, and involves the ingestion of large particles by large vesicles called phagosomes (diameter > 250 nm).²²¹ Smaller particles, ranging from a few up to hundreds of nanometres are internalized by pinocytosis or macropinocytosis, which occurs in almost all cell types. Energy-dependent clathrin-mediated endocytosis is probably the primary characterized mechanism for the cellular uptake of NPs.²²² Greulich *et al.* investigated the uptake of 50 nm PVP-coated AgNPs by human mesenchymal stem cells and their intracellular distribution.²²³ Their results demonstrated that clathrin-mediated endocytosis and macropinocytosis were the primary uptake mechanisms. In another study, Haase *et al.* suggested that THP-1 macrophages may internalize AgNPs through both phagocytic and non-phagocytic mechanisms.¹⁶⁴ TEM imaging revealed aggregated AgNPs in vesicles but also individual particles distributed throughout the cytoplasm without being surrounded by membrane envelopes. The authors speculated that aggregated AgNPs are incorporated *via* phagocytosis while single NPs enter cells *via* non-phagocytic routes. Other authors have also suggested that NPs are able to enter cells by direct penetration of the cell membrane *via* diffusion, membrane fluidity, passing through ion-channels or by adhesive interactions (electrostatic forces, Van der Waals- or steric interactions),^{224, 225} but these mechanisms are controversial. Moreover, there is currently limited understanding on the cellular fate of NPs after they enter cells. After endocytosis, NPs should normally reside in membrane-bound vesicles,^{226, 227} *i.e.* endosomes that would later evolve into phagolysosomes or autophagosomes. Some studies, however, also report the presence of NPs in the cytosol,¹⁷⁴ in mitochondria,²²⁸ and in the nucleus.^{70, 164, 165} The mechanisms through which NPs can escape from endosomes are not understood, but seem to depend on the core composition,²²⁹ surface composition,²³⁰ charge²³¹ and shape of the NPs.^{232, 233}

The surface charge of NPs can largely influence the uptake mechanism by cells. In general, due to the negatively charged character of the cell plasma membrane, cationic NPs are internalized more efficiently than neutral or anionic NPs.²³⁴ Macropinocytosis seems to be the dominant mechanism for the uptake of positively charged NPs, while a clathrin- and caveolae-independent endocytosis may mainly contribute to the uptake of negatively charged NPs.²³⁵ For example, bare AuNPs (positively charged) were taken up by macropinocytosis and clathrin- and caveolin-mediated endocytosis, whereas PEG-coated AuNPs (negatively charged) mostly entered cells by caveolin- and/or clathrin-mediated endocytosis, but not by macropinocytosis.²³⁶ The shape of NMs is another important factor that can determine their uptake mechanism. This can have important implications on the long-term consequences of nanomaterial exposure; for example since frustrated phagocytosis is considered as an important factor in the initiation of an inflammatory response due to exposure to fibres with a high aspect ratio. Comparisons with asbestos fibres in the lung and the induction of

mesothelioma have been made to both AgNWs and multi-walled carbon nanotubes with high aspect ratios.^{19, 237} In a recent study, backscatter scanning electron microscopy (BSE) was used to investigate the cut-off length for the frustrated phagocytosis of AgNWs *in vitro* and *in vivo*.⁷⁵ While *in vitro* frustrated phagocytosis by THP-1 macrophages could be observed with fibres $\geq 14\ \mu\text{m}$, *in vivo* studies showed incomplete uptake at a fibre length of $\geq 10\ \mu\text{m}$. The same group had shown that inflammation in the pleural space after intrapleural injection of AgNWs in mice occurred at a length $\geq 5\ \mu\text{m}$.²³⁷ Therefore, the onset of inflammation could not be correlated with the onset of frustrated phagocytosis. However, the limited resolution of the technique used did not allow for any quantitative analysis of dissolution or changes to the AgNP morphology and chemistry after cellular uptake.

The amount and rate of uptake of AgNPs by the cells can differ by several orders of magnitude depending on the size, shape, surface charge, and surface functionalization of the particles.²³⁸ Analytical techniques such as ICP-MS, ICP-OES or AAS are often used to quantify the amount of AgNPs taken up by cells. However, attention should be paid to the protocol used for the digestion of tissue samples, since some procedures commonly used can lead to low Ag recoveries due to AgCl precipitation.²³⁹ Furthermore, the use of these techniques does not efficiently distinguish between AgNPs attached to the cellular membrane and those internalized by the cells. To overcome this limitation, AgNPs labelled with a fluorescein derivative (DTAF) were used to quantify cellular uptake by macrophages (RAW 264.7) after 3 hour exposure. TEM and confocal microscopy were used to confirm the localization of AgNPs in the cellular cytoplasm.²⁴⁰ Confocal microscopy was also employed by Cronholm *et al.*, who detected AgNPs in the cytosol in 80% of the investigated cells.²⁴¹ When developing a methodology based on fluorescently-labelled NPs, however, it is important to keep in mind that the attachment of a fluorochrome to the NP surface may alter the physicochemical properties of AgNPs, thus influencing their cellular uptake, or the label may detach from the NPs inside cells, leading to artefacts. Pratsinis *et al.* employed dark-field microscopy to visualize the intracellular localization of AgNPs of different sizes.¹⁹⁶ Both 5.7 nm and 16.8 nm AgNPs were efficiently taken up by mouse macrophages after 24 hour exposure. Their results also indicated that NP agglomeration had taken place following cell internalization. In another study, macrophages were exposed to 5 nm and 100 nm PVP-coated AgNPs.¹⁵⁶ Using TEM and optical ultra-resolution imaging, a lower amount of 5 nm PVP-coated AgNPs was detected inside macrophage cells compared to 100 nm particles. However, a higher cytotoxicity was induced by the smaller sized AgNPs (LD_{50} was 6.25 $\mu\text{g/mL}$ for 5 nm AgNPs and 25 $\mu\text{g/mL}$ for 100 nm AgNPs). The authors suggested that the techniques used didn't provide the spatial resolution needed to visualize the 5nm AgNPs inside cells. However, additional data on the solubility of AgNPs would be necessary in order to confirm whether the observed cytotoxicity was due to higher dissolution, as would be expected due to the increased surface area to volume ratio.

In order to visualize the distribution and chemistry of AgNPs inside cells, a combination of electron microscopy techniques (TEM, SEM and dual beam FIB combined with energy dispersive X-

Ray (EDX) analysis) has been applied in a number of studies. In a study by Park *et al.*, no presence of AgNPs was detected in damaged cells, suggesting that the acute toxicity of these NPs was due to their intracellular dissolution.²⁴² Van Winkle *et al.* observed an intracellular size reduction of AgNPs in a rat type 1-like alveolar epithelial cell line by TEM.²⁴³ On the contrary, Carlson *et al.* concluded that 55 nm AgNPs (unspecified “hydrocarbon” coating) did not dissolve after uptake by a rat alveolar macrophage cell line.¹⁵² TEM imaging showed that AgNPs remained at the same size, even when they were inside cellular compartments responsible for digestive processes, such as vacuoles. In another study, 20-40 nm AgNPs were found as agglomerates inside A549 lung cells.¹⁶⁵ Moreover, a change in particle morphology was observed by TEM, as smaller particles had formed close to the original AgNPs inside cells. The authors suggested that these small particles could have been formed due to the precipitation of silver salts/complexes following release of Ag^+ ions intracellularly, or resulting from a re-nucleation of AgNPs. Since no chemical analysis of these smaller particles was performed, however, these hypotheses could not be tested. In a recent publication, high resolution analytical TEM techniques were employed to elucidate the cellular uptake and reactivity of AgNWs inside a human alveolar epithelial type 1-like cell line (**Figure 2.11**).³ AgNWs were found in the cytoplasm and membrane-bound vesicles by bright field TEM (BF-TEM). However, staining agents, such as osmium tetroxide and potassium ferricyanide, which are frequently used during biological sample preparation for TEM (to enhance the electron density of cellular compartments and organelles), were shown to cause substantial changes to the morphology and chemistry of AgNWs. Therefore, heavy metal staining processes were eliminated and precautions were taken to avoid AgNW sulfidation in ambient air and in the cell culture medium.¹⁸⁹ High angle annular dark field scanning transmission electron microscopy (HAADF-STEM), a technique which is highly sensitive to local variations in the atomic number within a sample, and EDX spectroscopy were used to observe the morphological and chemical features of individual AgNWs within TT1 cells. These techniques provided compelling evidence that Ag^+ is released from AgNWs inside epithelial cells and that Ag^+ ions subsequently precipitate as Ag_2S . It was suggested that this process occurred by the action of sulfide species including H_2S , HS^- and S^{2-} inside cells. Cell viability studies showed no evidence of cytotoxicity and reactive oxygen species were not observed on exposure of cells to AgNWs. It was therefore proposed that Ag_2S formation reduces the amount of bioavailable free Ag^+ , significantly limiting short-term toxicological effects. These findings underline the importance of careful experimental design and sample preparation and the use of a combination of characterization techniques in order to understand transformations of AgNPs inside cells, and the consequences on the bioreactivity of the particles. One of the main challenges is the ability to measure the amount of “free” ionic and particulate Ag in the biological *milieu*. The use of ICP-MS to measure the concentration of Ag^+ ions or AgNPs within cells¹⁰⁶ is limited by the fact that ICP-MS detects the total metal content without distinguishing between metal oxidation states. Therefore, the development of methods for the quantification of oxidation rates of AgNPs inside cells is crucial for future research. Previously, fluorescent dyes and

confocal microscopy were employed to visualize the amount of ionic Zn^{2+} released from ZnO NWs inside human macrophages.²⁴⁴ The development of a sensitive and selective colorimetric Ag^+ detection method, however, is still under research. Application of similar methods would provide valuable insight into the mechanisms by which AgNPs exert their biological effects and help deconvolute the effects of Ag^+ ions and Ag particles.

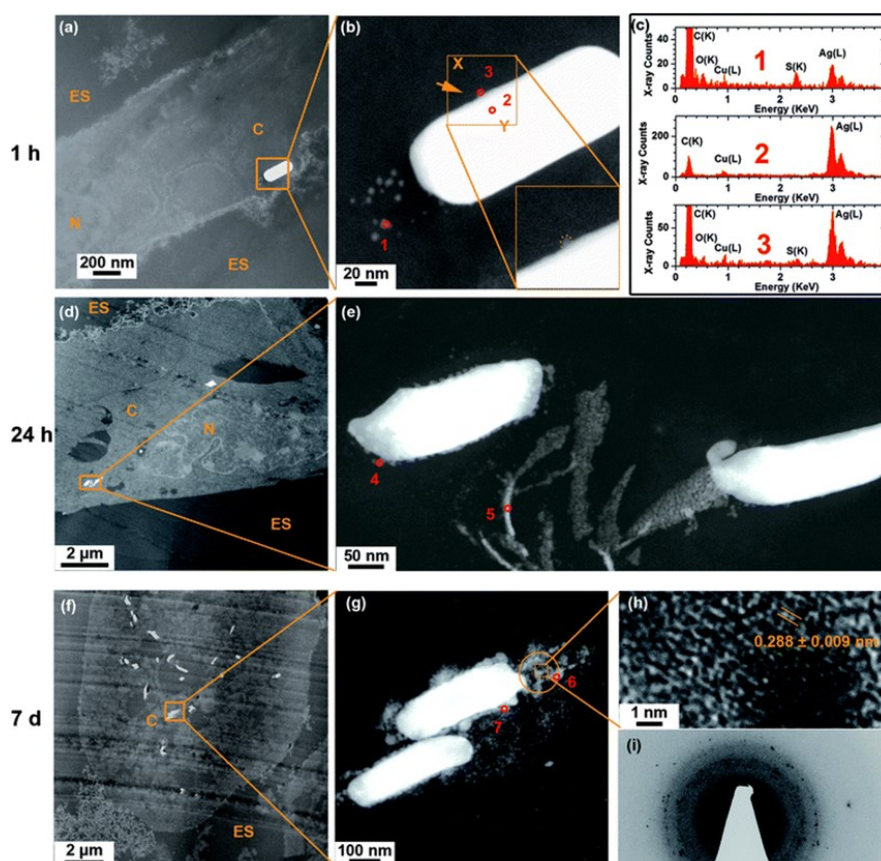


Figure 2.11 Changes in the morphology and chemistry of AgNWs as a function of time, after their uptake by TT1 epithelial cells at 1 hour (a–c), 24 hours (d–e) and 7 days (f–i), following a pulsed exposure, using unstained cell sections. (a) HAADF-STEM image of an AgNW inside the cell cytoplasm showing particles surrounding the tip of the AgNW. (b) A corresponding higher resolution HAADF-STEM image depicts the boxed area in (a). The insert in (b) shows a higher magnification image of the AgNW edge. (c) STEM-EDX spectra taken from the corresponding areas 1–3 marked in (b). Ag(L) peaks and S(K) peaks were detected in the STEM-EDX spectra of these small particles, indicating the formation of Ag_2S (d–e) HAADF-STEM images, where image (e) depicts the boxed area in (d), showing that dissolution and sulfidation of the AgNWs was more substantial after 24 hours (f–i) HAADF-STEM images, where (g) depicts the boxed area in (f). (h) An HRTEM image of the boxed area in image (g). The particles had a lattice spacing of 0.29 nm, close to the mono-clinic structure of Ag_2S (-112) (i) SAED pattern from the circled area in (g), using a selected area aperture size of ~ 130 nm. The interplanar spacings measured from the SAED patterns were consistent with bulk monoclinic Ag_2S . Reproduced from ³ by permission of The Royal Society of Chemistry.

2.3.5 Silver Nanoparticles as Antibacterial Agents for the Treatment of Bacterial Infections in the Lung

Recent advances in the research and development of engineered nanomaterials have enabled a wide range of novel applications for commercial and industrial uses. One field that nanoparticles are expected to revolutionise is medicine, through the design of complex structures that can be targeted to specific tissues, have mechanisms for controlled release and evade rapid clearance.^{51, 245} The lung is a particularly attractive target for nanomedicine, to treat pulmonary as well as systemic disease. Its large surface area and circumvention of first pass metabolism allow for high drug absorption. Moreover, inhalable drugs are non-invasive and can be self-administered, while their nano-size allows them to penetrate in the deep lung and reach the alveolar region more efficiently than larger particles. There are currently several types of nanoparticles being developed for respiratory applications, which aim to overcome the limitations of conventional drugs and be used for the treatment of numerous lung diseases, such as asthma, bacterial infections, emphysema, cystic fibrosis, cancer and others (**Table 2.5**).²⁴⁶ A major part of research on drug delivery nanoformulations has previously been devoted on “soft” nanoparticles, such as solid lipid nanoparticles, organic polymers and liposomes.²⁴⁶ Recently there has also been a move toward the use of “hard” inorganic nanoparticles, like carbon nanotubes or noble metal nanoparticles.¹ In both cases, nanoparticles have often been used as vehicles for the administration of active pharmaceutical ingredients in the lungs. Silver has been used as a disinfectant since antiquity;²⁴⁷ therefore silver nanoparticles are receiving increasing attention for their inherent antibacterial properties and have already been incorporated in several consumer products (*e.g.* wound dressings, antibacterial textiles, cosmetic and hygiene products, antibacterial sprays, nasal drops). Consequently, AgNPs represent an exciting opportunity for the development of platforms for the treatment of pulmonary bacterial infections.

Among lung diseases, invasive microbial infections affecting the lungs are increasing in incidence and diversity.²⁴⁸ Although the lung is constantly exposed to potential infectious agents, the extraordinary natural defence mechanisms of the respiratory tract may, in most cases, prevent serious infections. The most common causes of bacterial lung infections include *Streptococcus pneumoniae*, *Haemophilus species*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*.²⁴⁹ After infection, bacteria bound to the surface of macrophages are rapidly internalised into phagosomes, which engage with endosomal trafficking processes and gradually acquire the characteristics of terminal phagolysosomes, an event concomitant with the death of the microbe. Pulmonary macrophages have a key role in the defence against respiratory infections produced by viruses, bacteria, mycobacteria and fungi. Together with dendritic cells, they are active producers of cytokines and leukotrienes, and have important pro- and anti-inflammatory roles in the alveoli and airway submucosa, respectively.²⁵⁰ However, some pathogens, such as *Legionella* spp. and *Mycobacteria* spp., can alter lysosome

trafficking²⁵⁰ and phagolysosome fusion²⁵¹ or even block host macrophage apoptosis by a TNF receptor-dependent mechanism^{252, 253} in order to survive *inside* cells.

Type of Nanoparticle	Application	Reference
Solid Lipid Nanoparticles	Asthma	254
	Lung Cancer	255
	Cystic fibrosis	256
	Tuberculosis	257
	Insulin delivery	258
	Pulmonary hypertension	259
Polymeric Nanoparticles	Pulmonary fibrosis	260
	Lung Cancer	261
	Anti-oxidant delivery	262
	Lung infections	263
	Anticoagulant delivery	264
	Pulmonary hypertension	265
	Gene delivery	266
Liposomes	Asthma	267
	Anti-oxidant delivery	268
	Bacterial infections	269
	Pulmonary arterial hypertension	270
	Surfactant therapy	271
Polymeric liposomes	Gene delivery	272
	Protein and peptide delivery	273
Lipid nanoparticles	Asthma	274
	Lung infections	275
Dried nanoparticles	Anti-inflammatory	276
	Lung cancer	277
	Pulmonary infections	278
Self-assembly nanoparticles	Antibody delivery	279
Carbon nanotubes	Cancer	280
Silver nanoparticles	Anti-proliferative effects in cancer cells	281, 282
Gold nanoparticles	Lung cancer diagnosis	283
	Anti-cancer drug delivery	284
	Simultaneous magnetic resonance imaging (MRI) and therapy	285
	Photodynamic therapy	286

Table 2.5 Examples of nanoparticle systems for pulmonary applications. Reproduced from ⁴⁸¹ with permission from The Royal Society of Chemistry.

In the last two decades, the rate at which bacteria are becoming resistant to current antibiotic treatments has increased substantially, leading to an urgent need for the development of new

antibiotics (World Health Organisation, 2014). Silver nanoparticles are considered as potential therapeutic agents which could have a significant impact on respiratory research and medicine, by offering a multifunctional platform that could simultaneously diagnose and treat bacterial infections.²⁸⁷⁻²⁸⁹ The antibacterial properties of AgNPs have been shown to depend on several physicochemical properties of the particles, including their size, shape, chemistry and surface coating.^{194, 290-292} Currently, the mechanisms by which AgNPs are able to reduce the activity of bacteria are believed to arise due to a synergistic effect, including both direct particle-specific biological effects and also the release of Ag^+ ions.^{195, 293} However, several discrepancies in the published literature need to be addressed in order to draw accurate links between the physicochemical properties of the particles and the observed biological effects. Moreover, the same properties that make AgNPs excellent antimicrobial agents may also exert adverse effects on pulmonary structure and function. In fact, Ag^+ ions released from AgNP surfaces have been shown to damage proteins by desulphurisation, generate reactive oxygen species (ROS)¹²⁵ or interfere with NO redox equilibria in airway smooth muscle cells in the lung²⁹⁴. This oxidative potential may increase the permeability of the lung epithelium to AgNPs, resulting in DNA damage, lipid membrane damage, chromosomal aberrations and cell-cycle arrest.^{126, 295}

The effects of AgNPs on bacteria are commonly evaluated by various *in vitro* viability measurements, such as minimum inhibitory concentration (MIC), fractional inhibitory concentration (FIC), zone of inhibition test, and the LIVE/DEAD bacterial assays. These assays can provide valuable information about the antibacterial action and potential cellular toxicity of AgNPs. However, more detailed mechanistic investigations of the cellular interactions with AgNPs are necessary in order to provide insights about how to optimise the design of AgNPs for the treatment of bacterial infections. Several reports have illustrated that biophysical interactions may occur between AgNPs and bacteria, such as biosorption, AgNP breakdown or aggregation and cellular uptake.^{296, 297} AgNPs can increase the permeability of bacterial cell membranes, penetrate into the cytoplasm and disturb cellular functions.²⁹⁸

The application of three-dimensional (3D) analytical electron microscopy in this area could be invaluable, because it can provide high spatial resolution information of both the morphology and chemistry of AgNPs. Since the physicochemical properties of AgNPs may change in the cellular environment, sufficient characterization should take place not only for as-synthesized particles but also at the point of exposure, in order to understand what agent of Ag actually interacts with the cells. Moreover, spatially resolved maps of the anatomical distribution and physico-chemistry of AgNPs at the sub-cellular level and observation of changes in the cellular ultrastructure could help deciphering the antibacterial mechanisms exerted by nano-Ag. Therefore, nanoscale imaging methods have the potential to provide vital information about these systems and, when used in conjunction with complementary quantitative studies or other techniques such as low resolution optical imaging, could provide a holistic understanding of the system interactions.

2.3.5.1 *Phagocytic Mechanisms and Morphological Alterations in Bacteria-infected Macrophages*

Since infection is a localized disease, the first step in the design of actively targeted nano-therapeutics would be to understand where bacilli reside in infected lungs. The use of microscopy techniques can provide crucial information about the topography and the biological processes taking place in infected lungs. Bacterial phagocytosis by immune cells is the crucial step in the host defence against microbial invaders.²⁹⁹ The phagosome is a central mediator of both the homeostatic and microbial functions of macrophages. This phagocytic vesicle is programmed to degrade internalized material with high efficiency, through rapid acidification and fusion with lysosomes, which are rich in proteolytic and lipolytic enzymes. However, bacilli have developed mechanisms to escape from phagosomes and enter into autophagosomes for survival and replication.³⁰⁰ For instance, Yuan *et al.* demonstrated that autophagosomes play a regulatory role in the clearance of *Pseudomonas aeruginosa*, a bacterium that frequently infects immunodeficient individuals.³⁰¹ In their work, the accumulation of typical autophagosomes with double membranes in infected alveolar macrophages (MS-H) was identified using transmission electron microscopy (TEM) (**Figure 2.12A**). Together with TEM imaging, the results of bio-analytical assays led to the conclusion that *P. aeruginosa*-induced autophagy represented a protective host mechanism.³⁰¹ Recently, the kinetics and mechanical pathways by which macrophages may interact with, and internalize bacteria, was investigated in depth by Moller *et al.* using a combination of microscopy techniques, including scanning electron microscopy (SEM), differential interference contrast (DIC) microscopy with interference reflection (IRM) and 3D confocal microscopy (CM).³⁰² Their findings revealed a multistep process in which macrophages used filopodia as phagocytic tentacles to sense and form long-lived interactions with surface-adhering bacteria (*Escherichia coli*). In the same work, fluorescence microscopy showed that *Legionella pneumophila* could enter macrophages through a cholesterol-sensitive pathway, thus avoiding immediate digestion inside lysosomes by interacting with the autophagy pathway of the host cells.³⁰²

Mycobacterium tuberculosis (*M.tb*) is the causative agent of tuberculosis, one of the most devastating infections in the lung.³⁰³ It is well-known that *M.tb* bacilli may spread infection by arresting phagosome maturation.³⁰³ Infected macrophages present an increase in the retention of host lipids, which provides a potential source of nutrients that could be accessed by bacteria. Confocal microscopy and TEM imaging revealed the presence of a great number of lipid droplets throughout the cytoplasm of infected macrophages (**Figure 2.12B**).³⁰⁴ Apart from inhibiting phagosome maturation as a survival strategy, *M.tb* may interfere in processes linked to cell death, such as apoptosis. Apoptosis is considered as a mechanism whereby the host cell can contain the infection and eliminate it before it can spread further throughout the body.³⁰⁵ As shown in **Figure 2.12C-E**, cells infected with virulent (H37Rv) *M.tb* strains present morphological perturbations in the ultrastructure

of mitochondria, such as increased electron density, clear definition of the cristae and inter-mitochondrial fusion. These findings suggest an augmentation in the functional activity of mitochondria, which could be linked to an inhibition of the apoptotic response.³⁰⁶ Lee *et al.* investigated the characteristics and determinants of macrophage cell death caused by *M.tb*, using TEM and SEM (**Figure 2.12F-G**).³⁰⁵ Their study provided evidence that cell death caused by virulent *M.tb* was distinct from classical apoptosis. The authors suggested a mechanism based on the permeabilization of lysosomal membranes, followed by the destruction of lipid bilayers and a concomitant degradation of several phospholipid species.³⁰⁵

In conclusion, the use of confocal and electron microscopy techniques can provide important information about key biological signals involved in bacterial infection. Cellular mechanisms taking place in infected host cells, such as autophagy or inhibition of phagosome maturation, can be observed using high resolution imaging. Together with data obtained from cytotoxicity assays (*e.g.* mitochondrial activity measurements), imaging revealing the ultrastructural morphology of cellular organelles, can provide a broader understanding about the pathways of cell death. Moreover, the use of techniques such as immunolabelling, could help identify molecular ligands involved in the cellular uptake of bacteria. This information could have a pivotal role in the identification of the appropriate targets for the development of antibacterial drugs based on AgNPs. Finally, in future studies, analytical TEM techniques will be indispensable in the study of the intracellular localization, and the morphological and chemical stability of AgNPs applied to bacteria-infected cells.

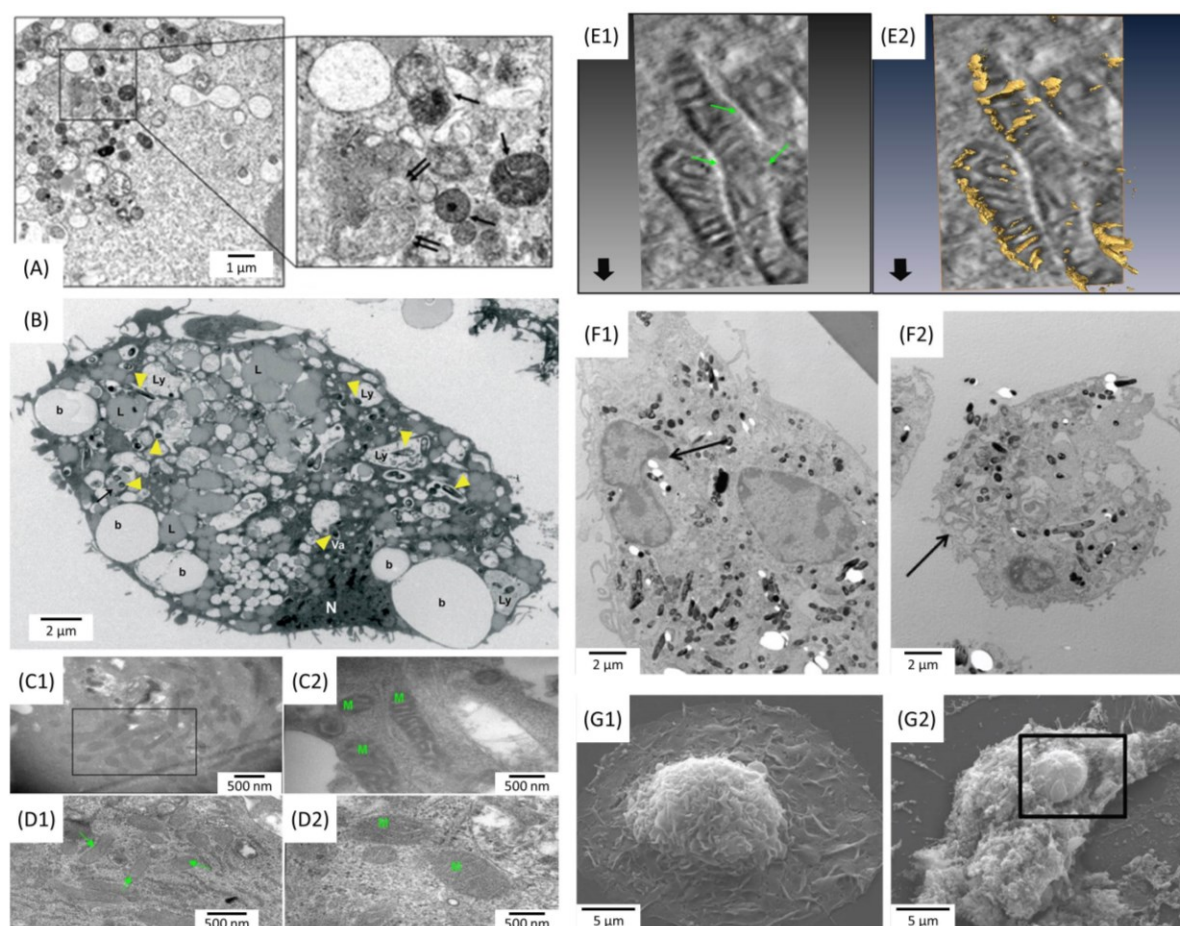


Figure 2.12 Electron microscopy images showing morphological alterations in bacteria infected macrophages. (A) Formation of autolysosomes (single arrows) and autophagosomes (double arrows) in *Pseudomonas aeruginosa*-infected MS-H cells after 1 hour (MOI=10:1). Journal of Cell Science by Company Of Biologists. Reproduced with permission of Company of Biologists Ltd.³⁰¹ (B) Distribution of beads (b), lysosomes (Ly), lipid droplets (L), nuclei (N) and *Mycobacterium tuberculosis* bacilli (arrowheads) in infected human monocyte derived macrophages (HMDM) after 5 days (MOI=5:1). Reprinted with permission from³⁰⁴. Copyright 2012 John Wiley & Sons Ltd. (C) Ultrastructural changes in the mitochondria of THP-1 cells infected with a virulent *M.tb* strain for 24 hours (MOI=10:1). Electron dense and elongated mitochondria, with prominent cristae were observed in infected cells, compared to uninfected THP-1 cells (D). Reprinted with permission from³⁰⁶. Copyright 2013 Nature Publishing Group. (E) Electron tomography images showing inter-mitochondrial fusion and breaches in the mitochondrial cell wall. Reprinted with permission from³⁰⁶. Copyright 2013 Nature Publishing Group. (F) Peripheral nuclear condensation and partial nuclear protrusion from condensed areas in bone marrow derived macrophages (BMDM) infected with *M.tb* (MOI=25:1), after 3 h (F1); cells at the late phase of death with damaged plasma membrane (F2). Reprinted with permission from³⁰⁵. Copyright 2011 Lee *et al.* (G) Extensive loss of the plasma membrane, exposing the underlying cytoskeleton and intracellular organelles in *M.tb*-infected macrophages (G2) compared to uninfected cells (G1). Reprinted with permission from³⁰⁵. Copyright 2011 Lee *et al.*

2.3.5.2 Unravelling the Antibacterial Mechanisms of Silver Nanoparticles

Since AgNPs are known to dissolve and release Ag^+ ions under physiological conditions, there has been much debate in the literature as to whether the reactivity of AgNPs arises due to an ionic^{307, 308} or particulate effect^{87-89, 157}, or both. The following section discusses the mechanisms by which the release of Ag^+ ions can be correlated to their antibacterial activity and the influence of the AgNP-cell interface on these processes.

Dissolution of Silver Nanoparticles and Effects of Ag^+ Ions on Cell Membrane Permeability

Recent evidence has suggested that AgNPs exert their antimicrobial properties by the localised release of Ag^+ ions in the acidic and aerobic environment of *E.coli*.¹⁹⁵ The bacterial membrane has an acidic pH and oxidising environment, which (according to equation 2.1) will accelerate AgNP dissolution and release of Ag^+ ions. This release could disrupt the outer membrane of bacteria, enhancing AgNP and Ag^+ ion uptake leading to a reduction in cell viability. This hypothesis has recently been supported by different authors, who used a sensor sensitive to Ag^+ ion release to show that the toxicity of AgNP could be explained entirely by accumulation of intracellular Ag^+ ions.^{194, 195}

Silver salts alone, such as AgNO_3 , can also kill bacteria and damage the surrounding tissue.^{288, 293} Therefore, it is assumed that one of the most important pathways through which AgNPs exert their antimicrobial properties is based on the effect of free Ag^+ ions.^{195, 293} Xiu *et al.* demonstrated that AgNPs themselves did not affect the biological activity of bacteria, only the ionic Ag^+ .³⁰⁸ In their study, the antibacterial properties of polyethylene glycol (PEG) coated AgNPs of three different diameters (2.8 ± 0.47 nm, 4.7 ± 0.20 nm and 10.5 ± 0.59 nm) were evaluated under aerobic and anaerobic conditions, that preclude Ag^0 oxidation. Under anaerobic conditions, AgNPs did not exert any effects on the viability of *E. coli*, whereas prolonged air exposure induced higher toxicity. This work showed that AgNPs do not show antibacterial effects in anaerobic conditions that preclude Ag^0 oxidation and Ag^+ ion release³⁰⁸. However, by removing the oxygen from the system, the redox active surface of AgNPs could lose its capability for generating reactive oxygen species (ROS), which has also been linked to their toxicity,³⁰⁹ as discussed in the following paragraphs. By not taking this factor into account, this study cannot completely isolate particulate and ionic effects. The above findings suggest that the antibacterial activity of AgNPs could be controlled by modulating Ag^+ release, possibly through the manipulation of oxygen availability, coating agents or NP size.

The mechanism by which Ag^+ ions permeate the cell wall, is thought to be due to a strong interaction between ionic Ag^+ and thiol groups of cysteine residues of proteins present in the bacterial cell wall. This may lead to the disruption of these proteins^{310, 311} and the accumulation of Ag^+ ions in

the cell wall.³¹² It has also been proposed that Ag^+ ions can destabilize the membranes of bacteria, leading to the formation of pits in the bacterial cell wall,^{313, 314} increasing its permeability and facilitating the internalization of AgNPs and Ag^+ ions.¹⁹⁵ Based on theoretically calculated and experimentally estimated Ag^+ ions affinities, other amino acids such as arginine and histidine should be better ligands for Ag^+ ions, but these interactions have not been widely investigated.²⁸⁸ The interaction of Ag^+ ions with enzymes of the respiratory chain reaction, such as NADH dehydrogenase,³¹⁵ could lead to the uncoupling of respiration from ATP synthesis. Ag^+ ions may also bind to transport proteins, causing proton leakage and collapse of the proton motive force.^{316, 317}

Ag^+ ions released from AgNPs or chemisorbed on its surface could also be responsible for the catalytic generation of ROS by serving as electron acceptor.³¹⁸ ROS are natural by-products of the metabolism of respiring organisms and, while small levels can be controlled by the antioxidant defences of the cells, excess ROS production may produce oxidative stress.⁸ ROS mainly target lipids, DNA, RNA and proteins, causing severe consequences, such as membrane breakdown and impairment of the mitochondrial function or of the DNA replication machinery. In bacteria, Ag^+ ions would likely induce ROS generation by impairing the respiratory chain enzymes, through direct interactions with thiol groups in these enzymes or with superoxide radical scavenging enzymes, such as superoxide dismutases.^{316, 318, 319}

Some authors have also suggested ROS generation directly from the AgNPs. In one study, where the toxic effects of Ag^+ ions, AgCl and AgNPs on nitrifying bacteria were investigated, the authors reported that all forms of Ag were able to induce intracellular ROS generation.³⁰⁹ The bacterial inhibition of each form of Ag was correlated with the corresponding concentration of intracellular ROS. However, at the same concentration of ROS, AgNPs were more toxic than the other forms of Ag, indicating that additional mechanisms contributed to the overall toxicity.³⁰⁹ The formation of the superoxide anion, but not of hydroxyl radicals, observed for Ag^+ ions,³¹⁸ was also observed for *E. coli* treated with AgNPs.³²⁰ In contrast to these findings, other authors claim that ROS production has a negligible contribution to the antimicrobial properties of AgNPs. Sintubin *et al.* showed that only a small amount of ROS were induced by the presence of AgNPs and an even smaller amount was produced in the presence of Ag^+ ions alone.³²¹

Taken together, these findings could support the hypothesis that the bacterial properties of AgNPs arise mainly due to Ag^+ ion release from their surface. Morones *et al.* supported this hypothesis, demonstrating that Ag^+ ions were more toxic than AgNPs to *S. aureus* bacteria, in relation to bacterial growth.²⁹⁶ TEM imaging revealed that the ultrastructural changes observed in AgNP-treated bacteria were similar to those induced by Ag^+ ions: detachment of the cytoplasm membrane from the cell wall and formation of a low density region, rich in agglomerated DNA,³²² which protects DNA molecules from damage induced by external stimuli. Similarly, low molecular weight proteins can also accumulate around the nucleus as a response to Ag^+ ion attack.³²³

Direct Particle-Cell Wall Interactions

Although the principal mode of antibacterial activity of AgNPs involves the release of Ag^+ ions, studies have demonstrated that the mechanism by which AgNPs interact with bacteria involves a combination of both chemical and physical interactions.¹⁹⁴ The small size and extremely large surface area of AgNPs allows them to make strong contact with the bacterial cell wall.³²⁴ Several studies have reported that AgNPs are able to adhere to the plasma membrane, change its permeability and penetrate inside the cytoplasm.^{293, 297, 309, 317, 325} Studies have shown that a synergistic effect is required, where Ag^+ ions are required for the antibacterial effects of AgNPs, but cell-NP contact increases Ag^+ release and increases the amount of cellular uptake of particle associated Ag^+ ions.^{194, 195} A report by McQuillan *et al.* supports the hypothesis that dissolution at the cell membrane is the primary mechanism of action of silver nanoparticles.²⁹³

Several other studies have demonstrated that AgNPs are more toxic to bacteria than the Ag^+ ions released from the corresponding AgNPs in the extracellular media.^{88, 291, 326, 327} Taglietti *et al.* pointed out that two factors need to be considered, the release of Ag^+ ions in the exposure medium, referred to as a “long-distance mechanism”, but also a “short-distance mechanism” at the particle-bacterial membrane interface.³²⁸ Some authors suggested that this short-distance effect arises due to enhanced dissolution of the AgNPs close to the bacterial cell wall.¹⁹⁴ Recent studies have provided new insight that may link the discrepancies existing on the relative effect of AgNPs and Ag^+ ions on toxicity.^{194, 195} Bondarenko *et al.* examined the effect of three types of AgNPs (uncoated, PVP-coated and protein-coated) against six bacterial strains (Gram-negative: *E. coli*, *P. fluorescens*, *P. putida* and *P. aeruginosa* and Gram-positive: *B. subtilis* and *S. aureus*). Their data demonstrated that direct contact between the bacterial cell and AgNPs enhanced the toxicity of AgNPs because the amount of *internalized* Ag^+ ions released from the particle surface increased, which could be driven by the proton motive force of the bacterial membrane that decreases the local pH to as low as 3.¹⁹⁵ Similarly, Ivask *et al.* analysed the amount of bioavailable Ag inside bacterial cells using bacterial bioreporters and showed that the concentrations of bioavailable Ag were substantially higher than the amount predicted based on the concentration of (abiotic) dissolved silver from the same NPs.¹⁹⁴ These findings suggest that the particle-cell interface plays an essential role in the antibacterial action of AgNPs and therefore support a synergistic effect between AgNPs and Ag^+ ions where the AgNPs can deliver a localised ionic dose to the cell.¹⁹⁵ The low local pH around bacterial membranes is expected, according to equation 6, to lead to higher AgNP dissolution rates. However, in a previous work, low pH values promoted the aggregation and coarsening of 20 nm citrate-capped AgNPs.⁷⁹ As discussed in the following section (“Particle size”), this would compromise the ability of AgNPs to bind on bacterial cell walls.³²⁹ Therefore further investigation is necessary in order to deconvolute the effects of each parameter on the bioreactivity of AgNPs.

Particle size is considered as one of the main physicochemical properties that affect the bactericidal behaviour of AgNPs, as explained by equation 6.²⁹¹ Metal particles with smaller diameters present a very high surface area compared to larger particles, potentially increasing their overall reactivity.³³⁰ Morones et al., for instance, demonstrated that the binding strength of particles to bacteria was dependent on the surface of interaction.²⁹⁶ Four types of Gram-negative bacteria (*E. coli*, *V. cholera*, *P. aeruginosa* and *S. typhus*) were exposed to AgNPs in the range of 1–100 nm and examined by complementary analytical electron microscopy techniques, including TEM in combination with elemental mapping analysis and high angle annular dark field (HAADF) scanning transmission electron microscopy (STEM). The combination of these techniques provided spatially resolved maps showing the morphological changes of bacteria following incubation with AgNPs, and the anatomical position of AgNPs inside the cell. Only the smallest particles, with diameters of 1–10 nm, were found on the surface of the cell wall and inside the bacteria. The presence of very small (~1 nm) AgNPs was detected using HAADF-STEM, a technique in which images are formed by electrons that have been scattered to high angles, due to Rutherford scattering. As a result, the intensity of the images is related to Z^2 , which provides high contrast between metallic silver and organic material.³³¹ The authors suggested that the mechanism through which AgNPs were able to penetrate into *E. coli* was mediated by changes to the structure of the cell wall, which led to an increase in its permeability.²⁹⁷ In another report, however, AgNPs from 20 nm up to 80 nm were able to penetrate through the inner and outer membrane of *P. aeruginosa*, suggesting that bacteria may also internalize larger particles.²⁹⁸ The data produced in both studies did not provide enough details about the structural damage induced to the cell membrane or possible differences in the uptake mechanism of AgNPs of different sizes. Moreover, the aggregation states of AgNPs in the media used in the two studies were not characterized, which could be another factor that affects their uptake. The effect of aggregation was demonstrated for *E. coli* exposed to 20–30 nm citrate-coated AgNPs, which were compared to citrate- and polycyclic aromatic hydrocarbon (PAH)-coated gold nanoparticles (AuNPs). TEM and field emission scanning electron microscopy (FEG-SEM) revealed that the binding of Ag or AuNPs to bacteria was different depending on their aggregation state. PAH-coated AuNPs were found to be more strongly bound to cell walls and penetrated more readily inside bacteria than citrate-capped AgNPs, which tended to aggregate more readily.³²⁹ Consequently, further research is needed in order to understand if the interaction of AgNPs with bacterial membranes or their internalization is size-dependent and what is the role of aggregation. Determining the optimal particle size for enhanced interaction between AgNPs and bacteria will enable an increase of their antibacterial activity while keeping the dose as low as possible. The use of 3D imaging techniques (*e.g.* electron tomography, focused ion beam scanning electron microscopy) would be beneficial for this purpose, as they can provide valuable information about the interaction dynamics of NPs with and within the cells, which can be limited or lost in the case of 2D imaging.

Effects of the cell wall structure. Although particle size might play an important role in the penetration of AgNPs inside bacteria, structural differences in the organization of bacterial cell wall may also influence this interaction. Gram-negative bacteria have a thin peptidoglycan layer (~2–3 nm) between the cytoplasmic membrane and the outer membrane.³³² In contrast, Gram-positive bacteria lack the outer membrane, but have an outer peptidoglycan layer about 30 nm thick.³³³ The thicker cell wall of Gram-positive bacteria acts as a barrier protecting the cell from penetration of AgNPs or Ag⁺ ions into the cytoplasm. Therefore, Gram-positive bacteria are expected to be more resistant to the action of AgNPs. The bacterial strain may also modulate the bioavailability of Ag⁺ ions liberated from AgNPs by altering: (i) the extracellular dissolution of AgNPs via bacterial exudates (organic acids, peptides, biosurfactants) or (ii) the cellular uptake of Ag⁺ ions *via* cell-NP interactions which are controlled by the structure and charge of the cell wall.²⁹³

Effects of surface coating and charge. The differences between the cell wall structure of Gram-positive and Gram-negative bacteria suggest that the surface chemistry of AgNPs could also affect their antibacterial activity.^{334, 335} The surface charge and coating of AgNPs is a significant factor that could govern the particle-membrane interactions and subsequently the antibacterial effects of AgNPs, supporting the hypothesis that particle-cell contact may indeed play a role in modulating the reactivity of AgNPs.²⁹³ Bondarenko *et al.* compared PVP and protein (casein)-coated colloidal AgNPs and showed that the antibacterial potency of collagen coated AgNPs was greater than PVP coated AgNPs, which was attributed to differences between the affinity of each nanoparticle type to the cell wall.¹⁹⁵ Xiu *et al.* also showed that the survival rates of *E. Coli* to AgNPs was higher for PVP-AgNPs compared to PEG-AgNPs, however the particle diameters were not comparable.³⁰⁸ In one study, electron microscopy demonstrated that the bacterial cell wall of *E. coli* tended to attract and accumulate positively charged PAH-coated AuNPs rather than negatively charged citrate-capped AgNPs.³²⁹ The carboxyl phosphate and amino groups on the cellular membrane of Gram-positive and Gram-negative bacteria give them a negative surface charge.³³⁶ The bacterial cell wall would consequently form an electrostatic barrier that limits its interaction with negatively charged AgNPs, but enhances it with positively charged AgNPs.³³⁷ This is supported by a recent study, in which positively charged AgNPs were shown to tightly interact with bacterial surfaces, thus resulting in higher concentrations of bioavailable Ag⁺ ions from these particles. Moreover, positively charged AgNPs interfered with the normal function of the bacterial electron transfer chain and were responsible for ROS generation at the cell membrane.¹⁹⁴

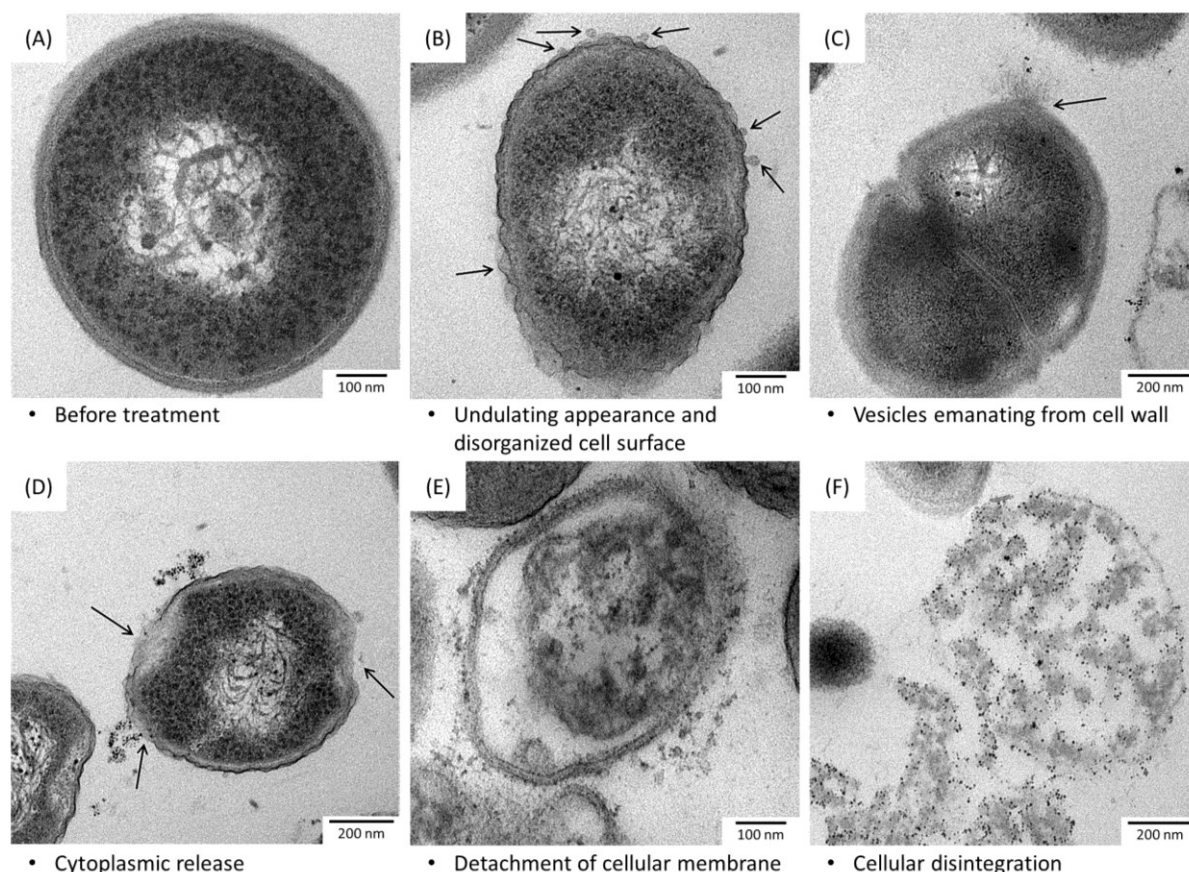


Figure 2.13 TEM images of Gram-positive bacteria (*S. aureus*) (A-E) and Gram-negative bacteria (*P. aeruginosa*) (F), before (A) and after (B-F) exposure to AgNPs, showing ultrastructural damage in bacterial cells induced by AgNPs. Reprinted with permission from ³³⁸. Copyright 2013 Elsevier Ltd.

Positively charged surface coatings have been shown to play a key role in accelerating cell wall breakdown and cytoplasm release.³²⁹ For example, cationic polymer-stabilized AgNPs bind to the negatively charged bacterial surface via electrostatic interactions.³³⁷ However, the potential of using these coatings for biomedical applications remains an obstacle due to the high cytotoxicity of the polymers.^{339, 340} One alternative to overcome this problem would be the use of peptides or polysaccharides containing amine groups.¹⁶⁸ In one study, a pronounced destruction of the bacterial cell wall was observed in *P. aeruginosa* exposed to chitosan-coated AgNPs using SEM.¹⁶⁸ Bacitracin A (BA) and polymyxin E (PE) are polypeptides with cationic macrocyclicamido groups, which are known as antimicrobial peptides (AMPs). These peptides were used by Mei *et al.* in the synthesis of functionalized AgNPs for the treatment of both Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (*S. aureus* and *B. amyloliquefaciens*).³³⁸ The immobilization of both peptides on the AgNP surface increased their antibacterial activity up to a 10-fold without symptoms of bacterial resistance. Fluorescence microscopy and TEM revealed that BA/PE-AgNPs were localized on the surface of bacteria and inside the cytoplasm. The membrane appeared seriously damaged, showing an undulating appearance and vesicles emanating from the cell wall, while some specimens were more

severely damaged and showed cytoplasmic release (**Figure 2.13**). The major target sites for antimicrobial agents are often present at the outermost layers of the bacterial cell. The cell wall of Gram-positive bacteria is rich in teichoic acids, whose main function is to provide rigidity by attracting cations such as Mg^{2+} .³⁴¹ AMPs are considered excellent antimicrobial agents^{342, 343} because their macrocyclicamido groups can participate in metal-ligand p-bonding, producing a complex with Ca^{2+} and Mg^{2+} cations. Therefore, it has been suggested that BA and PE on the AgNP surface can chelate Mg^{2+} and Ca^{2+} and extract them from the original binding sites in teichoic acids, thus damaging the bacterial cell wall.³³⁸ Moreover, the same study showed the presence of BA/PE-NPs in the bacterial nucleoid. These findings suggested that AgNPs could bind to ribosomes and chromosomes, resulting in an inhibition of ribosomal function and a suppression of DNA replication, ultimately causing cell death.²⁹⁶ The important role that organic surface coronas surrounding nanoparticles may play towards controlling nanomaterial properties for biological applications has also been demonstrated by Daima *et al.*²⁹² AgNPs were modified by the surface corona of biologically active polyoxometalates (POMs), which enhanced the damage to bacterial cells due to a synergistic antibacterial action of AgNPs and POMs. SEM micrographs revealed significant physical damage to treated bacterial cells, such as big holes in the bacterial structure or even complete disintegration. The modified AgNPs were toxic to both Gram positive and Gram negative bacteria, while being biocompatible with PC3 epithelial mammalian cells, suggesting their potential towards specific antimicrobial targeting.²⁹²

Therefore, surface coatings will affect the dissolution rates, bioavailability and aggregation state of AgNPs *in vitro* and *in vivo*. AgNPs aggregation or dispersion in cell culture media can be controlled by modifying their surface properties (*i.e.* hydrophobic or hydrophilic coatings), altering their uptake inside cells by changing their contact with the cell membrane. In fact, some publications have demonstrated how polymer stabilizers may play an important role in determining the toxicity of AgNPs by reducing the exposure of cells to particles.³³⁴ The surface properties of AgNPs may govern to a large extent their interaction with bacterial cell walls via electrostatic attraction, by complexation of metal cations localized on the cell wall, or through the interaction with sulphur-containing membrane proteins. Regardless of the exact mechanism involved, it is clear that AgNP and bacterial cell wall interactions increase the permeability of the cell wall and consequently lead to its disruption.

Published literature has provided enough evidence that the release of Ag^+ ions from the surface of AgNPs may be regarded as the main mechanism of antibacterial activity of AgNPs. However, it is becoming increasingly clear that dissolution of AgNPs in the exposure medium cannot wholly account for the observed antimicrobial effects. Additional effects at the particle-bacterial membrane interface and inside the cells seem to play a role in the action of AgNPs. Therefore, a synergistic effect between Ag^+ ions and AgNPs must be considered in order to obtain accurate conclusions on the antibacterial pathways. Recent findings illustrate the importance of membrane interactions in the antimicrobial activity of AgNPs, as they can translocate across, and become

internalized, directly through the cytoplasm or inside vesicles, where their subsequent dissolution driven by an oxidising and acidic pH at the plasma membrane could lead to very high local concentrations of Ag^+ ions.¹⁹⁴ Further research is necessary in order to understand the exact physicochemical and cellular mechanisms underlying these interactions and how they can be exploited in the design of therapeutic AgNPs.

2.3.6 Conclusions

In summary, the toxic impact of AgNMs in the lungs is being studied extensively.⁵⁰ Toxic outcomes, including oxidative stress, lipid peroxidation, inhibition of mitochondrial activity, damage of DNA and cell apoptosis, have been demonstrated and suggested to result from the release of Ag^+ ions upon the interaction of AgNPs with lung tissue.^{21, 70-74, 344} However, the bioreactivity of one-dimensional AgNWs in the lungs is not well understood. Several papers have already shown that AgNWs are able to access the alveoli in the deep lung *in vivo*.^{75 345} Consequently, AgNWs have raised concerns due to the comparisons with asbestos fibres in the lung and the induced mesothelioma.^{75, 346} *In vitro* work suggested that AgNWs were more toxic than spherical AgNPs on alveolar epithelial cells.⁷¹ AgNWs were also shown to produce dose-dependent inflammation in *murine* lungs and responses that dependent on both AgNW length and dissolution rates.³⁴⁵

However discrepancies still exist in published data. The lack of consistency could be in part due to a lack of characterization of the fate of AgNWs in biologically relevant environments. AgNMs are highly dynamic systems and their properties can dramatically change when incubated in biological media. As recent work has highlighted, sulfidation of AgNWs in cell culture media¹⁸⁹ as well as inside human alveolar epithelial type 1-like cells³ reduced AgNW toxicity. For AgNWs that reach the alveolar region of the lung, the first point of contact will be the lung lining fluid (LLF). Although inhaled AgNWs that deposit in the alveoli are expected to adsorb LLF components, the effects of LLF on the bioreactivity of AgNWs have not been previously explored. Currently, few data exist on the stability of AgNMs in general in the LLF, or in individual LLF components. Hence, to draw accurate conclusions about the bioreactivity of AgNWs in the lungs, there is an urgent need to examine their physicochemical properties at the *point of exposure*, in order to understand the characteristics of the system that the cells encounter. Accurate characterization of the particles should rely on several complementary techniques across biological and physical sciences disciplines. Furthermore, characterization of AgNWs should also extend to the particle-cell interface. Investigating possible transformations of the particles within the cellular environment can provide valuable information about the interactions of AgNWs with cellular components and the mode of their biological action. Correlating this information with the results of biological assays could lead to an improved

understanding of the properties of AgNWs that drive their bioreactivity, which may ultimately guide the regulation of this material and provide guidelines for their safe handling.

2.4 The Bioreactivity of Zinc Oxide Nanomaterials in the Lungs

Metal oxide nanostructures are receiving increasing attention in nanotechnology research because they offer distinct advantages over their bulk counterparts in a large variety of applications. Nanostructures of ZnO, in particular, have attracted interest because of their unique piezoelectric, semiconducting and catalytic properties.³⁴⁷ ZnO is a direct bandgap semiconductor that is transparent in the visible, has high infrared reflectivity and is chemically and thermally stable. Additionally, ZnO can easily be doped to provide novel magnetic properties. These distinctive optoelectronic properties of ZnO nanostructures allow for the development of novel nanosensors, nano-transducers, photocatalysts and nanogenerators and have made ZnO one of the most important products in nanotechnology, besides carbon and silicon NMs.^{348, 349} ZnO has a rich family of nanostructures, including particles, rods, wires, plates, dendrites, tubes and hierarchical structures. Among them, one-dimensional ZnO nanostructures, such as nanowires, are increasingly being studied due to the novel properties observed with the formation of high aspect ratio structures.³⁴⁹ Despite the fact that the functional properties of ZnONWs are being exploited and developed, little is known about their bioreactivity. With the continuous growth in the production of ZnONWs and their implementation in commercial products, occupational and public exposures are expected to inevitably increase. Consequently, there is an urgent need to address their potential hazards and establish the conditions for their safe use.

2.4.1 The Bioreactivity of Zinc Oxide Nanomaterials *In Vivo*

At present, epidemiological data on human exposure and health effects of ZnONMs are lacking. Ultrafine ZnO particles are known to reach the alveoli and cause pulmonary inflammation and symptomatic responses in the lung.^{350, 351} This manifests as metal fume fever in welders.³⁵² However, only a few studies have investigated the *in vivo* pulmonary toxicity of ZnONMs.

According to Sayes *et al.*, rats exposed to ZnONPs (50-70 nm) by intratracheal instillation expressed potent but reversible pulmonary inflammatory responses.³⁵³ However, the comparison of *in vivo* and *in vitro* measurements demonstrated little correlation, indicating that *in vitro* cellular systems need to be further developed and validated in order to provide useful screening data on the relative toxicity of inhaled particles. Wang *et al.* examined the toxic effects of ZnO nanoparticles (20 nm) following inhalation.³⁵⁴ A significant increase in zinc content was observed in liver tissues, while histopathological examination showed that nanoparticles caused a severe damage in liver and lung

tissues. This led to the conclusion that ZnONPs may translocate from the lung into the blood circulation after exposure. Additionally, the deposition in the lungs and the speed of penetration were shown to be more rapid for ZnO than iron oxide (Fe_2O_3) NPs.

Cho *et al.* found that ZnONPs (10 nm) intratracheally instilled into female wistar rats were inflammogenic to the lungs both acutely (24 hours) and chronically (4 days).³⁵⁵ In a similar study by the same group, ZnONPs induced pulmonary eosinophilia, goblet cell hyperplasia, severe fibrosis and airway epithelial injury.³⁵⁶ Similar pathologies were also observed after instillation of dissolved Zn^{2+} into rat lungs, which indicated that the main cause of the toxic effects could be the dissolution of ZnO in the acidic environments of lysosomes. This could cause lysosomal destabilization and cell death, leading to the subsequent pathogenicity observed. In support of this paradigm, doping ZnONPs with iron, which results to a slower rate of dissolution, led to a decreased acute pulmonary inflammation and cytotoxicity.³⁵⁷ However, some discrepancies remain in the published literature regarding the respective role of particulate and ionic zinc. In a more recent study, *in vitro* and *in vivo* exposures were performed on ZnONPs as well as the aqueous extracts (ZnOAEs) obtained from their dissolution.³⁵⁸ ZnOAEs and ZnONPs had similar toxic and pro-inflammogenic effects on the human alveolar type 2-like epithelial cell line A549, but the strength of the effects was higher for ZnONPs. Following pulmonary instillation in rats, both ZnONPs and ZnOAEs acutely elicited similar profiles of inflammation and toxicity but eosinophil recruitment was only observed with ZnONPs. There was also a difference in chronic effects, as ZnOAEs did not cause any sustained inflammatory effects by 4 weeks whereas ZnONPs caused a chronic eosinophilic infiltrate into the bronchoalveolar lavage (BAL). These data suggested that the effects of soluble ions were confined to the acute phase of inflammation, probably because they are rapidly cleared by adsorption to systemic circulation.³⁵⁹ The fact that Zn^{2+} ions produced false-positive effects *in vitro* that were not borne out *in vivo*, indicates the need for more sophisticated *in vitro* cell culture systems and the designation of more relevant (chemically, biologically and physiologically) end points to test for *in vitro* toxicity.³⁵³

The ability of ZnONPs to cause cardiopulmonary impairments has been highlighted in a recent work, where Sprague-Dawley rats were exposed to ZnO nanoparticles (ZnONPs) *via* intratracheal (IT) instillation and inhalation of occupationally relevant concentrations.³⁶⁰ In contrast to results of previous studies, instilled ZnONPs accumulated predominantly in the lungs over 24 hours, with only trivial amounts of zinc measured in the heart, liver, kidneys and blood. Instilled ZnONPs altered zinc balance and increased the levels of total cells, neutrophils, lactate dehydrogenase (LDH) and total protein in BAL and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in blood after 72 hours. Inhaled ZnONPs induced systemic inflammation and oxidative imbalance. Moreover, cardiac inflammation and development of fibrosis were detected 7 days after exposure, while degeneration and necrosis of the myocardium occurred after 30 days.³⁶⁰

2.4.2 The Antibacterial Activity of Zinc Oxide Nanomaterials

Over the last two decades, there has been an increased interest in the use of inorganic antimicrobial agents for the control of microbes. Metal oxides, in particular, have attracted attention due to their ability to withstand harsh processing conditions. The antibacterial activity of ZnO nanoparticles has been reported for both Gram-positive and Gram-negative bacteria.³⁶¹⁻³⁶³ The antibacterial activity of ZnONPs was shown to be greater than that of TiO₂ and SiO₂ nanoparticles.³⁶⁴ In most cases, the results suggest a higher toxicity on Gram-positive bacteria than on Gram-negative bacteria.³⁶³⁻³⁶⁵ However, Nair *et al.* observed the opposite effect for ZnO nanoparticles capped with poly ethylene glycol (PEG).³⁶² Antibacterial activity only increased slightly with decreasing particle size but more prominently with particle concentration.^{362, 366} ZnONPs (13nm) completely inhibited *E. Coli* and *S. Aureus* growth at concentrations above 13.4 mM and 1 mM respectively.³⁶⁵ Zhang *et al.*, on the other hand, reported a strong antibacterial activity of ZnONPs (93 nm) on *E. Coli* even at 1.25 mM.³⁶⁷

ZnO nanorod antibacterial coating approaches are also being investigated, for the development of antimicrobial fabrics for facemasks or tissues,³⁶⁸ or for implant modifications, to reduce bacterial adhesion and viability, and thus implant-associated infection. Wang *et al.* showed a decreased survival rate of *S. Aureus* and *E. Coli* on ZnONW arrays with different orientations.³⁶⁹ Jansson *et al.* evaluated the bacterial adhesion and viability of common implant-associated pathogens, *P. aeruginosa* and *S. epidermidis*, on ZnONW surfaces compared to sputtered (flat) ZnO substrates and glass substrates.³⁷⁰ Adherent *P. aeruginosa* on ZnONW surfaces were reduced compared to glass and sputtered ZnO, but the number of adherent *S. epidermidis* on ZnONWs was equivalent to glass. Both ZnONW and sputtered ZnO surfaces demonstrated a significant bactericidal effect on adherent *P. aeruginosa*, killing a 2.5-fold and 1.7-fold more bacteria compared to glass. A more pronounced bactericidal effect of ZnO substrates on *S. epidermidis* was found, with sputtered ZnO and ZnONW substrates killing a 20-fold and 30-fold more than glass, respectively. More recently, ZnONWs grown on glass substrates exhibited significant antimicrobial effects to *B. Subtilis* and *E. Coli* and prevented biofilm formation.³⁷¹ *B. subtilis* was more sensitive to the presence of ZnONWs than *E. coli*, with a 100% and 95% cell inactivation within 2 hours of exposure to the ZnONWs, respectively.

Initially, the generation of reactive oxygen species (ROS) was considered as the main mechanism responsible for the antibacterial activity of ZnONMs,^{361, 366, 367} but lacked solid supporting evidence. More recently, the results of many studies support that their antibacterial activity may be attributed to the release of Zn²⁺ ions from ZnONMs.³⁷²⁻³⁷⁵ The role of dissolution on the bioreactivity of ZnO is discussed in further detail in Chapter 2.4.4.

2.4.3 The Bioreactivity of Zinc Oxide Nanomaterials *In Vitro*

Meanwhile, the toxicity of ZnONPs has been reported for various *in vitro* mammalian cell systems.³⁷⁶ For instance, exposure of mouse neuroblastoma Neuro-2A cells to ZnONPs (50-70 nm) led to cell death, with IC₅₀ values between 50 and 100 µg/ml.²² ZnONPs caused a significant decrease in mitochondrial function while TEM imaging revealed nanoparticles attached to the cell surface as well as some internalization into the cells. The same study showed that ZnONPs were more toxic than other metal oxide nanoparticles (TiO₂, Fe₃O₄, Al₂O₃ and CrO₃). Similarly, ZnO nanorods (20-70 nm × 100-200 nm) were more toxic than Fe₂O₃ and Y₂O₃ NPs on human aortic endothelial cells.²³ The inflammatory response of the cells initiated at a threshold concentration of 10 µg/mL and was dose-dependent above this value, with an IC₅₀ of 50 µg/ml. Another comparative study revealed that ZnONPs were more toxic than carbon black, single walled carbon nanotubes and SiO₂ on primary mouse embryo fibroblast cells.³⁷⁷ In accordance with previous findings, cell viability decreased in a dose-dependent manner above a threshold exposure dose of 10 µg/mL and was linked to the generation of intracellular oxidative stress.

Since *in vivo* work indicates that airway exposure to ZnONPs poses an important hazard, a large part of the *in vitro* studies have focused on pulmonary exposure models. Along with alveolar macrophages, the human bronchial epithelial cell line (BEAS-2B) and the human alveolar adenocarcinoma cell line (A549) are used most frequently. The cytotoxicity of ZnONPs has been shown to be mediated through reactive oxygen species (ROS) generation and oxidative stress,^{378, 379} apoptosis^{380, 381} or genotoxicity.^{382, 383} For example, 20 nm ZnONPs induced a concentration- and time-dependent cytotoxicity in BEAS-2B cells, as well as increased LDH release, oxidative stress and intracellular [Ca²⁺].³⁷⁹ Moreover, the expression of four genes involved in apoptosis and oxidative stress were increased. Wu *et al.* examined the effect of ZnONPs on the expression of a pro-inflammatory mediator, interleukin-8 (IL-8), in human bronchial epithelial cells and BEAS-2B cells.³⁸⁴ ZnO exposure (2-8 µg/mL) increased IL-8 mRNA and protein expression, and this induction was mediated by p65 phosphorylation and IκBα phosphorylation and degradation. Furthermore, Ng *et al.* explored the activation of cellular DNA damage pathway, through the tumour suppressor p53 gene, by ZnONPs.³⁸⁵ They showed that the p53 pathway was activated in human neonatal foreskin fibroblasts upon ZnONP treatment, with a concomitant increase in cell death, suggesting that cellular responses like apoptosis require p53 as the molecular master switch towards programmed cell death.

The role of particle size on ZnONP toxicity has been investigated with mixed results. According to one study, the size of ZnONPs influenced the mitochondria activity and chemokine production of A549 cells, with smaller particles being more toxic than larger ones.³⁸⁶ Lin *et al.* demonstrated that the exposure of A549 cells to ZnONPs (70 and 420 nm) induced a dose-dependent cytotoxicity over a narrow concentration range of 8-18 µg/ml.³⁸⁷ The cells presented elevated ROS levels, resulting in intracellular oxidative stress, lipid peroxidation, cell membrane leakage and

oxidative DNA damage. However, cell viability and ROS levels were not particle size-dependent. Along a similar line, Yuan *et al.* showed that ZnONPs of three different sizes (20-40 nm) were toxic to human embryonic lung fibroblasts in a dose-dependent but not a size-dependent manner.³⁸⁸ Other authors have suggested that the secondary aggregate size may be more important than the primary ZnONP size in determining their toxicity profile.³⁸⁹ Smaller secondary aggregates of ZnONPs were found to be more efficient than larger secondary aggregates in inducing mitochondrial dysfunction, generating elevated intracellular ROS and eventually leading to cell apoptosis in RAW 264.7 murine macrophages.³⁹⁰

The main paradigm explaining the toxicity of ZnONPs is their dissolution and the release of Zn^{2+} ions. Brunner *et al.* found that virtually all human mesothelioma MSTO-211H and rodent 3T3 fibroblast cells died after being incubated with 19nm ZnONPs at concentrations above 15 $\mu\text{g/mL}$.³⁹¹ They inferred that this sharp concentration dependence of the cytotoxic response may be due to the presence of a critical Zn^{2+} concentration in the cell culture medium and to the toxic effects of aqueous Zn^{2+} .³⁹² In an attempt to quantify the role of dissolved Zn^{2+} ions to the cellular toxicity, Xia *et al.* studied the solubility of ZnONPs (13 nm) in water and two cell-free culture media (MDEM and BEGM).³⁷⁸ ZnONPs readily dissolved in the culture media, with more than 80% of the maximum dissolved Zn^{2+} concentration being reached within 3 hours. They also showed that non-dissolved ZnONPs entered caveolae in BEAS-2B cells, whereas in RAW 264.7 murine macrophages they entered lysosomes, where the acidic pH facilitated their further dissolution. However, the concentrations of Zn^{2+} ions were not determined under the experimental conditions and their contribution to cytotoxicity was not clear. Song *et al.* studied the dissolution of ZnONPs in RPMI-1640 cell culture medium and found the equilibrium concentration of Zn^{2+} to be around 10 $\mu\text{g/mL}$ when 40 $\mu\text{g/mL}$ of particles were used.³⁹³ Their results implied that dissolved Zn^{2+} in the cell culture medium or inside cells played the main role in ZnONP toxicity. Deng *et al.* demonstrated that ZnONPs (10, 30, 60 and 200 nm) were toxic to mouse neural stem cells at concentrations higher than 12 $\mu\text{g/mL}$ in a dose-dependent manner, and that ZnCl_2 at the same concentrations had similar toxic effects to the cells.³⁸⁹ Iron doping of ZnONPs, which changes the material matrix to slow Zn^{2+} release, decreased ZnO cytotoxicity.³⁹⁴ Correspondingly, coating 32–95 nm ZnONPs with a TiO_2 shell reduced mitochondrial activity (MTT reduction), membrane damage (LDH release), IL-8 production and ROS generation in A549 cells.³⁹⁵ ZnONPs with thicker shells were less cytotoxic, presumably because of a lower rate of Zn^{2+} release by the ZnONPs. Similarly, the encapsulation of ZnO nanorods in a nanothin amorphous SiO_2 coating resulted to slower dissolution kinetics and significantly lower DNA damage in lymphoblastoid cells.³⁹⁶

Gilbert *et al.* used high resolution X-ray spectromicroscopy and high elemental sensitivity X-Ray microprobe analyses to determine the fate of ZnO and iron-doped ZnONPs following exposure to BEAS-2B cells.³⁹⁷ The cells accumulated zinc from the cell culture solution and only organic-complexed, ionic Zn^{2+} was present intracellularly one hour following exposure. This finding strongly

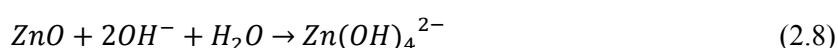
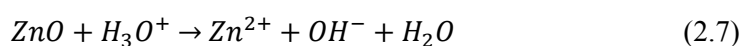
indicates that ZnO toxicity is caused by free or complexed Zn^{2+} within cells. However, their study did not directly show whether the uptake of Zn^{2+} or ZnONPs is the dominant pathway for internalizing zinc. Other reports indicate that the cytotoxicity of ZnONPs is independent of the amount of extracellular soluble Zn^{2+} in the cell culture medium and that direct nanoparticle contact with the cells is important.^{357, 398, 399} Müller *et al.* showed that the toxicity of ZnONWs (100-300 nm $\times$ 2-10 μm) on human monocyte derived macrophages (MDMs) was similar to that of ZnCl_2 .²⁴⁴ Quantification of the amounts of Zn^{2+} released in simulated bodily fluids of extracellular pH (ex-SBF, pH 7.4) and lysosomal pH (lyso-SBF, pH 5.2) by ICP-MS, revealed that ZnONWs dissolve rapidly in lyso-SBF but are relatively stable in ex-SBF. Confocal live microscopy and transmission electron microscopy confirmed that MDMs uptake ZnO nanowires by phagocytosis and that the acidic pH of the lysosomes triggers ZnONW dissolution. Confocal live microscopy also revealed that an increase in intracellular Zn^{2+} concentrations was followed by cell death, which had features of both apoptosis and necrosis.

The effect of shape on ZnONM bioreactivity has not been extensively investigated. ZnO nanorods (6 $\times$ 8 nm and 7 $\times$ 19 nm) were more toxic to A549 cells than spherical ZnONPs (6, 25, and 38 nm).³⁸⁶ The authors suggested that the contact area between a single NP and a single cell was more important in determining toxicity than the total surface area of a NP. Exposure of A549 cells to ZnO nanorods (50 $\times$ 140 nm) induced cytotoxicity, ROS generation, oxidative stress, and activation of caspase-3 and caspase-9 in a dose- and time-dependent way.³⁸⁰ ZnO nanorods induced apoptosis involving the p53, survivin, bax/bcl-2 and caspase pathways. A few studies have examined the toxicity of ZnO nanorod-coated substrates. Li *et al.* investigated the biocompatibility of ZnO wire surfaces (1 μm $\times$ 200 μm) on HeLa (cervical cancer) and L-929 (subcutaneous connective tissue) cell lines.⁴⁰⁰ Cell viability at 48 hours was reduced only for concentrations higher than 100 $\mu\text{g/mL}$, and was 75% for HeLa and 50% for L-929 cells. HeLa cells were shown to phagocytose some of the broken ZnO wires. In contrast to previous findings where mesenchymal stem cells were able to survive on silicon NWs for several days,⁴⁰¹ the total number of adherent cells and adherent live NIH 3T3 fibroblasts, human umbilical vein endothelial cells and bovine capillary endothelial cells decreased significantly after 24 hours on ZnO nanorod (50 nm $\times$ 500 nm) surfaces.⁴⁰² The authors suggested that the engulfment of ZnO nanorods and delivery of toxic material into the cell could be the source of toxicity. In a similar study, murine macrophages initially settled and spread on ZnO nanorod and flat ZnO surfaces.⁴⁰³ However, a significant degree of necrosis was apparent after 6.5 hours of incubation. After 16 hours, the number of live cells adherent on flat ZnO was 52% but only 12% on ZnO nanorods, indicating the role of both nanotopography and the material on cell viability. Apart from the study by Müller *et al.* discussed in the previous paragraph,²⁴⁴ however, there is a gap in the literature regarding the pulmonary toxicity of freestanding high aspect ratio ZnONWs.

2.4.4 The Role of Zinc Oxide Nanomaterial Dissolution on their Bioreactivity

Zinc is an important element in biological systems. As an essential trace element, it is involved in a wide number of biological processes such as metabolism, cell proliferation and differentiation, signal transduction and control of gene expression.⁴⁰⁴⁻⁴⁰⁶ It is a component of over 300 enzymes, as either a catalytic or regulatory cofactor or in a structural capacity.⁴⁰⁴ A severe zinc deficiency in humans can lead to a range of conditions such as dermatitis, anorexia, neurosensory disorders and cell-mediated immune dysfunction. A genetic disorder that affects enteral zinc absorption, *acrodermatitis enteropathica*, can be fatal if left untreated.⁴⁰⁷ Oral zinc supplementation has proven beneficial in a number of infectious diseases, particularly in acute lower respiratory infections, leprosy and tuberculosis⁴⁰⁶ while the topical application of zinc oxide was found to facilitate the healing processes of chronic and surgical wounds.⁴⁰⁸ It is also becoming increasingly clear that Zn^{2+} , like Ca^{2+} , is an essential signalling molecule for a number of physiological processes, with cellular zinc homeostasis being tightly controlled. Alterations in brain zinc levels are now thought to contribute to neurodegeneration in conditions such as Alzheimer's disease, amyotrophic lateral sclerosis and ischemia.⁴⁰⁹⁻⁴¹¹

However, the role of ZnONP dissolution in their toxicity against mammalian cells has not been completely elucidated. The phase diagram of ZnO indicates that it is an amphoteric, which can dissolve according to either reaction (2.7) or (2.8) depending on the solution pH:⁴¹²



The rate of dissolution can also be affected by several factors, such as size, doping, surface coating or the presence of biological components. **Table 2.6** summarizes the results of dissolution studies performed on ZnONMs in relation to toxicological studies. Moreover, the dissolution of 1D ZnO nanostructures is not well understood. In the wurtzite crystal structure, the top (001) plane of ZnO nanorods is comprised of either O^{2-} or Zn^{2+} and is polar. The (110), (100), and (010) side planes are comprised of an equal number of O^{2-} and Zn^{2+} and are thus electrically neutral. The surface energies of (100), (110) and (002) faces calculated using an *ab initio* (all-electron) approach were 2.32 J/m², 4.1 J/m² and 5.4 J/m², respectively.⁴¹³ The polar faces, having the highest surface energy, are metastable and may therefore be dissolved preferentially. However, such selective dissolution is not always observed. In one study, ZnO nanorods with diameters of 100 nm or smaller could not be etched in an alkaline solution.⁴¹⁴ This may be due to the fact that the energy of the polar faces is smaller only by a factor of 2.3 compared to the most stable faces. Small electrochemical changes at

the interface between the solution and ZnO may change the surface reactivity. Illy *et al.* suggested that the selective dissolution of ZnO nanorods may be explained in terms of both the anisotropy of the ZnO surface energy as well as diffusion limitations on the dissolution reaction as a result of the nanoscale morphology.⁴¹² They described a mechanism where rod diameter and chloride stabilization of the (002) planes play a critical role in determining the nanostructure morphology following dissolution. Consequently, ZnO rods with a small diameter (<100 nm) are dissolved homogeneously whereas anisotropic dissolution is observed for rods with a higher diameter (>100 nm), which can lead to the formation of ZnO tubular structures.⁴¹²

Although zinc has been shown to induce cytotoxicity and apoptosis in mammalian cells, few of the studies have linked this to a mechanistic paradigm such as oxidant injury. Considering the number of cellular processes and signalling pathways zinc is involved in, it is not surprising that zinc dyshomeostasis can induce cell death. A rise in the intracellular Zn^{2+} concentration is often associated with high levels of ROS production.³⁷⁸ These oxygen radicals could originate at the particle surface but also from biological substrates such as damaged mitochondria.³⁷⁸ Several studies in isolated mitochondria have shown that Zn^{2+} inhibits cellular respiration. Moreover, it is possible that Zn^{2+} may exert extra-mitochondrial effects that contribute to ROS generation. Cellular oxidant injury could promote further Zn^{2+} release, for example from metallothioneins.⁴¹⁵⁻⁴¹⁷ Increased zinc could also inhibit key enzymes in the glycolytic pathway, leading to ATP depletion in cells.^{418, 419} Finally, high levels of Zn^{2+} could contribute directly to mitochondrial PT pore opening and cytochrome c release, resulting in apoptosis and/or necrosis in target cells.^{420, 421}

Size	Dissolution Medium	Analytical Methodology and Analysis Technique	Summary of Observations	Ref.
30 nm	Algal test medium	Dialysis membrane ICP-AES	- No difference in dissolution rate and equilibrium Zn^{2+} concentration for bulk and nano ZnO particles (19% dissolution).	422
50–70 nm	Osterhout's medium	Recombinant metal sensor bacteria (E. coli MC1061)	- No difference in dissolution between nano and bulk ZnO particles (up to 80% dissolution).	423
20–30 nm	Natural seawater	Centrifugal filtration ICP-AES	- Dissolution decreased with increasing NP concentration (70% dissolution for starting concentrations between 0.1–1 mg/L and 32% for starting concentration of 10 mg/L).	424
50–70 nm	LB Broth	Membrane filtration ICP-AES	- Dissolution rate decreased with increasing NP concentration (~ 3.2% dissolution).	425
6 and 16 nm spheres- 240 × 50 nm and 860 × 300 nm rods	Artificial seawater	Centrifugation and filtration Graphite furnace atomic absorption spectrometry (GFAAS)	- No significant effect of starting NP concentrations. - Dissolution kinetics not significantly different between particle sizes or shapes. - 4.1–4.9% dissolution, with highest equilibrium solubility for smallest spherical particles and lowest for largest rods	426
4, 15 and 241 nm	Aqueous solutions (varying pH and humic acid contents)	Centrifugation ICP-AES	- Size dependent dissolution with equilibrium Zn^{2+} concentrations of 57 mg/L for 4 nm, 22 mg/L for 15 nm and 10 mg/L for 241 nm particles. - pH dependent dissolution (higher at lower pH). - The presence of humic acid increased dissolution.	427
4–130 nm	HEPES buffer (pH 7.5) with varying concentrations of citric acid	Centrifugal Ultrafiltration ICP-OES	- Dissolution decreased with increasing NP size, despite the NPs forming aggregates in solution (~ 1–3 μ m). - Citric acid significantly enhanced dissolution for all NP sizes.	428
20 nm	-Deionized water (DI) - Tris buffer with varying pH (pH 7, 8, and 9) -Artificial seawater (ASW ± Suwannee River fulvic acid (SRFA), cysteine)	Membrane filtration GFAAS	- No significant effect of starting NP concentrations. - Higher solubility in DI compared to ASW. - pH dependent dissolution (higher at lower pH). - Tris buffer enhanced dissolution, possibly by changing the NP aggregation state or serving as a Zn^{2+} binding ligand. - SRFA decreased the dissolution rate and the total amount of Zn^{2+} released, but cysteine had the opposite effect.	429
100–300 nm × 2–10 μ m wires	-Extracellular simulated body fluid SBF (ex-SBF, pH 7.4) - Lysosomal SBF (lyso-SBF, pH 5.2)	Centrifugal Ultrafiltration ICP-MS	- ZnONWs were relatively stable in ex-SBF, with 4.45% dissolution after incubation at 37 °C for 30 min. - ZnONWs dissolved rapidly in lyso-SBF, with complete dissolution after the same incubation conditions.	244
13 nm	- Ultrapure water - DMEM with 10% FBS - BEGM (contains growth factors, cytokines and supplements)	Centrifugation ICP-MS	- Dissolution followed the order: DMEM (90 μ M) > BEGM (80 μ M) > H ₂ O (60 μ M). - Organic components enhanced dissolution, with up to 80% of total dissolved Zn within 3 h.	378

Table 2.6 Summary of ZnO dissolution studies reported in the literature.

Size	Dissolution Medium	Analytical Methodology and Analysis Technique	Observations	Ref.
19 nm	- Ultrapure water ($\pm$ 0.85% v/w NaCl) - 5 mM PBS - Minimal Davis medium (MDM) - LB medium	Centrifugation and filtration AAS	- Equilibrium solubility followed the order: LB (68 mg/L) > MDM (38 mg/L) > NaCl (14 mg/L) > H ₂ O (6.9 mg/L) > PBS (0.57 mg/L). - Nano and bulk ZnO dissolution nearly the same in ultrapure water. Dissolved Zn increased with increasing starting ZnO concentration.	374
25, 40 and 70 nm	- Ultrapure water - Environmental Protection Agency moderately hard water - DMEM and RPMI-1640 ($\pm$ BSA)	Membrane filtration ICP-AES	- Equilibrium solubility followed the order: DMEM (34 mg/L) > H ₂ O (7.18-7.40 mg/L) > RPMI-1640 (5 mg/L) > moderately hard water (0.7-1 mg/L) - BSA enhanced NP dissolution.	430
40 nm	10 mM sodium nitrate (NaNO ₃) solutions with varying concentrations of dipotassium phosphate (K ₂ HPO ₄)	Centrifugation and membrane filtration ICP-OES	- Low concentrations of phosphate rapidly and substantially reduced Zn ²⁺ release. - In the presence of phosphate, particle morphology changed to anomalous and porous material, containing mixed amorphous and crystalline phases of ZnO and zinc phosphate.	431
30 nm	10 mM NaNO ₃ solutions with varying concentrations of sodium sulphide (Na ₂ S)	Centrifugal Ultrafiltration ICP-AES	- Increasing Na ₂ S concentrations reduced the rate and extent of Zn ²⁺ release. - Dissolution and reprecipitation led to the formation of zinc sulphide (ZnS) NPs (< 5 nm). - The solubility of partially sulfidized ZnO NPs is controlled by the remaining ZnO core and not quenched by the formation of a ZnS shell.	432

Table 2.6 (continued) Summary of ZnO dissolution studies reported in the literature

2.4.5 Conclusions

Inhalation of airborne ZnONMs is being widely investigated, as it represents a key route of human exposure, both from the perspective of intentional (diagnostic and therapeutic applications) and unintentional scenarios. In summary, ultrafine ZnO is known to cause pulmonary inflammation,^{351, 352} while ZnONPs have been shown to cause cardiopulmonary impairments.³⁶⁰ *In vitro*, the toxicity of ZnONPs has been reported for various mammalian systems,³⁷⁶ including lung epithelial cells and alveolar macrophages.^{378, 379} Their cytotoxicity was mediated through reactive oxygen species (ROS) generation and oxidative stress,^{378, 379} apoptosis^{380, 381} or genotoxicity.^{382, 383} The main paradigm explaining the toxicity of ZnONPs is their pH-dependent dissolution in the acidic environment of phagosomes following uptake in cells, and the release of toxic Zn²⁺ ions.²⁴⁴

Despite the fact that the use of ZnONWs is becoming more widespread, their potential adverse effects to human health upon exposure are still unclear³⁷⁶. In a previous study, ZnONWs were toxic to human MDMs at similar concentrations as ZnCl₂.²⁴⁴ A rise in intracellular Zn²⁺

concentrations was correlated to cell death, demonstrating that ZnONW toxicity was due to dissolution rather than the high-aspect nature of the wires. Therefore, there is an urgent need to investigate the bioreactivity of ZnONWs with the pulmonary epithelium, in order to elucidate the mechanisms that control their toxicity. Given the central role of dissolution in the bioreactivity of nano-ZnO and the fact that several factors have been shown to affect the release of ionic Zn^{2+} (e.g. size,⁴²⁷ shape,⁴¹² pH,⁴²⁷ complexing anions^{428, 431} and organic components^{378, 427}), characterization of ZnONWs at the *point of exposure* to cells must take place in order to draw accurate conclusions about their bioreactivity. However, no data exist on the stability of ZnONWs in the presence of phospholipids or proteins from the LLF, which represents a first contact site with the pulmonary epithelium, or the effects of LLF on the bioreactivity with lung epithelial cells. Further research is needed in this area in order to improve our understanding of the mechanisms that control the bioreactivity of ZnONWs, which is essential for the regulation and safe use of this material.

3 Experimental Techniques

3.1 Electron Microscopy

Electron microscopy (EM) employs a beam of energetic electrons, which is controlled and focused by a set of electrostatic and electromagnetic lenses, as the source of sample illumination.⁴³³ Electrons are generated from either thermionic sources (e.g. tungsten/LaB₆ filaments) or field emission sources. Due to the much smaller wavelength of electrons compared to visible light, electron microscopes have a much higher spatial resolution than light microscopes. The maximum resolution d that may be achieved through the lens is proportional to the wavelength λ of the incident beam:

$$d = \frac{\lambda}{2n\sin\alpha} \quad (3.1)$$

where n is the refractive index of the medium and α is the half-angle of the maximum cone of light that can enter or exit the lens. According to de Broglie's principle for the particle-wave duality, the wavelength λ of a particle is inversely proportional to its momentum p :

$$\lambda = \frac{h}{p} \quad (3.2)$$

where h is Planck's constant. The momentum p is related to the kinetic energy E of the particle by:

$$p = \sqrt{2m_0E} \quad (3.3)$$

where m_0 is the rest mass of an electron. Since $E=eV$, where e is the elementary charge and V is the potential through which the electrons are accelerated:

$$\lambda = \frac{h}{\sqrt{2m_0eV}} \quad (3.4)$$

Taking into account relativistic effects for particles travelling at speeds close to the speed of light in vacuum c , the wavelength of the electrons can be described by:

$$\lambda = \frac{h}{\sqrt{2m_0eV\left(1+\frac{eV}{2m_0c^2}\right)}} \quad (3.5)$$

Therefore, the wavelength of the electrons is related to the acceleration potential. In transmission electron microscopy, electrons typically have wavelengths in the range of 10^{-3} nm to 10^{-2} nm. Compared to the wavelength of visible light (400 -700 nm), this much smaller wavelength explains the higher resolution achieved by electron microscopes.

Upon interaction of a primary electron beam with a specimen, several different signals can be generated (**Figure 3.1**), such as secondary electrons (SE), backscatter electrons (BSE), transmitted electrons (if the specimen is sufficiently thin), Auger electrons, characteristic X-rays and photons.⁴³³ Using the appropriate detectors, these signals can be collected and amplified, allowing electron microscopes to provide not only morphological but also compositional and crystallographic information about specimens.

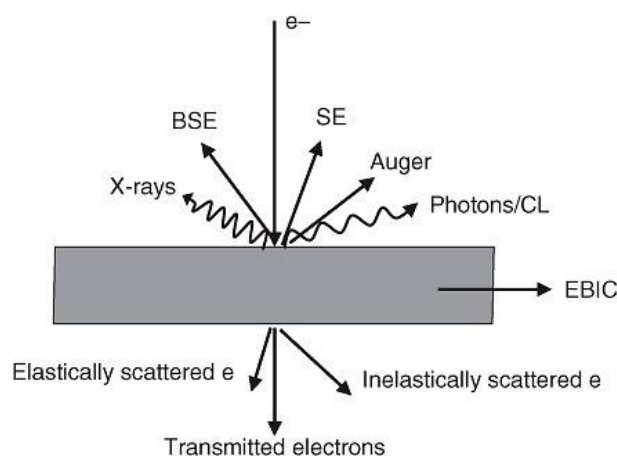


Figure 3.1 Overview of the signals generated when an electron beam interacts with a thin specimen. In the case of a thick specimen, there are no transmitted electrons and the signal gets absorbed within the material. BSE: backscatter electrons, SE: secondary electrons, CL: cathodoluminescence, EBIC: electron beam induced current. Reprinted with permission from ref.⁴³⁴. Copyright 2013 John Wiley & Sons Ltd.

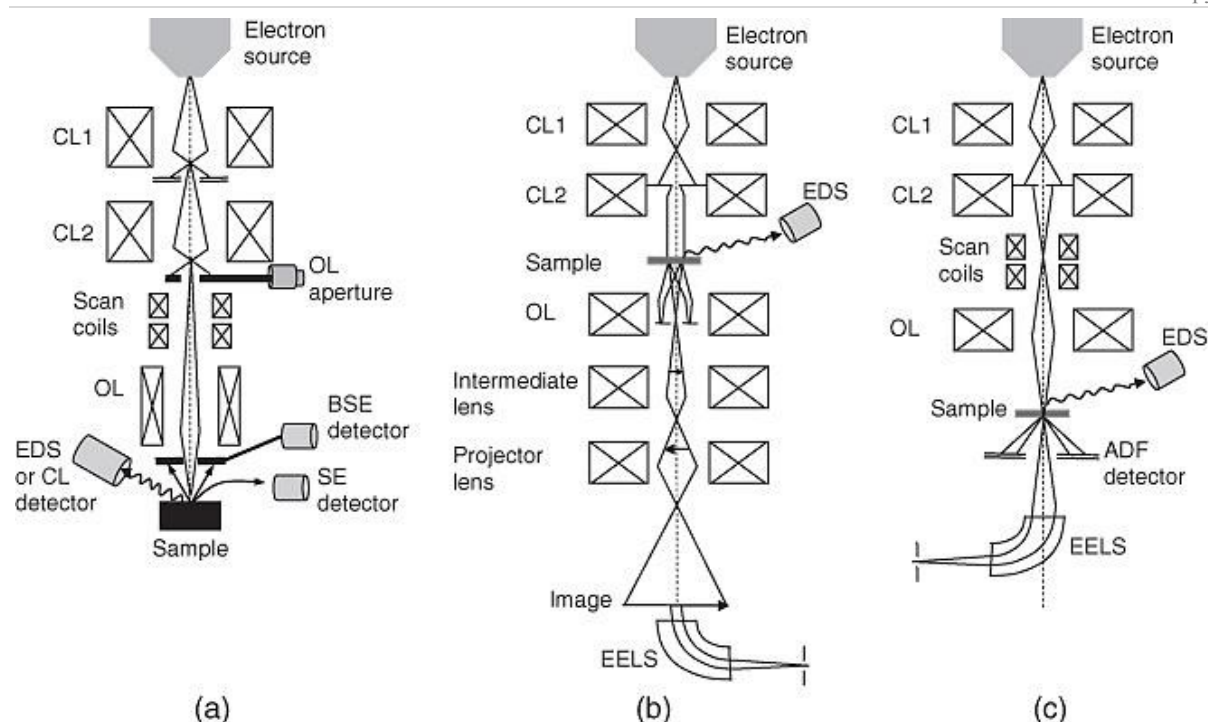


Figure 3.2 Schematic diagrams of the instrumental configuration of a scanning electron microscope (SEM; a), a transmission electron microscope (TEM; b) and a scanning transmission electron microscope (STEM; c). CL: condenser lens, OL: objective lens, BSE: backscatter electrons, SE: secondary electrons, EDS: energy dispersive X-rays, CL: cathodoluminescence, EELS: electron energy loss spectroscopy, ADF: annular dark field. Reprinted with permission from ref. ⁴³⁴. Copyright 2013 John Wiley & Sons Ltd.

3.1.1 Scanning Electron Microscopy

The scanning electron microscope (SEM) operates by raster scanning a fine electron probe of a few nanometres in size over a region of the sample (**Figure 3.2a**).⁴³⁴ SEM instruments typically operate between tens of volts and 30 kV, and are usually used for the examination of bulk materials, although thin specimens may also be examined in transmission mode. Spatial resolution is determined by the diameter of the electron probe, which is a function of both the electron source size and the lens configuration. However, the beam-specimen interaction volume further limits spatial resolution. Depending on detector configuration, SEM instruments can collect secondary, backscattered and transmitted electron signals. Moreover, signals like X-ray emission, cathodoluminescence, electron backscatter diffraction (EBSD) and electron beam induced current (EBIC) can be detected. The most common SEM mode is the detection of secondary electrons, whose number depends on the angle at which the electron beam meets the surface of specimen, *i.e.* on surface topography.⁴³⁴

3.1.2 Transmission Electron Microscopy

In transmission electron microscopy (TEM), very thin specimens (less than 100 nm thick) are irradiated by a beam of high-energy electrons (between few tens of kV up to 1 MeV) (**Figure 3.2b**). The resolution of TEM is about an order of magnitude higher than that of SEM. The resolution of a non-aberration corrected TEM with a field emission source is around 1-2 Å, whereas contemporary aberration corrected systems have a resolution down to 0.5 Å. In TEM, crystallographic information can be obtained by using either selected area or convergent beam diffraction modes. Elemental characterization can be provided by either energy dispersive X-ray spectroscopy (EDX) or electron energy loss spectroscopy (EELS).⁴³³

The contrast mechanism traditionally used for imaging biological samples by TEM is mass-thickness contrast. Electrons that pass through the sample are scattered off-axis by elastic scattering events. Electrons that interact with the electron clouds of atoms in the sample are scattered at relatively small angles and may still contribute to the image, but electrons that are scattered due to interactions with atomic nuclei are scattered to a higher angle and are less likely to contribute to the image formation. The cross-section of scattering depends on the atomic number, Z , and on the density and thickness of the sample. In bright field imaging, the direct beam is used to generate bright field TEM (BFTEM) images. Areas of the sample with higher mass-thickness produce areas of lower intensity in the resulting TEM image than areas of lower mass-thickness. The use of an objective aperture to select the angular range of electrons that contribute to the image may greatly improve image contrast. Mass-thickness contrast from cell organelles can be enhanced by staining the cells with heavy metals such as osmium tetroxide.³³¹

3.1.3 Selected Area Electron Diffraction

Selected area electron diffraction (SAED) can be applied to examine the crystal structure of specimens in EM.⁴³⁵ The electron beam is limited by a diffraction aperture to illuminate a circular region (usually 100-200nm in diameter). The majority of electrons will travel through the sample creating a bright central spot. Since the wavelength of high-energy electrons is a few thousandths of a nanometre and the spacing between atoms in a solid is about a hundred times larger, the atoms act as a diffraction grating to the electrons. Therefore, a fraction of the electrons will be scattered to particular angles, determined by the crystal structure of the sample. As a result, a SAED pattern is obtained, which can be used to identify crystal structures and measure lattice parameters.

3.1.4 High-Resolution Transmission Electron Microscopy

High-resolution transmission electron microscopy (HRTEM) is an imaging mode that allows to study properties of materials on the atomic scale.⁴³⁵ Image formation in HRTEM relies on phase contrast. Phase-contrast images are formed by removing the objective aperture or by using a very large objective aperture. This ensures that not only the transmitted beam, but also the diffracted ones are allowed to contribute to the image. Optical path differences result to the formation of an interference pattern, which is also influenced by microscope parameters such as chromatic and spherical aberrations, focus, coherence and energy spread. Consequently, the interpretation of phase-contrast images is not straightforward.

3.1.5 Scanning Transmission Electron Microscopy

In scanning transmission electron microscopy (STEM), the electron beam is focused to a probe, which is scanned through a selected region of the specimen (**Figure 3.2c**).⁴³⁴ The probe size, which defines STEM resolution, depends on the quality of the source and the microscope lens system. The signal detection relies on the angular distribution of the scattered electrons with the ability to detect both electrons that are elastically scattered through high angles (>50 mrad: high angle annular dark field (HAADF) detector) and electrons that are inelastically scattered through small angles (<10 mrad: bright field (BF) detector; 10-50 mrad: annular dark field (ADF) detector). The HAADF-STEM imaging mode is highly sensitive to the atomic number of the sample. Since HAADF-STEM relies on collecting electrons that have been scattered to high angles by Rutherford scattering, the intensity of a HAADF-STEM image depends on Z^n ($n \sim 2$). Therefore, this Z-contrast imaging is highly attractive for the detection of inorganic nanoparticles and heavy metal-stained organelles inside cells.

3.1.6 Energy Dispersive X-ray Spectroscopy

Energy dispersive X-ray spectroscopy (EDX) is an analytical technique that can be used for the elemental analysis or chemical characterization of a sample in EM.⁴³⁵ The underlying principle for the operation of EDX is the detection of X-rays that are generated when the electron beam interacts with the sample. The beam may excite an electron in an inner shell of an atom, ejecting it from the shell and creating an electron hole. This interaction leaves the atom in an excited state and in order to return to a stable state, an electron from an outer, higher-energy shell fills the electron hole. This process involves a reduction in energy through the emission of an X-Ray. As each element has a unique atomic structure, the emitted X-rays are unique to each material. The number and energy of the emitted X-rays are measured by an X-ray detector, which records an energy spectrum of intensity

versus energy. The characteristic energy distribution in the spectrum provides information about the composition of the sample, while the intensity of the peaks provides information about the amount of each element present in a sample.

3.1.7 Sample Preparation

One of the challenges for imaging nanomaterials within cells or materials-cell interfaces lies in sample preparation.³³¹ Sample preparation for TEM should ensure minimal alteration to the cellular ultrastructure but also to the distribution and chemistry of nanoparticles within cells. The most common procedure for preparing TEM samples at room temperature is fixation, dehydration and embedding followed by ultramicrotomy. With chemical tissue fixation, cellular structures are preserved in a state (both chemically and structurally) as close to living tissue as possible. Aldehydes (*i.e.* glutaraldehyde or formaldehyde) are commonly used as crosslinking fixatives, which act by creating covalent chemical bonds between proteins in tissue. Consequently, soluble proteins are anchored to the cytoskeleton, which imparts additional rigidity to the tissue. Fixation is followed by dehydration in ethanol and acetonitrile, and embedding in a plastic epoxy resin. With ultramicrotomy, ultrathin resin embedded sections containing the cell-nanomaterial interface are mechanically sectioned using a diamond knife.

3.1.8 Staining

To increase contrast in biological material and enhance fixation, heavy metal stains (*i.e.* osmium tetroxide, uranyl acetate, lead citrate) can be used.³³¹

Osmium tetroxide is a strong oxidant that cross-links lipids, therefore it acts as a secondary fixative of membranes but also increases contrast in TEM images, because of the high atomic number of the osmium atoms. In the staining of the plasma membrane, osmium tetroxide binds to the head regions of phospholipids, thus creating contrast with the neighbouring cytoplasm.

In uranyl acetate (UA) staining, uranyl ions bind to proteins and lipids with sialic acid carboxyl groups (*e.g.* glycoproteins) and to nucleic acid phosphate groups of DNA and RNA. Therefore UA provides contrast in membranes, nucleic acids and nucleic acids containing protein complexes, such as ribosomes. The ability of uranyl ions to bind to phosphate groups also allows its use for staining the headgroups of phospholipids.

Lead citrate enhances the contrasting effect for a wide range of cellular structures such as ribosomes, lipid membranes, cytoskeleton and other compartments of the cytoplasm. The enhancement of the contrasting effect depends on its interaction with reduced osmium, since osmium

allows the attachment of lead ions to the polar groups of molecules. Lead citrate also interacts, to a weaker extent, with UA and therefore lead citrate staining is employed after UA staining.

3.2 Laser scanning confocal microscopy

Laser scanning confocal microscopy (LSCM) represents one of the most significant advances in optical microscopy, mainly by allowing visualization deep within both living and fixed cells and tissues, and enabling the collection of sharply defined optical sections from which 3-dimensional reconstructions can be created. In a conventional widefield optical epifluorescence microscope, secondary fluorescence is emitted by the specimen through the whole excited volume, obscuring the resolution of features that lie in the objective focal plane. The problem is intensified in thicker specimens ($> 2\ \mu\text{m}$), which exhibit such a high degree of fluorescence emission that most of the fine detail of the sample is lost. The principal idea in the confocal approach is the use of spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus. Even though resolution is somewhat enhanced with LSCM over conventional widefield techniques, it is still considerably less than that of TEM, therefore LSCM can be considered as a bridge between these two techniques.

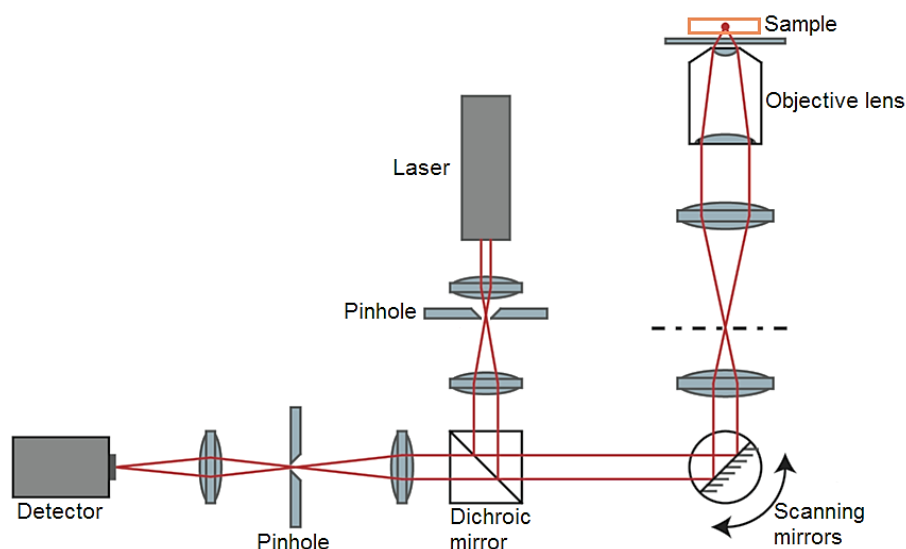


Figure 3.3 Schematic diagram of the instrumental configuration of a laser scanning confocal microscope (LSCM).

In the setup of a LSCM (**Figure 3.3**), coherent light emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate (confocal) plane with a scanning point on the specimen. The laser light is directed by a dichroic mirror towards an assembly

of scanning mirrors. As the laser is scanned across the specimen in a defined focal plane, secondary fluorescence emitted from points on the specimen (in the same focal plane) is de-scanned by the same mirrors, passes back through the dichroic mirror and is focused as a confocal point at the pinhole aperture of the detector (*e.g.* photomultiplier tube). The significant amount of fluorescence emission that occurs at points above or below the objective focal plane is not confocal with the pinhole and forms extended Airy disks in the aperture plane. Since only a small fraction of the out-of-focus fluorescence emission is delivered through the pinhole aperture, most of this light is not detected by the photomultiplier and does not contribute to the resulting image. The resulting confocal 2-dimensional images represent thin cross-sections through the sample. Refocusing the objective shifts the excitation and emission points in the specimen to a new plane that becomes confocal with the pinhole apertures of the light source and detector. Collection of images of planes at various depths within the sample (known as z stacks) allows the formation of 3-dimensional reconstructions of the sample.

4 Effect of Lung Lining Fluid on the Dissolution, Stability and Uptake of AgNWs by Human Respiratory Epithelial Cells

4.1 Introduction

One-dimensional nanomaterials are attracting increasing attention due to their unique physicochemical properties.⁴³⁶ Silver nanowires (AgNWs) are considered as potential building blocks for the next generation of optical, electronic and sensing devices.⁴³⁷ The increase in production and use of AgNWs has led to growing concerns about the potential adverse effects on human health upon exposure to AgNWs. Although occupational exposures to AgNWs during production, packaging or processing may be higher, general public exposures are also possible, given the increasing use of AgNW-containing consumer products (*e.g.* spray coatings, personal electronics). One of the primary routes for exposure is inhalation of airborne AgNWs. Upon inhalation, AgNWs may preferentially deposit in intermediately to terminally situated lung airways, with a peak alveolar deposition between 10% and 20%. In the alveoli, where removal is dominated by relatively slow, macrophage-mediated clearance,⁴³⁸ AgNWs have the potential to contribute to the buildup of dose.

Consequently, AgNWs have raised concerns due to the comparisons with asbestos fibres in the lung and the induced mesothelioma.^{75, 346} *In vitro* work revealed that AgNWs were more toxic than spherical AgNPs on alveolar epithelial cells.⁷¹ Recently, AgNWs were shown to produce dose-dependent inflammation in *murine* lungs and responses dependent on both AgNW length and dissolution rates.³⁴⁵ The toxicity of AgNWs has not been thoroughly investigated and discrepancies still exist on the mechanism of biological action of AgNMs in general.^{85, 89} The lack of consistency could be in part due to the fact that most studies have not considered the fate of AgNMs in

biologically relevant environments and alterations to the physicochemical properties of as-synthesized NMs. This has been highlighted in recent work that revealed the sulfidation of AgNWs in cell culture media¹⁸⁹ as well as inside human alveolar epithelial type 1-like cells.³ Due to the extremely low solubility of Ag₂S, the Ag⁺ ion release rate will be substantially reduced; therefore reduced AgNW toxicity could be expected. Hence, in order to draw accurate conclusions about the bioreactivity of AgNWs, it is vital to characterize their physicochemical properties at the *point of exposure*. For AgNWs that reach the alveolar region of the lung, this first point of contact will be the lung lining fluid (LLF).

Inhaled AgNWs that enter and deposit in the alveoli may adsorb LLF components, affecting their subsequent cellular effects.^{50, 51} Studies indicate that the LLF layer may promote interaction of inhaled particles with the underlying epithelium, through wetting forces that draw the particles into the surfactant toward the alveolar wall.^{52, 53} The effects of the LLF on the physicochemistry of AgNWs will determine their interaction with proteins, cells and tissues in the lung. An altered aggregation state could modify particle transport, the amount of AgNWs internalized by cells and subsequent interactions within cells.⁵⁴ Previous work has demonstrated that AgNPs incubated with DPPC were coated by a phospholipid corona, which delayed oxidative dissolution of AgNPs and inhibited aggregation and coarsening.⁷⁹ However, few data exist on the stability of AgNMs in other components of the LLF or in LLF from an animal model, as well as the consequences on the bioreactivity of AgNWs with lung epithelial cells.

The purpose of this study is to characterize the impact of individual components of the LLF on the stability of AgNWs, grown *in-house* to ensure a full control of their initial physicochemistry. Phospholipids, as well as each class of surfactant-associated proteins, the hydrophobic SP-B/C and the hydrophilic SP-A/D, were separately added to AgNWs in an effort to delineate their effects on the properties of AgNWs. These effects were investigated at different pH values, representative of environments found in the lung and that mimic endocytotic conditions. Moreover, the cellular uptake and intracellular transformation of AgNWs with human alveolar epithelial type 1-like (TT1) and type 2 (AT2) cells was examined, following up to 24 hours of continuous exposure in the absence and presence of Curosurf® or harvested primary human AT2 cell secretions (HAS). Type 2 alveolar cells produce and continuously release pulmonary surfactant by exocytosis, therefore HAS contain both the complete lipid as well as the full protein complement of human pulmonary surfactant.

Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES), *in situ* optical microscopy and analytical transmission electron microscopy (TEM) techniques were used to investigate the dissolution, colloidal stability and surface chemistry of AgNWs. The use of correlative imaging techniques, which provide both dynamic and spatially resolved information about the chemistry of the AgNWs, enabled us to directly visualize the impact of the LLF components on the surface chemistry of AgNWs, agglomeration states as well as the structure of the lipid corona. The advantages of using TEM are its ability to provide spatially resolved information about the

distribution of crystal phases, the structure of the lipid corona and the crystallinity of small nanomaterials. ICP-OES was also used to quantify the amount of AgNWs taken up by TT1 and AT2 cells. Finally, a combination of high resolution and analytical TEM was employed to monitor the interaction of AgNWs with TT1 cells over time. The binding of HAS proteins on AgNWs and the effects of Curosurf[®] and HAS on the toxicity of AgNWs to TT1 and AT2 cells (cell viability, inflammatory mediator release and generation of reactive oxygen species) were studied by collaborators at the National Heart and Lung Institute and reported by Sweeney *et al.*⁴³⁹

4.2 Methods

4.2.1 AgNW Synthesis

Pure AgNWs were synthesized using a modified polyol process, originally developed by Xia *et al.*⁴⁴⁰ Ethylene glycol (EG) acts as both solvent and reducing agent, whereas poly(vinyl pyrrolidone) (PVP) is used as the capping agent. The reduction of Ag⁺ ions by EG leads to the formation of Ag nuclei at the early stages of the reaction. Due to the stronger affinity of PVP for the (100) facets than the (111) facets of the Ag nuclei, this passivation leads to one-dimensional growth and the formation of high aspect-ratio AgNWs. The process was optimized in order to: (i) Eliminate the generation of AgNPs from the synthesis product, as the presence of different populations of particles would confound the correlation between the observed effects and the physicochemical properties of the particles. (ii) Avoid the use of other transition metal impurities, such as Cu⁴⁴¹ or Fe,⁴⁴² previously used to control the product morphology in the polyol synthesis of Ag nanostructures, because their impact on the physicochemical properties of AgNWs is not well-understood. (iii) Eliminate the need to control the rate of injection of the reactants, making the synthesis less complicated.⁴⁴³ The optimization of the synthesis conditions is described in Appendix A.

Briefly, Ethylene Glycol (EG, Sigma-Aldrich, anhydrous, 99.8%) (2.5 mL) was placed in a double-neck round-bottom flask connected to a condenser. A stock solution of sodium chloride (NaCl) (0.05 M) was prepared by dissolving NaCl in EG by bath sonication. The appropriate amount of NaCl stock solution was added to the flask so that the concentration of NaCl in the final reaction volume was 60 μ M. The flask was heated in an oil bath at 160 °C for 30 minutes to remove trace amounts of water. Meanwhile, argon flow (Ar, BOC, Pure Shield Argon) and magnetic stirring were applied and maintained throughout the synthesis. Silver nitrate (AgNO₃, 25 mM, Sigma-Aldrich, >99%) and poly(vinyl pyrrolidone) (PVP, Sigma-Aldrich), with an average molecular weight $M_w \approx 360k$, were dissolved in EG (3.5 mL) by magnetic stirring in the dark. The molar ratio of PVP to AgNO₃ in the final reaction volume was 1.5 and the concentrations of PVP were calculated in terms of the repeating unit. To remove oxygen, the AgNO₃/PVP/EG solution was purged with Ar for 30

minutes. The AgNO₃/PVP/EG solution (3.5 mL) was added to the reaction flask drop-wise. After injection, the reaction mixture was refluxed at 160 °C and went through a number of colour changes until the mixture became stable at approximately 90 min. The reaction was quenched by cooling the flask in a room-temperature water bath. The reaction mixture was transferred to a centrifuge tube and diluted with acetone 5 times by volume. The AgNWs were collected by centrifugation at 4500 rpm for 10 min. The washing process was repeated by repeated cycles of centrifugation with ethanol and three times with deionized (DI) water, to ensure that residual EG, PVP and unreacted Ag⁺ ions were removed. To confirm that most Ag⁺ ions had been removed, their concentration in the synthesis product was measured by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES) after ultrafiltration and was found to be under the ICP-OES detection limit (*i.e.* <0.6 ppb). Finally, the sample was dispersed in deionized water (5 mL) and stored in a sealed glass container at 4 °C in the dark, to avoid exposure to contaminations and reactions induced by the ambient atmosphere (*e.g.* sulfidation due to gaseous hydrogen sulfide (H₂S), carbonyl sulfide (OCS) and carbon disulfide (CS₂) in the atmosphere). All the experiments in this work were performed using a single batch of AgNWs.

Commercial AgNWs are available from at least 9 companies, with dimensions that range between 20-200 nm in diameter and 2-200 µm in length.⁴⁴⁴ Therefore, the diameter of the AgNWs synthesized for this work is at the average of this range while their length is at the lower-end of the range. Most of previous *in vitro* and *in vivo* studies on AgNWs have been performed on commercially available AgNWs.^{75, 345, 445} For example, a recent work by Silva *et al.* compared the *in vivo* pulmonary effects post instillation of commercial AgNWs of two different dimensions (“Short” AgNWs, length 2.0 µm, diameter 33.1 nm and “Long” AgNWs, length 20.8 µm, diameter 64.7 nm).³⁴⁵ Both AgNWs produced dose-dependent inflammation indicative of foreign body responses in the lung, but different inflammatory responses depending on AgNW length or higher dissolution rates by the smaller AgNWs. Details about AgNW fabrication methods are rarely disclosed by manufacturers and few characterization data of the products are provided. However, differences in the synthesis procedures, AgNW dimensions or capping agents may lead to differences in dissolution rates, agglomeration kinetics and ultimately in toxicological responses.⁴⁴⁶ In this work, AgNWs were produced in-house in order to have a full control over their physicochemical properties, which were thoroughly characterized.

4.2.2 Animal LLF extraction

Male rats were anesthetized by injection of a lethal dose of ketamine/xylazine, and then sacrificed by exsanguination. Bronchoalveolar lavage (BAL) was collected using buffered saline (1x-10mL wash). Cells were removed from the BAL by centrifugation (300×g, 10 minutes at 4°C). The supernatant (2mL) was utilized to fractionate small aggregate (SA, supernatant) and large aggregate

(LA, pellet) portions of BAL. The LA was re-suspended in 0.9% saline. Protein content for both the small (31.5 µg/mL) and large (166.1 µg/mL) aggregate fractions was determined by the bicinchoninic acid assay (BCA), Thermo Scientific (Rockford, IL, USA). Phospholipid content of the large aggregate fraction was assessed by determining the concentration of organic phosphate (1.57 µg/µL).⁴⁴⁷ This protocol was approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC) (Protocol Number: 06-028). The study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

The animal LLF extraction was carried out by Dr. Danielle Botelho in the laboratory of Prof. Andrew Gow at the Department of Pharmacology and Toxicology, Rutgers University, United States.

4.2.3 AgNW incubations

AgNWs were incubated at a concentration of 25 µg/mL from the original stock solution (on a Ag atom basis as determined by ICP-OES) in a temperature-controlled dry-block incubator at physiological temperature (37 °C) for 1 hour up to 336 hours (2 weeks) in the dark. This dose was selected in order to provide direct comparisons with previous work on spherical AgNPs⁷⁹ and on the *in vitro* effects of AgNWs on human alveolar epithelial cells.³ Accurate dosimetry in laboratory evaluations of the effects of inhaled particles has been a subject of concern, but very few data on AgNMs are currently available to evaluate realistic occupational and consumer exposures.⁵⁰ In one study, nanomaterial concentrations, including AgNPs, measured in air in manufacturing and research and development laboratories were reviewed, to identify input levels for estimating the nanomaterial mass retained in the human lung using the multiple-path particle dosimetry (MPPD) model. Model results were then converted (using the surface area and volume delivered in different types of cell culture well plates) to solution mass concentrations for *in vitro* testing. For AgNPs, alveolar retention for a working-lifetime (45 years) exposure duration was similar to higher concentrations (~ 50-200 µg/mL), tested in *in vitro* studies in the literature. The alveolar retention for a 24 hour exposure duration was equivalent to lower doses (~ 0.1-1 µg/mL) previously tested. Therefore, a dose representative of the lower-end of those previously tested in *in vitro* studies of AgNMs was selected for this study,¹²⁵ whose primary aim is to deconvolute the effects of individual components of the LLF on the physicochemistry of AgNWs.

The presence of complexing anions such as Cl⁻ or S⁻ is expected to lead to the precipitation of insoluble silver species⁴⁴⁸, which would confound the measurement of free Ag⁺ ions, therefore non-interacting perchlorate buffers were selected as the dispersion medium. AgNWs were incubated in Sodium Perchlorate (NaClO₄·H₂O, Sigma-Aldrich, >99%) (0.1 M) and the pH of the buffers was adjusted to 7 or 5, using either Perchloric Acid (HClO₄, Sigma-Aldrich, 70%, 99.999% trace metals

basis) or Sodium Hydroxide (NaOH, Sigma-Aldrich, anhydrous, 99.99%). These pH values were selected to simulate characteristic environments found in the lung. The interstitial and alveolar extracellular fluids have a pH of ~ 7.4 ⁴⁴⁹ and the cell cytoplasmic pH is ~ 7.2 . In the endocytic pathway of the cells, the pH decreases progressively from the early endosomes (pH ~ 6.5) to late endosomes (pH <6.0) and ultimately lysosomes (pH <5.5).⁴⁵⁰

To study the effect of each component of the lung surfactant on the stability of AgNWs, various combinations of these components were incubated together. Human surfactant consists mostly of phospholipids, with the most abundant being DPPC, therefore DPPC was used to study the effect of phospholipids. The effect of the two hydrophobic surfactant-associated proteins, SP-B and SP-C, was investigated using Curosurf[®] or the large aggregate (LA) fraction of *murine* LLF. Curosurf[®] is a natural surfactant, prepared from *porcine* lungs and used for the treatment of endogenous pulmonary surfactant deficiencies by intratracheal administration. Curosurf[®] contains almost exclusively phospholipids but also about 1% of SP-B and SP-C, therefore resembles LA in its composition. Finally, the roles of SP-A and SP-D on the stability of AgNWs were studied by adding the small aggregate (SA) fraction of *murine* LLF. SA contains the more soluble components of the lung lining fluid, such as the non-specific lung proteins IgG and albumin, but also the collectins SP-A and SP-D. Atochina *et al.* showed that, in the bronchoalveolar lavage of control healthy mice, over 90% of phospholipids, 100% of both SP-B and SP-C and about 60% of SP-A, could be found in the large aggregate fraction. In contrast, the majority of SP-D and the remaining SP-A were found in the SA fraction.⁴⁵¹

A stock solution of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Sigma-Aldrich, semisynthetic, $\geq 99\%$) (10 mg/mL) in DI water was prepared by magnetic stirring overnight. DPPC, Curosurf[®] (poractant alfa, Chiesi Farmaceutici, S.p.A.) and LA were used at amounts that correspond to a total lipid concentration of 100 $\mu\text{g/mL}$. SA was used at amounts that correspond to a total protein concentration of 100 $\mu\text{g/mL}$. These concentrations were chosen to correspond to the average values observed in the surfactant obtained from the bronchoalveolar lavage fluid of healthy persons.⁴⁰ For the phospholipids that constitute most of pulmonary surfactant, the critical micelle concentrations (CMC) fall within the range of 10^{-10} to 10^{-9} M.⁴⁵² For DPPC, for instance, a CMC of 10^{-10} M is equivalent to 7.34×10^{-5} $\mu\text{g/mL}$, therefore the formation of micelles is expected in our experiments. However, in essentially all of the *in vitro* studies, and at estimated physiological concentrations, pulmonary surfactant is well above its CMC.⁴⁵²

4.2.4 Scanning Electron Microscopy (SEM)

The morphology and size distribution of the AgNWs were characterized using a LEO 1525 Field Emission Gun Scanning Electron Microscope (FEG-SEM, Carl Zeiss Microscopy GmbH, UK).

The SEM was operated in secondary electron mode at an accelerating voltage of 5 kV, using the InLens detector. Samples were prepared by drop-casting aliquots of the AgNW suspensions on a piece of silicon wafer and dried under ambient conditions in a fume cupboard and in the dark. Samples were stored under vacuum and in the dark. The size distribution of the AgNWs was characterized using SEM images and ImageJ software (<http://rsb.info.nih.gov/ij/>).

4.2.5 Transmission Electron Microscopy (TEM) Sample Preparation

To prepare TEM samples from the incubated AgNWs, aliquots were removed at each time point and, after washing three times with DI water to remove excess salts or organic surfactant, they were drop cast on 300 mesh holey carbon film TEM grids (TAAB) in the dark. The grids were blot-dried with filter paper and were immediately placed under vacuum and in the dark to avoid reactions induced by ambient atmosphere. The grids were imaged within a period of 2 weeks to ensure that no degradation of the wires takes place under long storage periods. This was confirmed by preparing TEM samples from as-synthesized AgNWs and performing TEM analysis after a storage period of up to 3 months under vacuum, with no changes in their physicochemical properties detected (more than 100 wires analyzed, Figure A1, Appendix A). To enhance phospholipid contrast, samples were positively stained for 40 s with 2% uranyl acetate in water.

4.2.6 TEM

Bright field transmission electron microscopy (BFTEM), high resolution transmission electron microscopy (HRTEM) and high angle annular dark field scanning transmission electron microscopy (HAADF-STEM), combined with selected area electron diffraction (SAED) and energy-dispersive X-ray spectroscopy (EDX) were carried out using a JEOL JEM-2100F fitted with an EDX detector (Oxford Instruments). The scattering intensity in HAADF-STEM is proportional to Z^n ($n \sim 2$), therefore this technique is highly sensitive to atomic number (Z) variations within the sample. An accelerating voltage of 200 kV was used for both TEM and STEM experiments. For STEM experiments, the inner and outer HAADF collection angles were 150 and 400 mrad, respectively, and the probe diameter was <1 nm.

4.2.7 Light Microscopy (LM)

Aliquots were removed from the incubated AgNWs, placed on a clean glass slide, coverslipped and immediately observed in reflectance mode, using a Leica DM2500 with reflected

axis LED light source and a Leica DFC295 camera. The objective lenses used were an Olympus 50x/aperture 0.75 and an Olympus 100x/aperture 1.25.

4.2.8 Statistics

The LM/SEM/TEM images presented and the statistics on particles were obtained by viewing several AgNWs ($n \geq 100$ for each sample), from multiple areas of three samples prepared under identical conditions.

4.2.9 Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES)

To determine the amount of free Ag^+ ions released from the incubated AgNWs at 1 hour up to 336 hours, aliquots were collected at each time point and centrifuged at $13000 \times g$ for 10 minutes through 2 kDa centrifuge membranes (Sartorius Stedim VIVACON 500) to remove the AgNWs. The amount of Ag^+ in the filtrates was measured by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES, Thermo Scientific, UK) with a silver detection limit of $0.6 \mu\text{g/L}$. Each experiment was repeated three times and the results are given as the mean $\pm$ standard deviation of the three repeats. The method of centrifugal filtration used leads to 100% of Ag^+ ion recovery, as has previously demonstrated in a control experiment where a solution of AgNO_3 in DI water was subjected to filtration and the total amount of Ag^+ added was measured by ICP-OES in the filtrate.¹⁸⁹ To confirm that secondary AgNPs are also filtered by the centrifuge membranes, AgNWs were incubated at pH 7 for 24 hours. The Ultraviolet-Visible (UV-Vis) spectrum of the sample was collected before and after filtration, with no peaks observed in the spectrum after filtration (Figure A2, Appendix A).

4.2.10 TT1 and AT2 cell models

Ethics Statement: The human lung tissue used in this work was surplus tissue obtained following resection for lung carcinoma. Written informed consent was obtained for all samples and the study was carried out with the approval of the Royal Brompton and Harefield Ethical Committee (Ref: 08/H0708/73).

The laboratory of Prof. Teresa Tetley has previously immortalised human AT1-like cells (derived from freshly isolated and cultured primary human AT2 cells) using transduction with the catalytic subunit of telomerase (human telomerase reverse transcriptase; hTERT) and a temperature sensitive mutant of simian virus 40 large-tumour antigen.⁴⁵³ These AT1-like (transformed type-1-like; TT1) cells, the first human AT1-like cell line to be produced, are negative for the AT2 cell markers

SP-C, alkaline phosphatase and thyroid transcription factor-1. Moreover, TT1 cells do not contain lamellar bodies. They display a thin, attenuated morphology containing vesicles, being caveolae positive. TT1 cells were cultured to confluence in either 24-well culture plates (for uptake study) or 96-well culture plates (for all other assays) using DCCM-1 tissue culture medium with 10 % newborn calf serum (NCS), 0.05% G418 and 10% PSG. An initial cell seeding of 1×10^5 /well (24 well culture plate) 0.1×10^5 /well (96 well culture plate) yielded a confluent monolayer after 48 hours. Growth medium was replaced with serum-free DCCM-1 24 hours prior to AgNW exposure. Cells were incubated in humidified 5% CO₂/95% air at 37°C.

Primary human AT2 cells were isolated from human lung of grossly normal appearance obtained following resection for lung carcinoma, as described by Witherden and Tetley.⁴⁵⁴ Cells were isolated from a minimum of three subjects per experiment, with a total of fifteen subjects used in this study. Briefly, lung tissue sections were perfused by injection of 0.15 M sterile sodium chloride saline solution until the draining lavage ran clear and the cell count was $< 1 \times 10^4$ cells/ml. The tissue was then perfused with trypsin (0.25% in Hanks balanced salt solution) and incubating at 37°C for 45 minutes; trypsin was replaced twice during the incubation period. The tissue was then finely chopped in the presence of NCS, shaken for five minutes with DNase (250 µg/ml) at room temperature and passed through a 400 µm filter followed by a 40 µm filter to remove large tissue debris. The cell-containing filtrate was then centrifuged at 1300 rpm for 10 minutes and the resulting pellet was re-suspended in DCCM-1 culture medium containing 50 µg/ml DNase. Cells were then incubated in tissue culture flasks for two hours (humidified incubator, 5% CO₂/95% air at 37°C) to allow differential adherence and removal of contaminating mononuclear cells. Non-adherent AT2 cells were removed after 2 hours and the cell suspension was centrifuged at 1300 rpm for 10 minutes. The resulting pellet was re-suspended in a DCCM-1 culture medium containing 10%NCS and 1% PSG and cells were seeded on 96 well culture plates at 0.1×10^6 cells/well or 24 well culture plates at 0.5×10^6 cells/well. The 96 well culture plates were pre-coated with 50 µl of 1% PureCol collagen solution (Leimuiden, Netherlands; Type I collagen) and allowed to air dry. Cells reached confluence after 48 hours. These cells have been thoroughly characterised using electron microscopy, which shows that the cells are cuboidal in morphology, have surfactant-containing lamellar bodies, tight junctions and microvilli. Furthermore they stained positively for the AT2 cell marker alkaline phosphatase and express surfactant proteins A and C and maintain their phenotype for up to six days.⁴⁵⁴

The cell work was carried out by Dr. Sinbad Sweeney in the laboratory of Prof. Teresa Tetley at the National Heart & Lung Institute, Imperial College London.

4.2.11 Preparation of Curosurf® or HAS pre-incubated AgNWs

Harvested primary human AT2 cell secretions (HAS) were prepared by first culturing primary human AT2 cells to confluence in 24 well plates. Because pulmonary surfactant lies in very close proximity to the cells, 900µl (of the 1ml total culture medium) were removed to waste, leaving the remaining 100µl (containing the pulmonary surfactant and other AT2 cell secretions) undisturbed. The cultures were then rinsed with RPMI 1640 culture medium and the HAS-containing rinses were pooled. Following this, no further processing of the HAS was performed. AgNWs were incubated at room temperature and with agitation for 1 hour with a 1:1000 dilution of Curosurf® in RPMI 1640 culture medium, or with undiluted HAS. The incubated AgNWs were then centrifuged at 10000 x g for 10 minutes and the pellet was washed twice with PBS by repeated cycles of centrifugation. Following washing, the pre-incubated AgNWs were suspended in RPMI 1640 culture medium, ready for cell exposure. HAS contain the complete lipid as well as the full protein complement of human pulmonary surfactant, and were used to investigate the effect on uptake of AgNWs in TT1 cells.

This work was carried out by Dr. Sinbad Sweeney in the laboratory of Prof. Teresa Tetley at the National Heart & Lung Institute, Imperial College London.

4.2.12 TT1 and AT2 treatment with AgNWs

TT1 cells were treated with AgNWs (bare or with Curosurf® or HAS) at concentrations ranging from 0.1 - 25µg/ml, prepared in RPMI 1640 culture medium. AT2 cells were treated with bare AgNWs at concentrations ranging from 1 - 25µg/ml, prepared in RPMI 1640 culture medium. Both TT1 and AT2 cells were treated for 4 and 24 hours after which the conditioned medium containing the extracellular AgNWs, was removed. Cells were washed three times with PBS, and fresh RPMI 1640 culture medium was added for a further 24 hours to determine the bioreactivity status of the cells one day post-exposure.

This work was carried out by Dr. Sinbad Sweeney in the laboratory of Prof. Teresa Tetley at the National Heart & Lung Institute, Imperial College London.

4.2.13 Quantification of Ag in culture medium and cells by ICP-OES

To quantify the amount of AgNWs taken up by TT1 and AT2 cells, TT1 cells were treated with AgNWs (bare or with Curosurf® or HAS) and AT2 cells were treated with bare AgNWs; the total amount of AgNWs added to TT1 or AT2 cells for the uptake study was 25µg (*i.e.* 1 ml of a 25 µg/ml solution of AgNWs, prepared in RPMI 1640 culture medium). Both TT1 and AT2 cells were exposed for 1 and 24 hours, after which the treatments were removed. The cells were rinsed three times with 1

mL of fresh RPMI 1640 culture medium. The initial treatments and subsequent washes, containing the extracellular AgNWs, were collected and stored at -21 °C until further processing. Cells were scraped with 1 ml of fresh RPMI 1640 medium and stored at -21 °C. Each experiment was repeated three times. Cell culturing and exposure to AgNWs was performed by Dr. Sinbad Sweeney.

The amounts of Ag associated with the cells (*i.e.* the amount of AgNWs bound to the plasma membrane plus the amount internalized), as well as the extracellular amounts of Ag, were determined by ICP-OES, following acid digestion. Digestion procedures using only nitric acid (HNO₃) have been shown to produce inaccurate results for Ag analyses, whereas digestions based on mixtures of HNO₃ and hydrochloric (HCl) acids lead to high Ag recovery rates.²³⁹ Therefore, the cell pellets and 400 µL aliquots of the extracellular medium samples were digested with 5 mL of a 1:3 mixture of HNO₃ (69%) and HCl (37%) overnight. The samples were diluted 10 times in ultrapure water (Milli-Q), yielding clear digest solutions, and analysed by ICP-OES.

4.2.14 TEM, HRTEM and STEM-EDX analysis of AgNW uptake

Following the same treatment as described in paragraph 3.2.13, cells were rinsed with fresh RPMI 1640 culture medium and then fixed in 2.5% glutaraldehyde in 0.1M PIPES buffer, pH 7.2 for 1 hour at 4°C. The fixatives were removed by washing the cells with 0.1 M PIPES buffer times. Cells were scraped, transferred to 1.5 mL Eppendorf tubes and centrifuged at 1000 g for 20 minutes to obtain cell pellets. The cell pellets were dehydrated in graded solutions of ethanol (50%, 70%, 95%, and 100%), for 5 minutes three times in each, and then washed three times, for 10 minutes each, in acetonitrile (Sigma). After dehydration, samples were progressively infiltrated with a Quetol-based resin, produced by combining 8.75 g quetol, 13.75 g nonenyl succinic anhydride, 2.5 g methyl acid anhydride, and 0.62 g benzyl dimethylamine (all from Agar Scientific). Samples were infiltrated in a 50% resin:acetonitrile solution for 2 hours, in a 75% resin:acetonitrile solution overnight and in 100% resin for 4 days, with fresh resin being replaced daily. The embedded samples were cured at 60 °C for 24 hours. Thin sections (90 nm) were cut from the resin blocks directly into a water bath using an ultramicrotome and a diamond knife with a wedge angle of 35°. Sections were immediately collected on bare, 300 mesh copper TEM grids (Agar Scientific), dried and stored under vacuum until TEM analysis. No heavy metal staining process was used, as osmium tetroxide and potassium ferricyanide have been shown to cause substantial changes in the morphology and chemistry of AgNWs.³

Bright field transmission electron microscopy (TEM) imaging was performed on a JEOL 2000 microscope operated at 80 kV. High Resolution Transmission Electron Microscopy (HRTEM) and Scanning Transmission Electron Microscopy (STEM) was performed at 80 kV on a FEI Titan 80–300 TEM/STEM, fitted with a Cs (image) corrector and a SiLi Energy Dispersive X-Ray (EDX) spectrometer (EDAX, Leicester UK). HRTEM and HAADF-STEM-EDX analyses were performed on

non-stained samples. HAADF-STEM experiments were performed with a convergence semi-angle of 14 mrad and inner and outer HAADF collection angles of 49 and 239 mrad, respectively. The probe diameter was <0.5 nm.

4.3 Results

4.3.1 Characterization of as-synthesized AgNWs

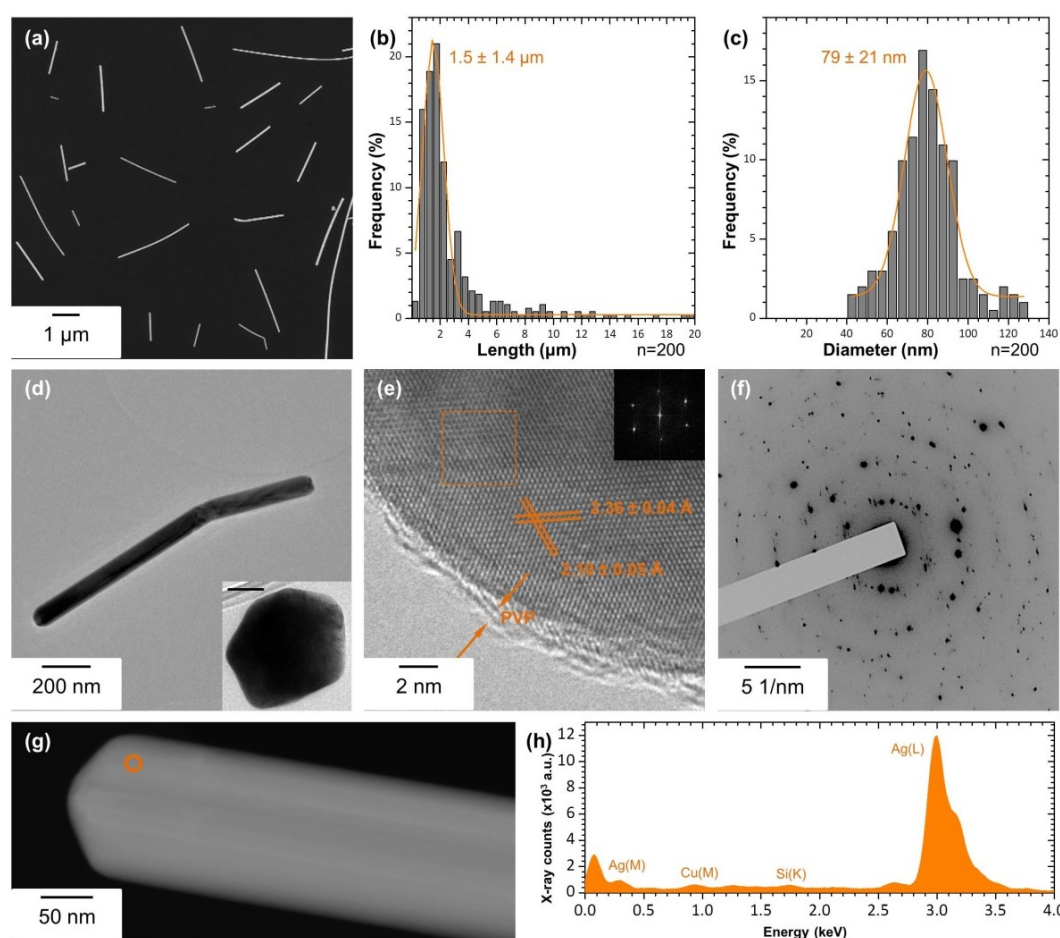


Figure 4.1 Physicochemical characterization of the as-synthesized AgNWs by SEM, TEM, SAED and STEM-EDX. (a) SEM image of as-synthesized AgNWs, and their length (b) and diameter (c) distributions. The curves represent the Gaussian fit to the data. (d) A low-resolution BFTEM image of AgNWs. Inset shows the cross section of AgNWs, scale bar 30 nm. (e) High-resolution BFTEM image of a single AgNW, showing its crystal structure and the PVP capping on its surface. Inset is the corresponding FFT pattern taken from the boxed area (f) SAED pattern taken from an area containing multiple AgNWs. (g) HAADF-STEM image of a single AgNW and (h) the corresponding STEM-EDX spectrum collected from the area circled in (g).

It is vital to characterize the chemistry and crystalline structure of AgNWs in ambient conditions, as previous work has shown that the chemistry of AgNWs changes if they are exposed to

the environment.¹⁸⁹ Scanning electron microscopy (SEM) of the as-synthesized product showed that it is composed only of nanowires (**Figure 4.1a**). The length distribution of AgNWs, measured from several representative SEM images using ImageJ software, had an average of $1.5 \pm 1.4 \mu\text{m}$ (**Figure 2.1b**), while the diameter distribution had an average of $79 \pm 21 \text{ nm}$ (**Figure 4.1c**). Bright Field Transmission Electron Microscopy (BFTEM) (**Figure 4.1d**) revealed a pentagonal cross-section of the wires, since AgNWs synthesized by the polyol process grow from five-fold twinned Ag seeds formed at the early stages of the reaction.¹⁷⁶ The lattice fringe spacings measured from phase contrast High Resolution TEM (HRTEM) images (**Figure 4.1e**), were 2.36 ± 0.04 and $2.10 \pm 0.04 \text{ \AA}$, which correspond to the interplanar spacing of bulk Ag (111) and (200) lattice planes, respectively (ref. # 01-087-0597). The interplanar spacings measured from SAED patterns (**Figure 4.1f**) were 2.35 ± 0.04 , 2.04 ± 0.02 , 1.43 ± 0.01 and $1.22 \pm 0.01 \text{ \AA}$, which are consistent with the bcc form of bulk Ag (ref. # 01-087-0597). The presence of an amorphous layer of $1.2 \pm 0.3 \text{ nm}$ on the surface of AgNWs (**Figure 4.1e**) corresponds to the PVP coating (Figure 1d). STEM-EDX shows that the AgNWs are composed of pure Ag (**Figure 4.1g, h**) and confirms the absence of impurities in the synthesized product. Finally, as-synthesized AgNWs had a negative surface charge of $-14.8 \pm 0.1 \text{ mV}$ in DI water.

4.3.2 Ag^+ ion release kinetics in LLF

One of the main mechanisms of AgNM toxicity is postulated to be their dissolution and release of free Ag^+ ions.^{57, 90} In order to understand discrepancies in previous studies, it is important to characterize the effect of relevant media on Ag^+ ion release kinetics. AgNW dissolution in the absence of LLF components was strongly pH-dependent (**Figure 4.2a**). At pH 7, Ag^+ release was negligible, and appeared to decrease over time, which could be correlated with the formation of AgNPs as discussed in **Chapter 4.3.5**. On the other hand, pH 5 resulted in a higher ion release rate, as has been reported for citrate-AgNPs.⁷⁹ In the presence of complete LLF at pH 7, no significant differences in the amounts of Ag^+ ions released were measured. In accordance to this finding, individual components of the LLF (DPPC $\pm$ SA, **Figure 4.2b** and Curosurf[®] $\pm$ SA, **Figure 4.2c**) had no effect on the amount or rate of Ag^+ ions released at pH 7. At pH 5, however, the presence of LLF led to a lower AgNW dissolution, with the amounts of Ag^+ ions measured from 24 hours onward being about half compared to the absence of LLF (**Figure 4.2a**). This decrease in dissolution was further investigated by incubating AgNWs in combinations of individual LLF components (**Figure 4.2b, c**). Both DPPC and Curosurf[®] imparted a retarding effect to AgNW dissolution, with the amount of Ag^+ released after 24 hours being about half compared with AgNWs in pH 5 buffer. A similar effect has previously been described for citrate-capped AgNPs with DPPC.⁷⁹ With DPPC, this retarding effect lasted for 1 week, when there was little difference in the amount of Ag dissolved compared to AgNWs without DPPC. The effect was more pronounced with Curosurf[®]: it took two

weeks for the amount of Ag^+ ions to reach the same concentration as with AgNWs in pH 5 buffer. Finally, addition of the SA, to both DPPC and Curosurf[®], at pH 5, resulted in a further decrease of the dissolved Ag^+ measured after 1 and 2 weeks. Under all conditions, the maximum concentration of free Ag^+ ions measured was $0.625 \mu\text{g/mL}$, which is very low and unlikely to be toxic to cells. According to Cronholm *et al.*, $1 \mu\text{g/mL}$ of Ag^+ (added as AgNO_3) did not cause a significant increase in cell death in human lung cell lines (A549 and BEAS-2B).²⁴¹

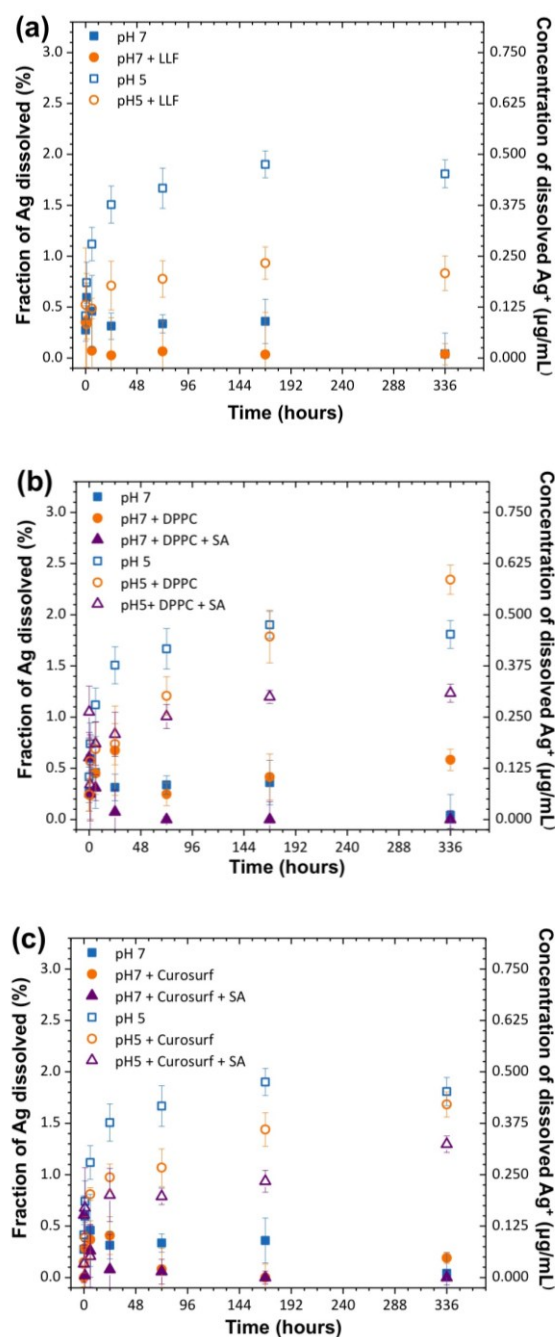


Figure 4.2 (a-c) ICP-OES analysis of the amount of free Ag^+ ions released from AgNWs in complete LLF (a) and individual LLF components (b, c). AgNWs were incubated in perchlorate buffer solutions (pH 7 and pH 5), in the presence and absence of LLF (a), DPPC $\pm$ SA (b) or Curosurf[®] $\pm$ SA (c), for 1 hour up to 336 hours (14 days).

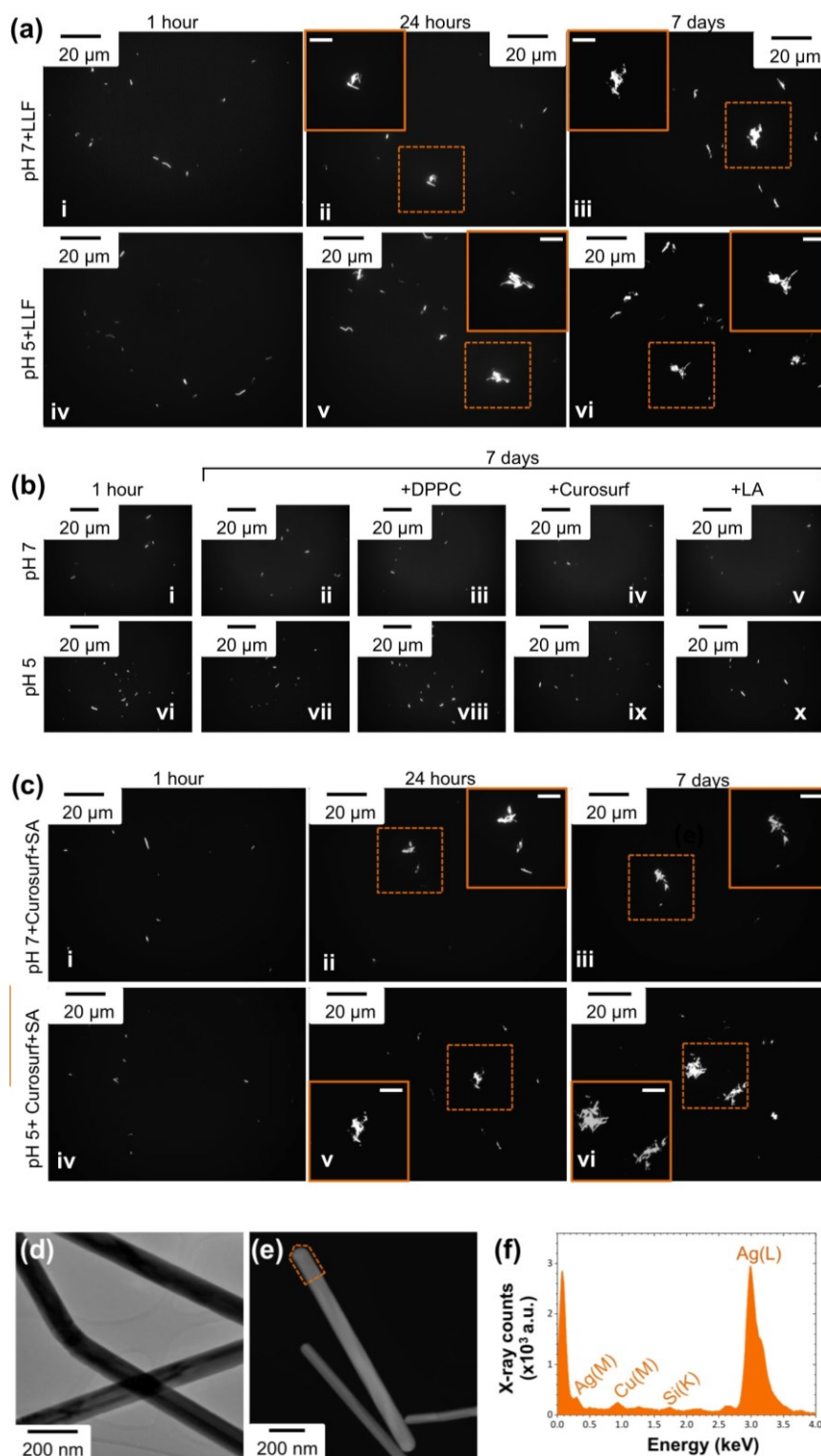


Figure 4.3 (a) Reflectance mode optical images of AgNWs incubated with LLF at pH 7 (i-iii) and pH 5 (iv-vi), for 1 hour (i, iv), 24 hours (ii, v) and 7 days (iii, vi). (b) Reflectance mode optical images of AgNWs incubated at pH 7 (i-v) and pH 5 buffers (vi-x), in the absence (i, ii, vi and vii) and presence of DPPC (iii and viii), Curosurf[®] (iv and ix) and LA (v and x). (c) Reflectance mode optical images of AgNWs incubated with Curosurf[®] and SA at pH 7 (i-iii) and pH 5 (iv-vi), for 1 hour (i, iv), 24 hours (ii, v) and 7 days (iii, vi). (d) A BFTEM image of AgNWs incubated at pH 5 for 14 days. (e) A HAADF-STEM image and (f) a STEM-EDX spectrum from the area marked in (d) of AgNWs incubated with SA for 24 hours.

4.3.3 Morphology and aggregation states in LLF

The aggregation states of AgNWs as a function of pH, incubation time and LLF components were investigated using optical microscopy while their morphological evolution was studied by TEM. Optical microscopy allowed to preserve the native aqueous environment of AgNWs and avoid drying-induced artefacts during sample preparation for electron microscopy. Incubation of AgNWs in complete LLF (**Figure 4.3a**), at both pH 7 (**Figure 4.3a, i-iii**) and pH 5 (**Figure 4.3a, iv-vi**) led to agglomeration of the wires, which was evident after 24 hours (**Figure 4.3a, ii and v**) and 7 days (**Figure 4.3a, iii and vi**). AgNWs were then incubated in individual LLF components, to examine which are responsible for the observed agglomeration. In the absence of biomolecules, incubation at both pH 7 (**Figure 4.3b, i and ii**) and pH 5 (**Figure 4.3b, vi and vii**) for 1 hour (**Figure 4.3b, i and vi**) up to 1 week (**Figure 4.3b, ii and vii**), led to no observable differences in the aggregation state of AgNWs; AgNWs appeared as single wires randomly dispersed in solution. The addition of DPPC (**Figure 4.3b, iii and viii**), Curosurf[®] (**Figure 4.3b, iv and ix**) or the LA (**Figure 4.3b, v and x**) had no effect on the apparent dispersion of AgNWs. These observations are in agreement with previous work, where small angle X-ray scattering (SAXS) data indicated that DPPC prevented AgNPs from agglomeration.⁷⁹ However, addition of the SA to Curosurf[®], at both pH 7 (**Figure 4.3c, i-iii**) and pH 5 (**Figure 4.3c, iv-vi**), resulted to agglomeration of the wires, observed from 24 hours of incubation (**Figure 4.3c, ii and v**). To compare with previous findings, where incubation of citrate-AgNPs at pH 5 resulted to aggregation and coarsening of the NPs,⁷⁹ TEM imaging was performed, but no pH-induced coarsening of the AgNWs was observed (**Figure 4.3d**). In contrast to citrate-capped AgNPs, AgNWs remain dispersed due to the steric stabilization by the PVP polymer capping, instead of the electrostatic stabilization provided by citrate ions.⁴⁵⁵ Analysis by STEM-EDX provided no evidence for changes in the crystal structure of the AgNWs in any of the LLF components. Most importantly, sulfur sources have previously been implicated in the formation of Ag₂S on the surface of AgNWs.^{189, 190} Incubation of AgNWs with SA, which includes the thiol-containing SP-A and SP-D proteins, did not result in precipitation of Ag₂S (**Figure 4.3d, e**, ≥ 100 AgNWs analyzed). This is in agreement with previous findings where, although AgNWs were readily sulfidized by inorganic sulfur species, they were not by sulfur containing amino acids or proteins, such as cysteine or bovine serum albumin.¹⁸⁹

4.3.4 Characterization of the phospholipid corona

TEM imaging provided further insights into the effects of phospholipids on the stability of AgNWs. Incubation with DPPC increased the thickness of the amorphous layer present around the AgNWs (**Figure 4.4b**) to an average of 7.6 ± 5.2 nm, compared to that of AgNWs in the absence of phospholipid components (**Figure 4.4a**), which was attributed to the coating of AgNWs by phospholipids.

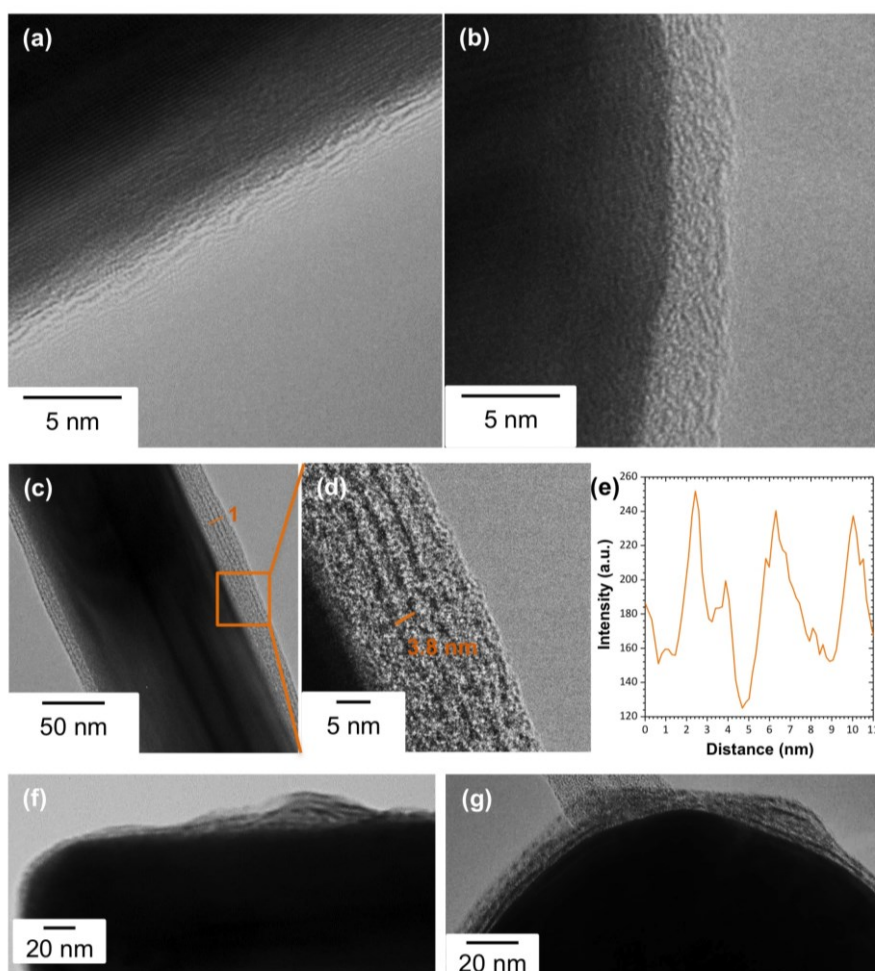


Figure 4.4 (a-d and f-g) BFTEM images of AgNWs incubated in pH 7 buffer (a), with DPPC (b-d), Curosurf[®] (f) and LA (g). In (c, d) and (f-g) samples were positively stained with uranyl acetate to enhance phospholipid contrast. (d) Magnification of the area boxed in (c), showing the phospholipid bilayers formed on the surface of AgNWs. (e) Intensity line profile collected from the line marked 1 in (c).

Their organization on the surface of AgNWs was revealed using uranyl acetate positive staining, which takes advantage of the high affinity of electron dense uranyl ions for the phosphate groups in phospholipids⁴³³, enhancing contrast. AgNWs incubated with DPPC, Curosurf[®] or the LA showed striations that are parallel to the long axis of the AgNWs (**Figure 4.4c, d, f, g**). The hydrophilic headgroup of the DPPC molecule is expected to produce dark contrast whereas the two hydrophobic fatty acid tails will show bright contrast.⁴³³ Therefore, a lipid bilayer structure was formed on the surface of AgNWs, with the hydrophobic tails of DPPC associated with each other and the hydrophilic head-groups oriented toward the aqueous environment and the PVP layer, which is also hydrophilic. The average bilayer thickness, measured from several intensity line profiles (**Figure 4.4e**), was 3.8 ± 0.5 nm, which is close to the hydrocarbon bilayer thickness of 4.16 nm calculated by Park *et al.*⁴⁵⁶ The hydrophobic fatty acid tails of the DPPC molecule are comprised by 16

hydrocarbons. Assuming that the carbon chains are stretched and a chain length between one carbon and the next of 1.3 Å, they estimated the maximum length of DPPC at 2.08 nm and thus 4.16 nm for a bilayer.⁴⁵⁶ The average DPPC bilayer thickness previously measured by AFM was 5.0 nm, and was shown to vary as a function of temperature and analysis technique.⁴⁵⁷ The DPPC bilayer thickness on citrate-AgNPs measured by TEM was 4.3 nm.⁷⁹ STEM-EDX analysis at the surface of AgNWs (**Figure 4.5a**) revealed the presence of phosphorous (**Figure 4.5b**), while STEM-EDX elemental mapping for silver (**Figure 4.5c**) and phosphorous (**Figure 4.5d**) confirmed that AgNWs were coated by phospholipids (**Figure 4.5e**).

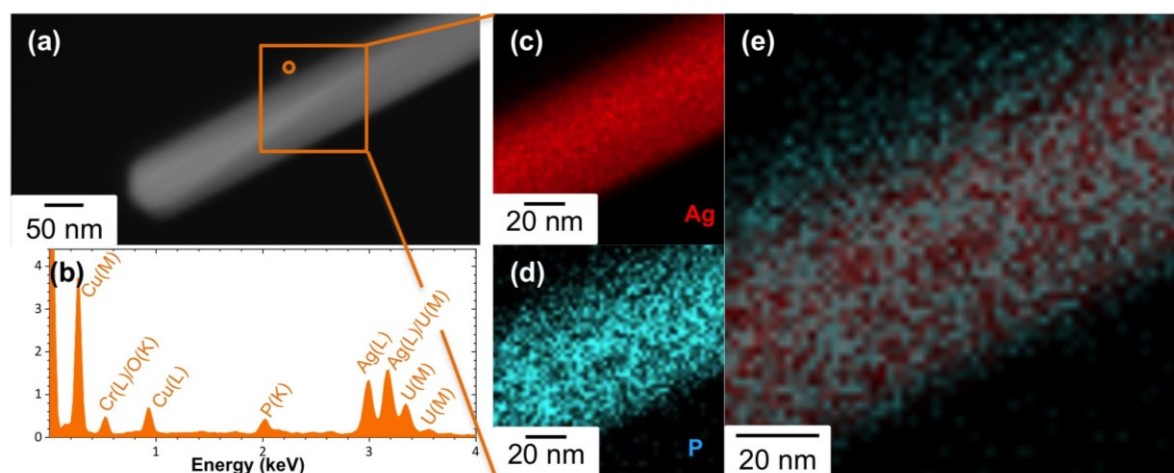


Figure 4.5 (a) HAADF-STEM image of a single AgNW and (b) corresponding EDX spectrum collected from the area circled in (a), confirming the presence of phosphorus on the surface of AgNWs. The samples were positively stained with uranyl acetate to enhance phospholipid contrast. (c-e) The boxed area in (a) was further characterized by STEM-EDX (c) silver (Ag) and (d) phosphorus (P) elemental mapping. (e) The overlaid image confirms that AgNWs are coated by phospholipids.

4.3.5 Generation of secondary AgNPs

The generation of a secondary population of NPs in all of the test solutions was also observed by TEM. After 24 hours, small particles appeared close to the PVP coating of AgNWs (**Figure 4.6a, b**), whereas in the presence of DPPC, inside the phospholipid layer (**Figure 4.6e-f**). After 7 days, a larger number of NPs were observed (**Figure 4.6c, d, Figure 4.7a**). Analysis of their chemistry by STEM-EDX revealed only the presence of Ag (**Figure 4.7b**) (the silicon (Si) peak is due to Si contamination of the carbon grids)⁴⁵⁸. Since the buffers used for the incubation of the AgNWs only contained perchlorate ions, precipitation of insoluble Ag compounds such as Ag₂O, AgCl, Ag₂S or Ag₃PO₄ could not have occurred in solution. STEM-EDX elemental mapping (**Figure 4.7c**) excluded the formation of these Ag compounds as a result of environmental contaminations during the experiments. HRTEM (**Figure 4.7d**) revealed a lattice spacing of 2.37 ± 0.07 Å, which corresponds to

the interplanar spacing of bulk Ag (111) (ref. # 01-087-0597), confirming that the particles are metallic AgNPs. More than 100 particles were analyzed in each sample, and all were shown to be AgNPs.

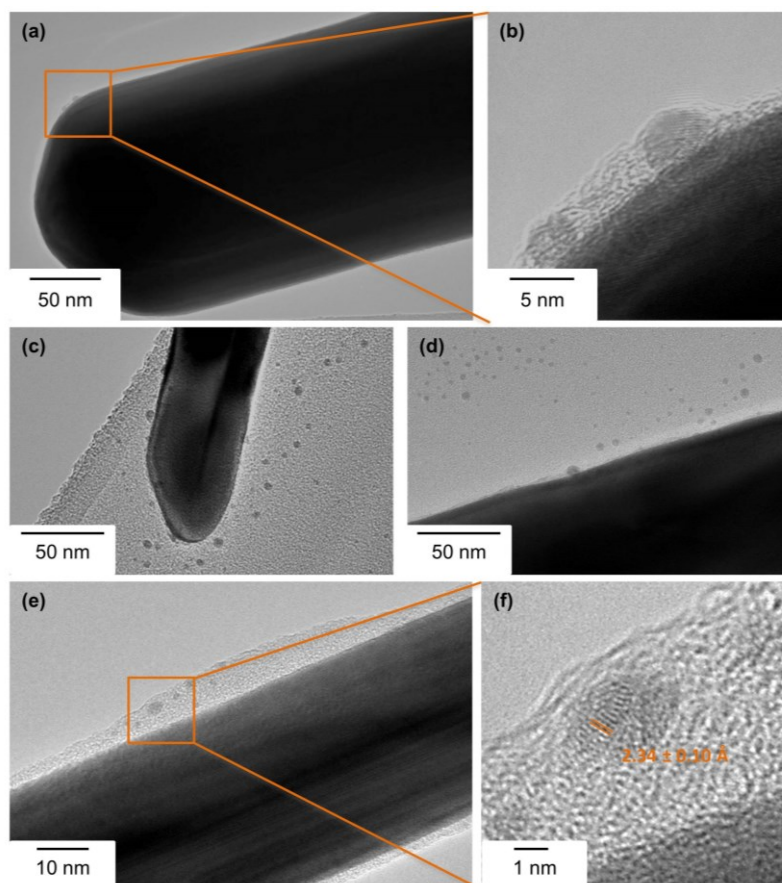


Figure 4.6 BFTEM images of AgNWs showing the generation of a secondary population of nanoparticles. (a) Small nanoparticles were formed at the surface of AgNWs after incubation in pH 7 perchlorate buffers for 24 hours. (b) The magnification of the boxed area in (a) shows that the NPs formed were trapped in the PVP capping layer of AgNWs. (c) AgNWs incubated at pH 7 for 7 days. A larger amount of nanoparticles was observed, close to the surface of AgNWs and in several areas of the carbon coating of the TEM grid. (d) AgNWs incubated at pH 5 for 7 days. (e) AgNWs incubated at pH 7 in the presence of DPPC for 24 hours. The amorphous layer around the AgNWs corresponds to the phospholipid wrapping of the wires. (f) The magnification of the boxed area in (e) shows that crystalline nanoparticles are present inside the phospholipid coating.

These AgNPs were never detected in as-synthesized samples, by neither SEM (**Figure 4.1a**) nor TEM/STEM (**Figure 4.1d, e, g**). When AgNWs were incubated in perchlorate buffers where dissolved oxygen had been removed by purging with argon to inhibit oxidative dissolution of AgNWs,⁷⁸ no NPs could be observed after 2 weeks, suggesting that the AgNPs are formed from a reprecipitation of dissolved Ag^+ ions. Since all the experiments were performed in the dark and no other reducing agent was present in solution, the reduction of Ag^+ ions to form AgNPs was attributed to the mild reducing capabilities of the PVP stabilizer.^{459, 460} Previous studies also reported that Ag^+

ions released from AgNPs were reduced by PVP and formed a secondary population of smaller AgNPs.^{461, 462} The size evolution of the particles (more than 300 particles measured in each case) as a function of time shows a slight increase after 2 weeks of incubation at pH 7 compared to 24 hours (Figure 4.7e). AgNPs formed at early incubation times probably serve as seeds that grow in size as more Ag^+ ions are released from AgNWs. AgNPs formed after 24 hours at pH 5 had a slightly larger size compared to pH 7, (Figure 4.7f), possibly due to the higher rate of Ag^+ ion release from AgNWs at pH 5. After 2 weeks, the size of AgNPs decreased, possibly due to dissolution of AgNPs themselves, which is faster at pH 5.

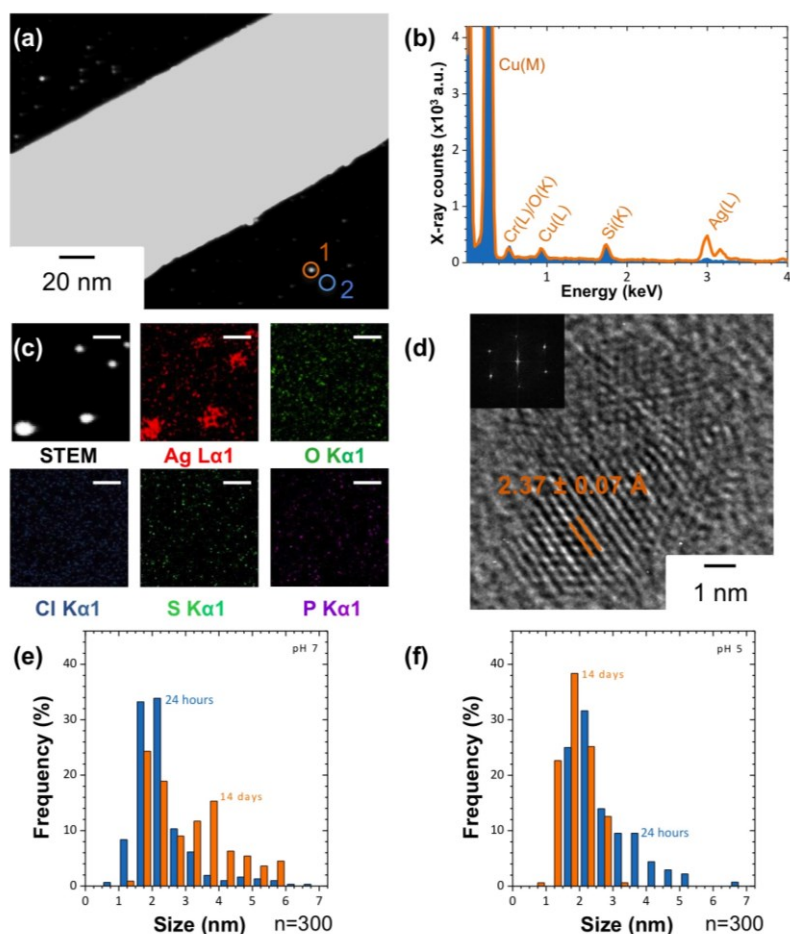


Figure 4.7 HAADF-STEM imaging, STEM-EDX elemental analysis and mapping and HRTEM characterization of the secondary nanoparticles from the original AgNWs. (a) HAADF-STEM image of AgNWs incubated in pH 7 perchlorate buffers for 7 days. The image reveals the presence of a large number of small particles on the surface and around the AgNWs. (b) STEM-EDX spectrum of a single nanoparticle, collected from the circled area 1 in (a) and comparison with the background spectrum, collected from the circled area 2 in (a). (c) HAADF-STEM imaging and EDX elemental mapping. Scale bar 10 nm. (d) HRTEM of nanoparticles formed after the incubation of AgNWs at pH 7 for 7 days. The lattice spacing of the nanoparticles corresponds to that of metallic Ag (111). (e-f) Size distribution of the AgNPs generated after the incubation of AgNWs at pH 7 (e) and pH 5 (f), for 24 hours and 14 days.

4.3.6 AgNW uptake by TT1 and AT2 cells

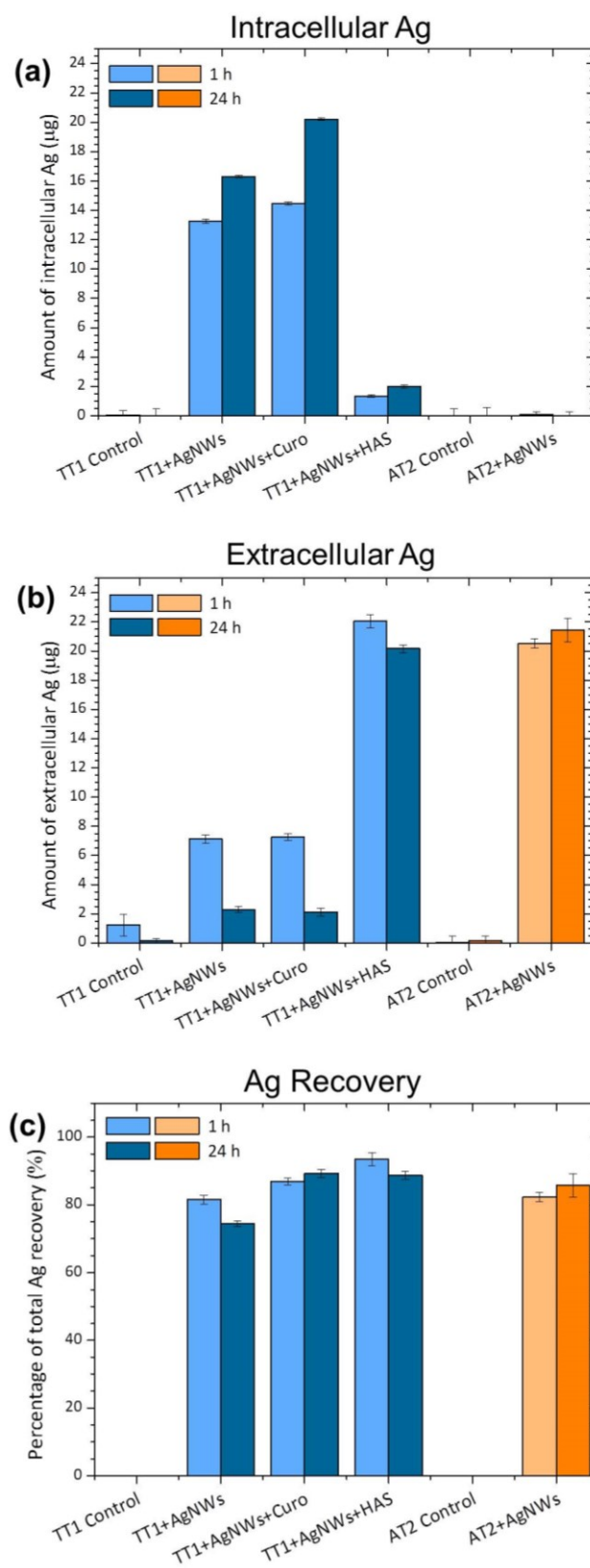


Figure 4.8 ICP-OES quantification of TT1 and AT2 intracellular (a) and extracellular (b) AgNWs following 1- and 24-hour treatments. (c) Percentage of recovery of total applied Ag.

The same AgNWs used in this work had previously been used in a study that showed their uptake by TT1 cells by TEM.³ AgNW uptake was rapid, as AgNWs had been found in the cytoplasm and membrane-bound vesicles of TT1 cells after a 1-hour exposure.³ Therefore, in the present work, the extent of AgNW uptake by TT1 and AT2 cells over 24 hours was quantified by measuring the intracellular amounts of Ag, following continuous exposures for 1 hour and 24 hours. Moreover, the effect of LLF on AgNW uptake was examined using two surfactant models: Curosurf[®] and HAS, which contains the complete lipid and full protein complement of human pulmonary surfactant. TT1 cells took up approximately 53% of the 25 µg of AgNWs applied following 1-hour exposure, and 66% of the 25 µg of AgNWs applied, following 24-hour exposure (**Figure 4.8a**). Pre-incubation of the AgNWs with Curosurf[®] resulted in a slightly higher uptake; TT1 cells took up approximately 58% of the 25 µg of AgNWs applied, following 1-hour exposure, and 84% of the 25 µg of AgNWs applied, following 24-hour exposure (**Figure 4.8a**). Strikingly, pre-incubating the AgNWs with HAS resulted in a significant reduction in uptake; TT1 cells took up only around 5 and 8% of the 25 µg of AgNWs applied, following 1- and 24-hour exposures, respectively. There was no AgNW uptake by AT2 cells after 1- or 24- hour exposures (**Figure 4.8a**).

In agreement with the ICP-OES quantification data, BFTEM imaging showed that bare AgNWs were taken up by TT1 cells following 1- and 24-hour exposures (**Figure 4.9a, b, g, h**). As previously demonstrated,³ some of these AgNWs entered the TT1 cells by piercing the cell membrane (**Figure 4.9b**). This “needle-like” mechanism of insertion into cells has also been reported for functionalized carbon nanotubes.⁴⁶³ After 24 hours, AgNWs were located in endosome/lysosome-like vesicles (**Figure 4.9g, h**). AgNWs that had been pre-incubated with Curosurf[®] were also internalised and showed very similar uptake processes to bare AgNWs, indicating endosomal uptake of the AgNWs (**Figure 4.9c, d, I, j**). AgNWs were located in endosome/lysosome-like vesicles as well as in the cell cytoplasm following 1-hour and 24-hour treatments of TT1 cells (**Figure 4.9c, d, i, j**). Both in the absence and presence of Curosurf[®], degradation in the morphology of AgNWs was observed, including hollowing-out and formation of small nanoparticles (**Figure 4.9h, j**). As described below, this degradation was further examined by STEM-EDX and HRTEM. In contrast to bare AgNWs and AgNWs pre-incubated with Curosurf[®], BFTEM imaging revealed very little uptake of AgNWs that had been pre-incubated with HAS for 1 hour (**Figure 4.9e, f**). After 24 hours, some AgNWs pre-incubated with HAS were found extracellularly to the TT1 cells, close to the cell membrane (**Figure 4.9k, l**). This low uptake of AgNWs + HAS corresponds with the ICP-OES data.

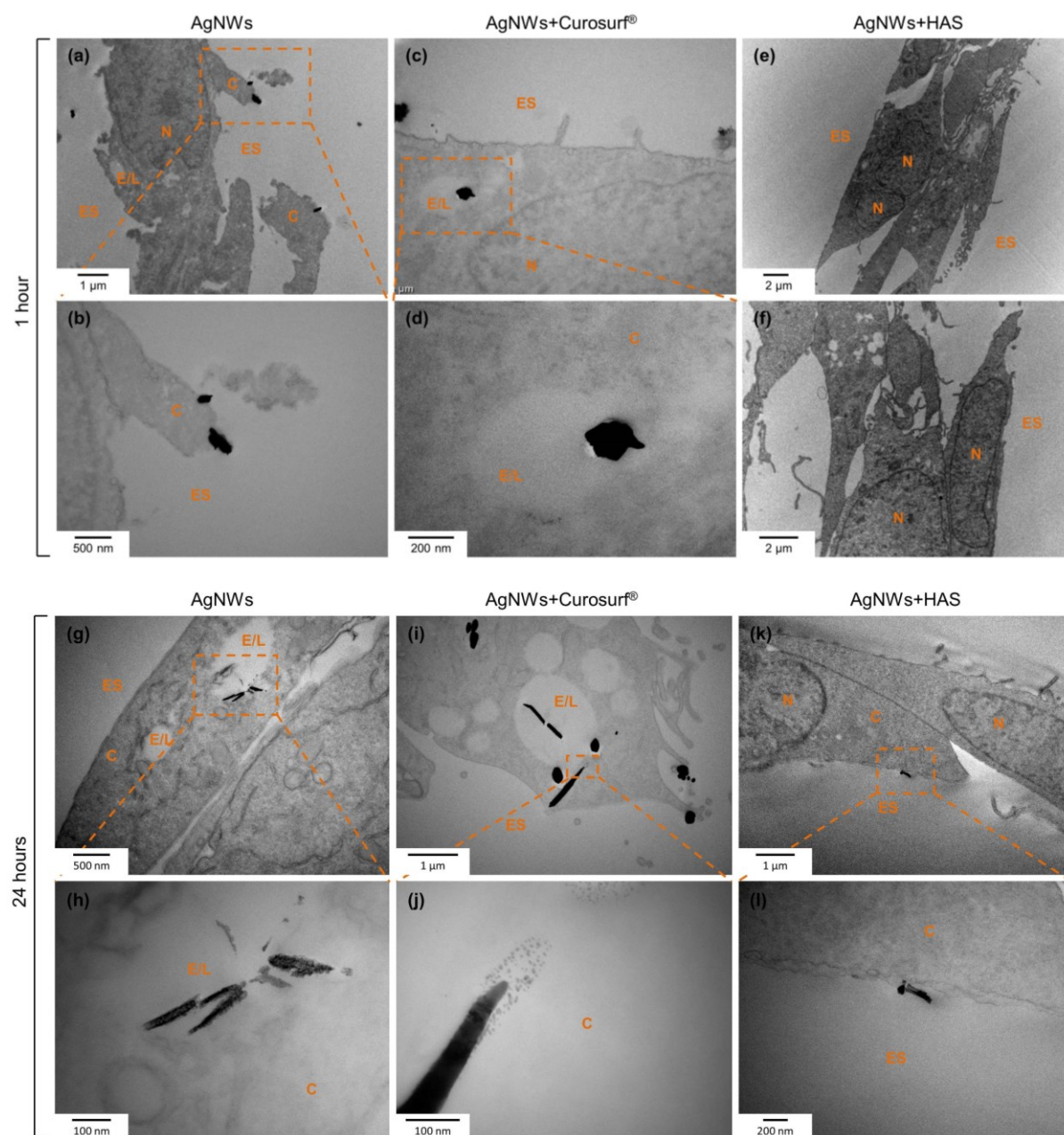


Figure 4.9 BFTEM imaging of AgNW uptake by TT1 cells. TT1 cells were exposed to AgNWs in the absence (a, b, g, h) and presence of Curosurf® (c, d, i, j) or HAS (e, f, k, l) for 1 hour (a-f) and 24 hours (g-l). (b, d, h, j and l) are magnifications of the areas squared in (a, c, g, i and k), respectively. N = nucleus, C = cytoplasm, E/L = endosome/lysosome, ES = extracellular space.

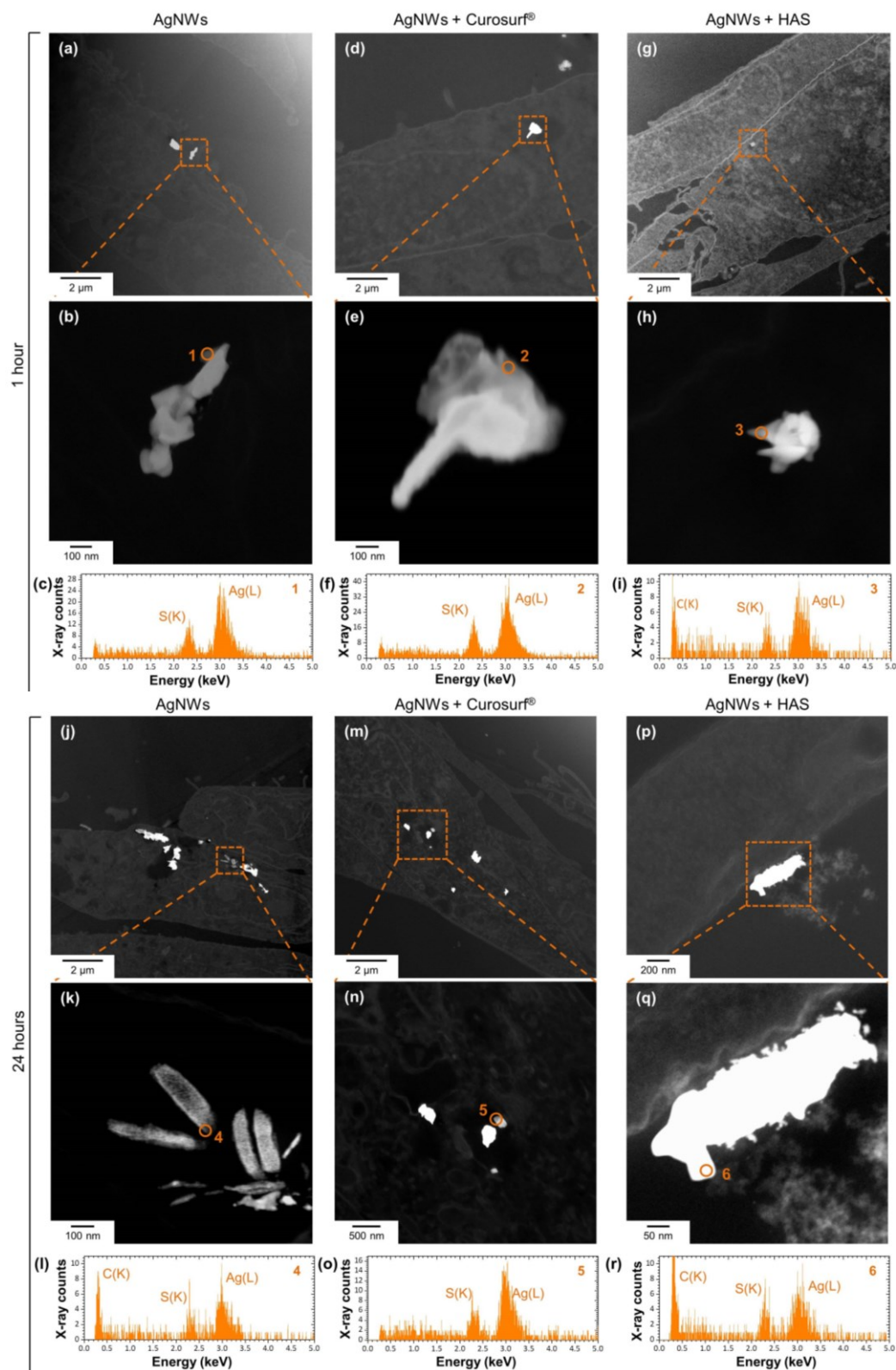


Figure 4.10 HAADF-STEM imaging and STEM-EDX analysis of TT1 epithelial cells exposed to AgNWs in the absence (a-c, k-l) and presence of Curosurf® (d-f, m-o) or HAS (g-i, p-r), for 1 hour (a-i) and 24 hours (j-r). The images show changes in the morphology of AgNWs after their interaction with TT1 cells. (b, e, h, k, n and q) are magnifications of the areas squared in (a, d, g, j, m and p), respectively. STEM-EDX spectra (c, f, i, l, o, r) were collected from the corresponding areas 1-6 marked in (b, e, h, k, n and q). The presence of sulfur in the EDX spectra indicates that the interaction of AgNWs with TT1 cells leads to dissolution and precipitation of Ag_2S .

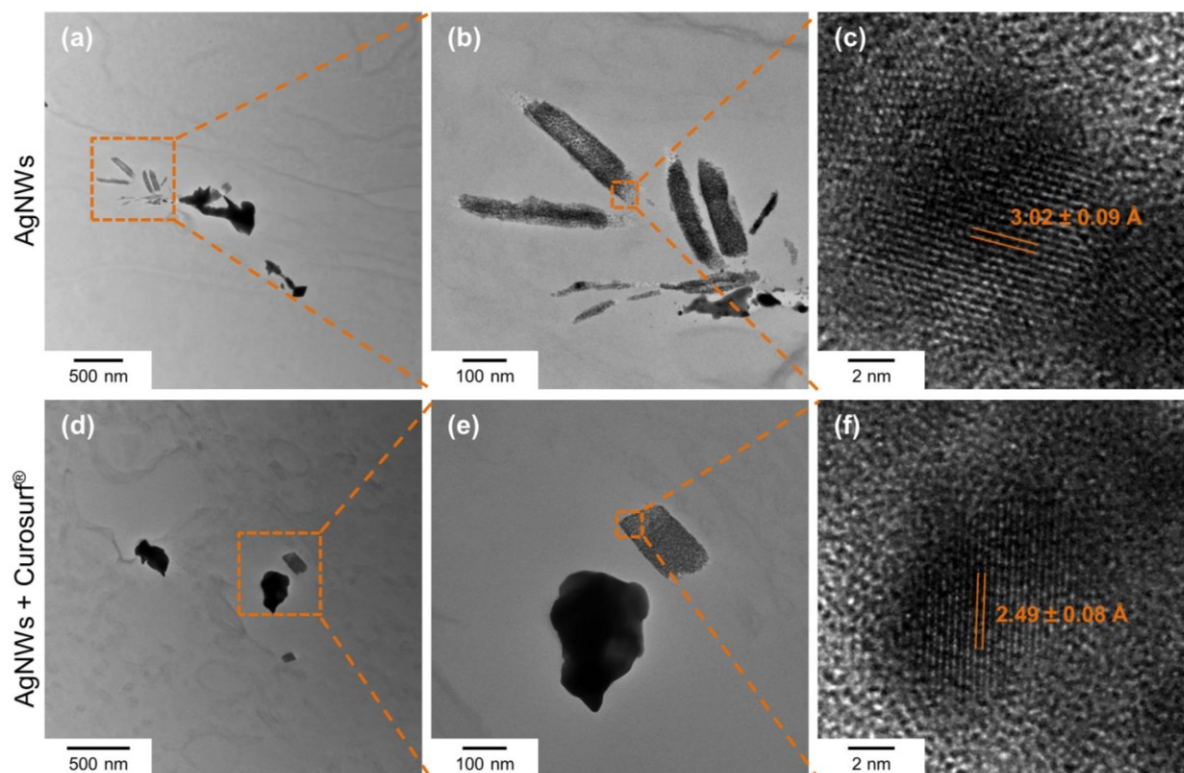


Figure 4.11 BFTEM (a, b, d, e) and HRTEM (c, f) images of TT1 epithelial cells incubated with AgNWs for 24 hours in the absence (a-c) and presence of Curosurf®. (a) and (d) were collected from the same areas shown in **Figure 4.10 (k)** and **(n)**, respectively.

HAADF-STEM imaging confirmed changes in the morphology of AgNWs (bare and with Curosurf®) taken up by TT1 cells after 1- (**Figure 4.10a, b, d, e**) and 24-hour exposures (**Figure 4.10j, k, m, n**). Ag(L) peaks and S(K) peaks were detected in the STEM-EDX spectra collected from the edge of the wires (**Figure 4.10c, f, l, o**), suggesting the formation of silver sulphide (Ag_2S). A previous study has shown that intracellular internalisation of bare AgNWs by epithelial TT1 cells resulted in the dissolution of the AgNWs and the precipitation of highly insoluble Ag_2S ($K_{\text{sp}} = 5.92 \times 10^{-51}$).³ This Ag_2S formation could act as a “trap” for free Ag^+ , thus significantly limiting the short-term toxicological effects of AgNWs. HRTEM imaging revealed that, following a 24-hour exposure to bare AgNWs, crystalline particles had precipitated, with a lattice spacing of $3.02 \pm 0.09 \text{ \AA}$, which is close to the spacing of the (111) planes of monoclinic Ag_2S (ref. # 00-014-0072) (**Figure 4.11a-c**). In the case of AgNWs + Curosurf®, the lattice fringe spacing measured was $2.49 \pm 0.08 \text{ \AA}$, which is close to the spacing of the (112) planes of monoclinic Ag_2S (ref. # 00-014-0072) (**Figure 4.11d-e**). These findings, confirm the precipitation of crystalline Ag_2S intracellularly. In accordance to BFTEM observations, HAADF-STEM showed very little uptake of AgNWs that had been pre-incubated with HAS for 1 hour (**Figure 4.10g, h**). After 24 hours, HAADF-STEM imaging showed that extracellular AgNWs were close to but separated from the plasma membrane (**Figure 4.10p, q**). Although pre-incubating AgNWs with HAS significantly reduced uptake of the AgNWs, they remained in close

proximity to the TT1 cells and STEM-EDX indicated that there was dissolution and precipitation of Ag₂S (Figure 4.10i, r).

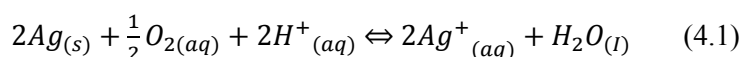
4.4 Discussion

The propensity for the physicochemical properties of AgNMs to alter, in some cases quite radically, depending on the environment they encounter, can significantly alter their bioreactivity with the environment, cells and tissues. Therefore, in order to understand the mechanisms of biological action of AgNMs, these transformations must be thoroughly examined. In the present work, by performing careful characterization using both *in situ* techniques to track dynamic information about the colloidal stability of the AgNWs and spatially resolved high resolution elemental analysis, the system parameters (*i.e.* pH, phospholipids, surfactant proteins, and capping agents) have been deconvoluted, and new insights on the effects of each parameter on the stability of AgNWs have been provided.

In summary, *ex vivo* adsorption of surfactant lipids on AgNWs has been shown for the first time. This lipid corona delayed the kinetics of Ag⁺ ion release from AgNWs. TEM imaging indicated that the adsorbed phospholipids form a coating on the surface of AgNWs and that the hydrophobic alkyl chains of the phospholipids are adsorbed onto the AgNWs while with the polar headgroups face away from the AgNWs toward the aqueous phase. Moreover, using *in situ* light microscopy, the small aggregate fraction of the LLF was demonstrated to be responsible for the agglomeration of AgNWs observed in LLF as well as the decreased release of Ag⁺ ions. The generation of a secondary population of AgNPs from the original AgNWs has also been shown, possibly due to the reduction of Ag⁺ ions by the PVP capping of the wires. Alterations of AgNW properties in the LLF were confirmed to have a significant impact on the uptake of AgNWs by TT1 cells. Pre-incubation of the AgNWs with HAS resulted in a dramatic decrease in AgNW internalization, which may be attributed to the SA-induced agglomeration of AgNWs observed in the LLF.

4.4.1 Effect of pH

The release of free Ag⁺ ions is often considered as the predominant mechanism of toxicity for AgNMs. In the endocytic pathway, the pH progressively decreases from about 7.1 to lower than 5.5 in lysosomes, which could affect the extent of AgNW dissolution in different cellular compartments. The dissolution of AgNPs is considered to be *via* a cooperative oxidation that involves both protons and dissolved oxygen.⁷⁸



In the above experiments, dissolution of AgNWs was carried out under ambient atmosphere and therefore dissolved oxygen is a possible factor in the oxidation of AgNWs. It is also clear from the data presented here, that the *kinetics* of AgNW dissolution *are* affected by the pH. Similar observations have been made for both citrate^{78, 79} and PVP capped AgNPs.⁴⁶² However, the maximum concentrations of Ag⁺ ions measured are not likely to significantly contribute to the toxicity of AgNWs.²⁴¹

4.4.2 Effect of phospholipids

The presence of phospholipids, in either simple (DPPC) or complex mixtures (Curosurf[®]), induced a retarding effect on the release of Ag⁺ ions at pH 5, which could be explained by the formation of phospholipid layers on the surface of AgNWs. This finding supports previous work, where DPPC coating of citrate-AgNPs delayed Ag⁺ ion release and prevented aggregating and coarsening.⁷⁹ It has previously been suggested that the DPPC coating acts as a diffusion barrier to molecular oxygen, decreasing the ion release kinetics. Here, it is suggested that the outermost surface of the lipid coating corresponds to the hydrophilic headgroup of DPPC. When strongly hydrophilic groups interact with the surrounding aqueous media, the sorption of the organic compounds facilitates the stability of the nano-Ag dispersion.¹⁸⁶ This observation may now explain previous findings where the bronchoalveolar lavage improved the dispersion of AgNPs.⁴⁶⁴ The adsorption of lipids on AgNWs could directly affect their uptake by cells and the intracellular responses. Research on other classes of nanomaterials has shown, for instance, that lipid adsorption led to increased internalization of TiO₂ NPs²⁰⁹ and SWCNTs²¹⁰ by macrophages. In another work, human LLF increased the uptake of polystyrene NPs by human alveolar epithelial cells, regardless of NP size or surface modification.⁴⁶⁵ Moreover, coating MWCNTs²¹¹ or SWCNTs²¹² with lipids evoked an increase in intracellular reactive oxygen species (ROS) production. However, in the present work, pre-incubation of AgNWs with Curosurf[®] did not significantly alter the internalization of AgNWs by TT1 cells. Furthermore, in agreement to this finding, Curosurf[®] had no significant effect on the inflammatory (**Figure 4.13**) and reactive oxygen species (ROS)-generating potential (**Figure 4.14**) of AgNWs, as reported by Sweeney *et al.*⁴³⁹

4.4.3 Effect of surfactant-associated proteins

The addition of SA further reduced the amount of Ag^+ ions released, which explains the decreased dissolution observed at pH 5 in the presence of complete LLF. This decrease in dissolution was not due to a chemical transformation of the AgNWs, since sulfidation by the thiol containing SP-A and SP-D was ruled out by HRTEM and STEM-EDX. However, SA induced agglomeration of the AgNWs, at both pH values tested, consequently lowering the available surface area and thus the dissolution rate. Recent work has suggested that incubation of various types of NPs with SP-A/D led to NP agglomeration,^{150, 213} possibly through the selective binding of SP-A and/or SP-D to the NPs.^{214, 465, 466} Sweeney *et al.* used the same AgNWs as the present work to examine protein binding from HAS collected from human AT2 cells, by Western blot analysis.⁴³⁹ Only 10% and 13% of the total protein available in HAS bound to 1 and 25 $\mu\text{g}/\text{mL}^{-1}$ of AgNWs respectively, following 1-hour incubation (**Figure 4.12a**). However, SP-A was specifically found to bind to AgNWs as indicated by both an increase in the amount of SP-A in the AgNW-containing pellet and a decrease in the amount of SP-A in the corresponding supernatant following centrifugation (**Figure 4.12b**). SP-D was also found to bind specifically to AgNWs (**Figure 4.12c**).⁴³⁹ Therefore, the agglomeration of AgNWs observed in the SA fraction of *murine* LLF may be attributed to the specific binding of SP-A and SP-D on AgNWs.

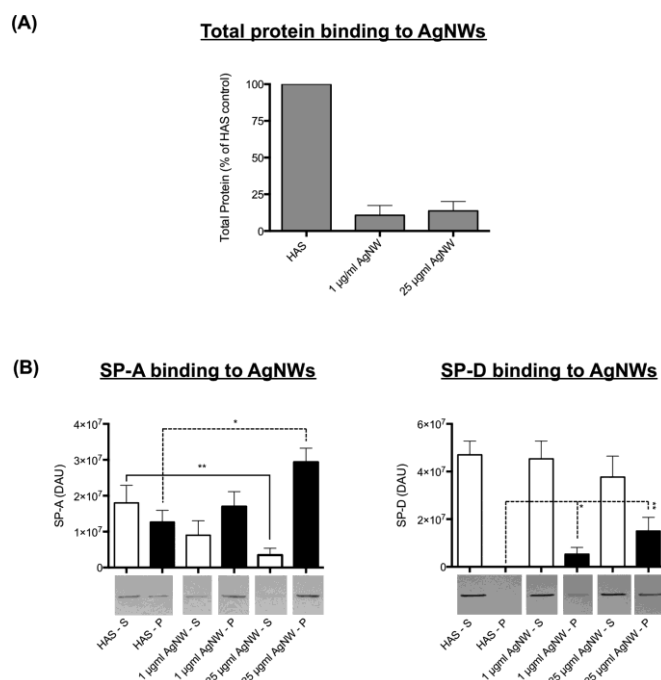


Figure 4.12 Total HAS protein and SP-A/SP-D protein binding to AgNWs. (A) Proportion of total protein available in HAS, bound to 1 and 25 $\mu\text{g}/\text{mL}$ of AgNWs. (B) Amount of SP-A (left) and SP-D (right) protein bound to 1 and 25 $\mu\text{g}/\text{mL}$ of AgNWs. The amount of SP-A or SP-D in the pellet (P) and the amount of SP-A or SP-D in the corresponding supernatant (S) is shown following centrifugation, in the absence and presence of AgNWs. Reprinted with permission from ⁴³⁹. Copyright 2013 The Royal Society of Chemistry.

LLF protein adsorption on AgNWs could have implications on the bioreactivity of AgNWs upon inhalation, since for other types of NPs this altered their cellular uptake, clearance, translocation and toxic effects.⁴⁶⁶ Changes in the agglomeration state of AgNWs may lead to different responses by different cell types. For example, lung epithelial (C-10) cells were found to be more resistant to AgNPs than macrophages (RAW-264.7).³³⁵ This was attributed to the fact that the aggregate size of AgNPs in the cell culture medium was more than 100 nm, which improved their uptake efficiency by macrophages but reduced it by epithelial cells.¹⁵⁴ In a recent work by Silva *et al.*, instillation of AgNWs in rats led to the deposition of AgNWs in the deep lungs.³⁴⁵ Both AgNW lengths tested produced significant numbers of Ag-positive macrophages at day 1.³⁴⁵ Here, SP-A/D induced agglomeration of the AgNW also within 24 hours; this finding could therefore explain both the high uptake of AgNWs by macrophages in the Silva study, but also the dramatic decrease of uptake by TT1 cells observed here. The toxicity of AgNWs against TT1 cells was further examined by Sweeney *et al.* and, although Curosurf[®] had no effect, HAS conferred a protective effect to TT1 cells exposed to AgNWs (**Figure 4.13** and **Figure 4.14**).⁴³⁹ Following 24-hour treatment with 10 and 25 µg/ml of AgNWs, the inflammatory chemokine human IL-8 release by TT1 cells significantly increased by a 2- and 1.8-fold, respectively, and remained elevated at day 1 post-exposure. Pre-incubation of AgNWs with HAS almost completely abolished the AgNW-induced IL-8 release, both during a 24-hour exposure period and at day 1 post-exposure. TT1 cell superoxide levels significantly increased following 4-hour treatment with 25 µg/ml of AgNWs, and following 24-hour treatment with 10 and 25 µg/ml of AgNWs. This increase remained at day 1 post-exposure. As seen for IL-8 release, HAS eliminated the AgNW-induced ROS levels, following both the 24 hour treatment period and at day 1 post-exposure.⁴³⁹ Because pre-incubation of AgNWs with Curosurf[®] did not reduce the material's toxicity, it is likely that the protein component and not the lipid component of HAS is responsible for its ability to reduce AgNW bioreactivity with TT1 cells. The evidence from the uptake data presented here and the bioreactivity study discussed above suggests that the protein component of HAS (*e.g.* SP-A and SP-D) may act to reduce AgNW bioreactivity by reducing AgNW uptake by TT1 cells.

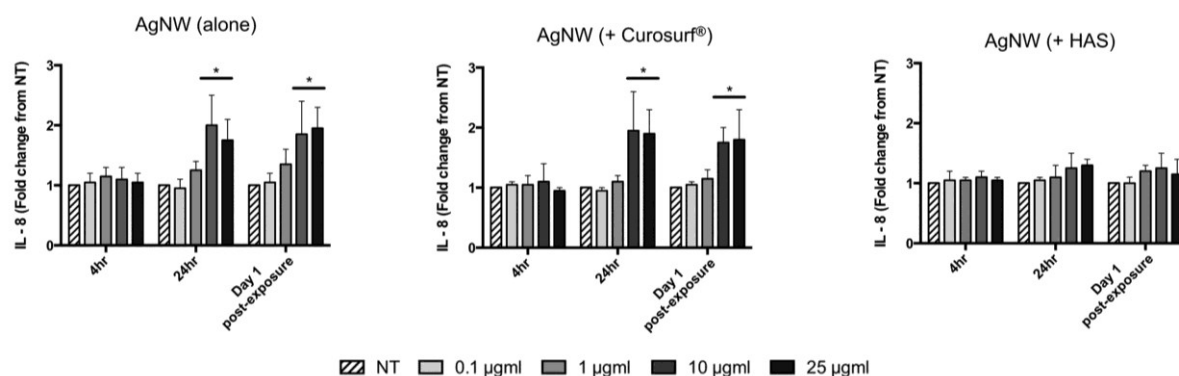


Figure 4.13 TT1 inflammatory mediator IL-8 release following 4- and 24-hour treatments with AgNWs (bare (left) or pre-incubated with Curosurf® (middle) or HAS (right)) and after a 1-day post-exposure recovery period. Increases in IL-8 release are presented in fold change ($n = 3$) $\pm$ SD; significant differences between non-treated and treated cells are indicated where $*p < 0.05$. Reprinted with permission from ⁴³⁹. Copyright 2013 The Royal Society of Chemistry.

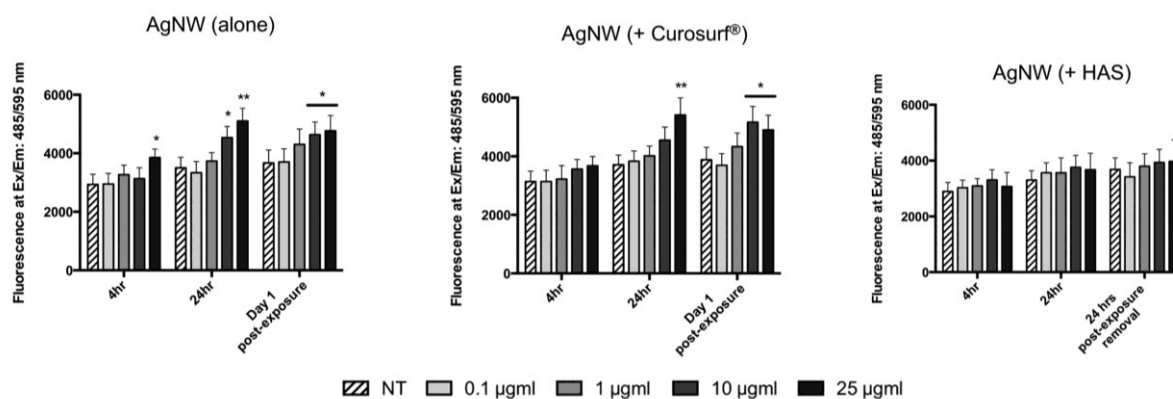


Figure 4.14 Reactive oxygen species (ROS) levels in TT1 cells, following 4- and 24-hour treatments with AgNWs (bare (left) or pre-incubated with Curosurf® (middle) or HAS (right)) and after a 1-day post-exposure recovery period. Superoxide levels are presented in fluorescence units at Ex/Em: 485/595 nm ($n = 3$) $\pm$ SD; significant differences between non-treated and treated cells are indicated where $*p < 0.05$. Reprinted with permission from ⁴³⁹. Copyright 2013 The Royal Society of Chemistry.

4.4.4 Effect of capping agent

The generation of a secondary population of AgNPs from the original AgNWs was observed following their incubation at pH 7 and pH 5 and in the presence of LLF components. PVP-capped AgNPs have also been shown to generate smaller AgNPs when exposed to high relative humidity⁴⁶¹ or in solution.⁴⁶² Surface coatings may have a vast influence on the toxicological potential of AgNMs, as has been recently highlighted in a study where citrate-AgNPs were more toxic than PVP-AgNPs, despite having similar sizes and dissolution rates.¹⁰⁶ The authors proposed that Ag^+ ions form complexes with PVP, which reduces their bioavailability and consequently cytotoxicity. In the present work we show that Ag^+ ions may also undergo PVP-mediated reduction to form secondary AgNPs.

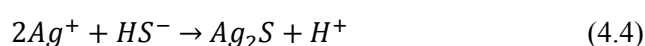
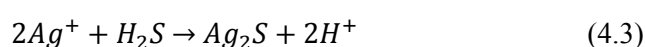
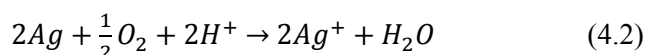
These secondary particles, having a much smaller size, could directly interfere with the function of cell membranes, allowing a larger number of AgNPs to penetrate inside cells.¹⁰⁶ Smaller AgNPs may also be able to reach internal organelles more easily, inducing more pronounced toxic effects.¹⁵² Due to their smaller size, they are also expected to have enhanced dissolution kinetics.⁷⁷

4.4.5 Intracellular transformation of AgNWs

Both in the absence and presence of Curosurf[®], AgNWs were observed intracellularly, in the cytoplasm and in membrane-bound vesicles after 1 hour and 24 hours. Moreover, precipitation of Ag₂S within the cell was detected. Chen *et al.* have suggested that Ag₂S formation acts as a “trap” for free Ag⁺, which could significantly limit the short-term toxicological effects of AgNWs. In accordance to this hypothesis, Sweeney *et al.* showed that TT1 cell viability, measured using a tetrazolium compound colorimetric assay (MTS), was not significantly changed following 4- and 24-hour exposures to 1-25 µg/mL AgNWs.⁴³⁹ The results presented here additionally suggest that the toxic potential of AgNWs to TT1 cells may further be limited through the SP-A/D-induced decrease in AgNW uptake. In contrast to other bio-persistent materials, such as asbestos, these results also indicate that AgNWs may be degraded within the cellular environment and transform to Ag₂S. This process could occur by the action of sulfide species, including H₂S, HS⁻ and S²⁻ inside the cells. These species exist in all human tissues, including the lung. H₂S is an important gasomediator resulting from tissue desulphydration of cysteine and is rapidly broken down by a combination of chemical and enzyme reactions. The free gas H₂S level in tissue is likely to be maintained on the order of nM concentrations; however, the total concentration of sulfide species is thought to be much higher (~µM).^{344, 467}

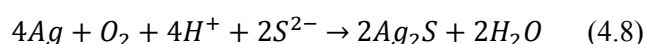
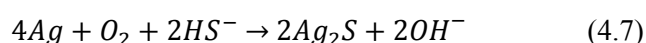
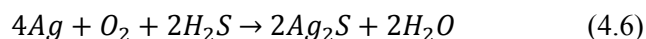
Chen *et al.* suggested that the cellular sulfidation of AgNWs may occur via two chemical reactions:

(1) Oxidative/precipitation (in-direct) pathway, *i.e.* oxidative dissolution of silver (reaction eq. 4.2) followed by sulfide precipitation (reaction eq. 4.3-4.5). This sulfidation process is effectively independent of sulfide concentration, due to the extremely low K_{sp} of Ag₂S, but determined by the oxidative dissolution rate of silver.⁴⁶⁸





(2) Particle-fluid reaction, *i.e.* a direct oxysulfidation route (reaction **eq. 4.6-4.8**), which is dependent on the sulfide concentration, such that the reaction rate increases as the sulfide concentration increases.



At the early stages of intracellular transformation, the sulfidation transformation process is likely dominated by the in-direct pathway. The Ag^+ ions released from the AgNW surface diffuse into the cellular environment; Ag^+ ions are rapidly re-precipitated as Ag_2S nanoparticles within the comparatively S-rich cytoplasm. With time, more Ag^+ ions dissolve, migrate within the microenvironment of the AgNWs, and precipitate as new Ag_2S nanoparticles or grow on the existing Ag_2S nuclei. On the other hand, S-species may also attack the AgNW surface through the direct oxysulfidation reaction, a relatively slower reaction that leads to the formation of small Ag_2S nanoparticles around the AgNWs surface. These small particles on the AgNW surface then act as nuclei, and grow into bigger Ag_2S particles and coalesce to form a shell-like structure, which may slow the dissolution rate of Ag^+ ions.

4.5 Conclusions

In conclusion, these findings show that different components of the LLF can significantly alter the aggregation state and surface chemistry of AgNWs. These alterations are shown to change the reactivity of AgNWs and how they are internalized and processed by the underlying epithelial cells. It is also demonstrated that the protein component of AT2 cell secretions dramatically reduces the uptake and bioreactivity of AgNWs by TT1 cells. It is suggested that the surfactant proteins SP-A and SP-D are likely to be involved in these mechanisms, which require further investigation. This study provides unique findings that are important for understanding interactions at the bio-nano interface of the alveolar unit and the impact these could have on subsequent epithelial–endothelial activity and nanoparticle translocation across the gas–liquid interface. More generally, the observations presented here highlight the need for detailed dynamic and static characterization at the

point of exposure in order to develop a holistic understanding into which parameters govern AgNM bioreactivity, as the *in situ* properties of AgNMs may prevail over their inherent properties. Improving our understanding of how the physicochemical properties of AgNMs change in the presence of biomolecules is paramount for appreciating their true environmental and human health impact.

5 Towards Imaging the Antibacterial Activity of AgNPs in *M.tb*-Infected Alveolar Macrophages

5.1 Introduction

Bacterial infections of the pulmonary system are increasing. Almost half of today's infections are caused by strains of bacteria that are resistant to existing antibiotics. One particular concern is the emergence of multidrug-resistant tuberculosis (MDR-TB). According to the World Health Organization, such reported cases in Africa vastly increased by 650% within seven years - from 2,445 in 2005 to 18,146 in 2012 (WHO, Global Tuberculosis Report, 2013). The problem is not limited to developing countries. In the US, at least 2 million people every year are infected by germs resistant to antibiotics, making these infections more frequent than cancer (Centres for Disease Control and Prevention, Antibiotic Resistance Threats in the United States, 2013). Despite the urgent medical need for new antibiotics against TB, the pipeline for such drugs has slowed down over the past decades. Meanwhile, the mismanagement of MDR-TB has led to the outbreak of extensively drug-resistant TB (XDR-TB), raising concerns for a future TB epidemic. Global health organization agencies are now calling for new approaches in the development of anti-TB treatments, in order to save millions of lives worldwide.

Treatment of pulmonary TB has been standing at the same strategic point as it had been when it started almost five decades ago. The most widely accepted approach involves the daily oral intake of a cocktail of antibiotics, but has a prolonged treatment time and is associated with a high percentage of side effects. This often results in low patient compliance, which in turn leads to an increased risk of treatment failure, relapse and the development of drug-resistant TB strains. Moreover, only a fraction of the anti-tuberculous drugs have sufficient tissue-penetrating properties to reach the bronchoalveolar spaces, which are the desired sites of drug action. Consequently, novel treatment options and modalities for TB are urgently needed. *Mycobacterium tuberculosis* (*M.tb*), the

bacterium that causes TB, utilises alveolar macrophages (AM) as its niche environment for survival and proliferation. Following phagocytosis, *M.tb* bacilli may spread infection by arresting phagosome maturation.³⁰³ Therefore, AMs are appropriate targets for inhalable drug delivery systems for anti-tuberculous therapy. To date, research on drug delivery systems focused on either polymeric or liposomal microparticles loaded with anti-TB drugs. These systems have been shown to reduce mycobacterial loads of AM and be many times more effective than the use of free drugs.^{469, 470}

As discussed in **Chapter 4**, the increasing commercialisation of silver nanomaterials (AgNMs) has led to the need to understand the occupational health, public safety and environmental implications of these materials. However, along with concerns about toxic effects, there is potential for enormous benefit via highly efficacious antibacterial modality. Silver nanoparticles (AgNPs) are considered as potential therapeutic agents that could have a significant impact on respiratory research and medicine, by offering a multifunctional platform that could simultaneously diagnose and treat bacterial infections.²⁸⁷⁻²⁸⁹ Due to their small size, AgNPs are expected to preferentially deposit in the alveolar region of the lungs. Their antibacterial properties have been shown to depend on several physicochemical properties of the particles, including their size, shape, chemistry and surface coating.^{194, 290-292} Currently, the mechanisms by which AgNPs are able to reduce the activity of bacteria are believed to arise due to a synergistic effect, including both direct particle-specific biological effects *and* the release of Ag⁺ ions.^{195, 293} One of the reasons which make AgNPs attractive as antibacterial agents is that bacteria are not likely to become resistant to silver like antibiotics, due to the wide range of possible interactions of Ag⁺ ions with biomolecules.²⁸⁸ However, several discrepancies in the published literature need to be addressed in order to draw accurate links between the physicochemical properties of the particles and the observed biological effects.

The effects of AgNPs on bacteria are commonly evaluated by various *in vitro* viability measurements. Biological assays can provide useful information about the antibacterial action of AgNPs. However, more detailed mechanistic investigations of the cellular interactions with AgNPs are necessary in order to provide insights about how to optimise the design of AgNPs for the treatment of bacterial infections. Some reports have indicated that biophysical interactions may occur between AgNPs and bacteria, such as biosorption, AgNP breakdown or aggregation and cellular uptake.^{296, 297} AgNPs have been suggested to increase the permeability of bacterial cell membranes, penetrate into the cytoplasm and disturb cellular functions.²⁹⁸ The application of analytical electron microscopy in this area could be invaluable, because it can provide high spatial resolution information of the morphology and chemistry of AgNPs. Moreover, spatially resolved maps of the anatomical distribution and physico-chemistry of AgNPs at the sub-cellular level and observation of changes in the cellular ultrastructure could help deciphering the antibacterial mechanisms exerted by nano-Ag. Therefore, nanoscale imaging methods have the potential to provide vital information about these systems and, when used in conjunction with complementary quantitative studies (*e.g.* cell metabolism

assays, bacterial proliferation assays, cytoplasmic membrane permeability assays), could provide a holistic understanding of the system interactions.

The aim of the present work is to form a basis for the potential use of AgNPs as antibacterial agents in *M.tb*-infected cells. Although the antimicrobial properties of AgNPs are being widely investigated, these properties have not been previously explored in the context of bacteria residing inside infected cells. First, the antibacterial effects of 20 nm citrate-coated AgNPs against *M.tb* bacilli were examined. AgNPs were synthesized and characterized in-house to ensure that their physicochemical properties were well-controlled. A colony forming unit (CFU) assay was employed to assess the viability of *M.tb* exposed to AgNPs. A set of analytical transmission electron microscopy (TEM) techniques was used to examine the ultrastructural damage in bacterial cells and the localization of AgNPs, to provide clues about the mechanisms (*e.g.* dissolution, adherence to the cell membrane or penetration inside the cytoplasm) by which the AgNPs induce cell damage. Furthermore, TEM was used to visualize the damage induced by *M.tb* infection in THP-1 macrophages, a human acute monocytic leukemia cell line. THP-1 cells can be differentiated into macrophage-like cells that mimic human macrophages,^{471, 472} and have been frequently used as a cellular model of alveolar macrophages in *in vitro* studies.^{75, 164} Finally, the potential antibacterial effects of AgNPs in *M.tb*-infected cells were examined by TEM, by simultaneously exposing THP-1 macrophages to both AgNPs and *M.tb*.

5.2 Methods

5.2.1 AgNP synthesis

Silver nanoparticles (AgNPs) were synthesised *via* chemical bath reduction, using sodium borohydride as the reducing agent and citrate as the stabiliser. Briefly, silver nitrate (AgNO_3 , 1.0×10^{-3} M, Sigma Aldrich, UK) and trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, 1.0×10^{-3} M, Fisher Scientific, UK) solutions were added to 250 mL of boiling DI water. A sodium borohydride (NaBH_4 , 1.0×10^{-3} M, Sigma Aldrich, UK) solution was then added drop-wise to the mixture over a period of 10 seconds. The colour of the solution immediately turned to yellow, indicating the formation of silver nanoparticles. Heating was continued for an additional 30 minutes, after which the solution was cooled to room temperature. In order to remove impurities and excess citrate, the synthesis product was washed by repeated cycles of centrifugation ($10000 \times g$) with de-ionized (DI) water. Finally, citrate-coated AgNPs were suspended in DI water in a sealed glass vial and stored at 4°C in the dark. AgNPs were synthesised and thoroughly characterized by Dr. Bey Fen Leo as described in ⁷⁹.

5.2.2 THP-1 cell culture

THP-1 cells, a human acute monocytic leukemia cell line (Cat. No. TIB-202), were obtained from American Type Culture Collection (ATCC) and cultured as per ATCC manual in RPMI 1640 supplemented with Penicillin/Streptomycin/L-Glutamine and 10% fetal bovine serum (Hyclone Laboratories, Inc. Logan, Utah) and 0.05 mM 2-mercaptoethanol. THP-1 cells were induced to differentiate into macrophage-like cells, which are often used as a model for human macrophages.⁴⁷¹
⁴⁷² Cells were maintained at 37°C in a humidified 5% CO₂ environment. Cells were passaged every 3-4 days. Cell culture was performed by Dr. Srijata Sarkar in the laboratory of Prof. Stephan Schwander at Rutgers School of Public Health, NJ, USA.

5.2.3 Preparation of AgNPs and *M.tb* for in vitro cell exposure

For *in vitro* THP-1 cell exposure experiments, AgNP samples were diluted in complete cell culture medium without antibiotics (RPMI 1640 supplemented with 10% pooled human AB serum (PHS)), followed by sonication in a Branson 3510 water bath sonicator for 2 minutes, prior to addition to the cell cultures. For THP-1 cell infection, an avirulent *M.tb* strain (H37Ra, ATCC catalogue number 25177) was used.

5.2.4 Colony forming unit (CFU) Assay

To evaluate direct, AgNP-mediated antibacterial effects, *M.tb* was grown in cell culture media in the absence (non-treated control) or presence of AgNPs. Ten µL of *M.tb* stock (CFU 1x10⁷/mL) were diluted to 1 mL with cell culture media (RPMI 1640 + 10% PHS) and incubated at 37°C in rotation on a revolver (Labnet International, Inc., Edison, NJ) in the presence of 0, 10 and 25 µg/mL of AgNPs or 0, 15.7 and 39.25 µg/mL of AgNO₃ for 24 hours. AgNO₃ concentrations corresponded to the amounts of Ag⁺ ions present in 10 and 25 µg/mL of AgNPs, respectively. Ten µL of the culture media samples were removed at 24 hours and serially 10-fold diluted in cell culture media without antibiotics (RPMI 1640 + 10% PHS), plated in triplicate onto 7H10 agar plates and incubated for 21 days at 37 °C.⁴⁷³ *M.tb* colonies were counted and bacterial colonies (CFU) determined using a stereo microscope at 40× magnification (Diagger Lab equipments, Vernon Hills, IL), and plotted as a function of the AgNP concentration. *M.tb* grown in NP-free culture media served as a control. The CFU assay was carried out by Dr. Srijata Sarkar.

5.2.5 Transmission electron microscopy (TEM)

To evaluate the antibacterial effects of AgNPs by TEM, *M.tb* were grown as described above in the presence of 10 µg/mL of AgNPs for 24 hours. To evaluate the cellular uptake of AgNPs, THP-1 macrophages were incubated at 37°C in a humidified CO₂ environment for 24 hours in the presence of 0 (non-treated control) and 10 µg/mL of AgNPs. To examine the morphological changes of infected cells as well as the effects of AgNPs on these cells, THP-1 cells were infected with *M.tb* at a multiplicity of infection (MOI) of ten bacilli per cell (MOI10), in the absence or presence of 10 µg/mL of AgNPs for 24 hours. Cell and bacteria exposures were performed by Dr. Srijata Sarkar.

Bacteria and cells were then rinsed with fresh RPMI 1640 culture medium and fixed in 2.5% glutaraldehyde in 0.1M HEPES buffer, pH 7.2 for 1 hour at 4°C. The fixatives were removed by washing the cells with 0.1 M HEPES buffer 3 times. Cells were scraped, transferred to 1.5 mL Eppendorf tubes and centrifuged at 5000 g for 20 minutes to obtain cell pellets. Then, the cell pellets were osmicated (1% osmium tetroxide (OsO₄) in 0.1 M HEPES). Potassium ferricyanide, which is usually used in osmication, was omitted from the staining process because it has been shown to significantly alter the physicochemistry of Ag nanomaterials.³ Following washing 3 times with DI water, the cell pellets were dehydrated in graded solutions of ethanol (50%, 70%, 95%, and 100%), for 5 minutes three times in each, and then washed three times, for 10 minutes each, in acetonitrile (Sigma Aldrich, UK). After dehydration, samples were progressively infiltrated with a Quetol-based resin, produced by combining 8.75 g quetol, 13.75 g nonenyl succinic anhydride, 2.5 g methyl acid anhydride and 0.62 g benzyl dimethylamine (all from Agar Scientific, UK). Samples were infiltrated in a 50% resin:acetonitrile solution for 2 hours, in a 75% resin:acetonitrile solution overnight and in 100% resin for 4 days, with fresh resin being replaced daily. The embedded samples were cured at 60 °C for 24 hours. Thin sections (90 nm) were cut from the resin blocks directly into a water bath using an ultramicrotome and a diamond knife with a wedge angle of 35°. Sections were immediately collected on bare, 300 mesh copper TEM grids (Agar Scientific, UK), dried and stored under vacuum until TEM analysis. In some cases, sections were post-stained with 2% uranyl acetate in 70% ethanol and lead citrate for 5 min each.

Bright field TEM imaging was performed on a JEOL 2000 microscope operated at 80 kV and fitted with an energy dispersive X-Ray (EDX) detector (Oxford Instruments, UK). High resolution transmission electron microscopy (HRTEM) and high angle annular dark field (HAADF) scanning transmission electron microscopy (STEM) was performed at 80 kV on a FEI Titan 80/300 TEM/STEM, fitted with a Cs (image) corrector and a SiLi EDX spectrometer (EDAX, Leicester UK). HAADF-STEM experiments were performed with a convergence semi-angle of 14 mrad and inner and outer HAADF collection angles of 49 and 239 mrad, respectively. The probe diameter was <0.5 nm.

5.3 Results

5.3.1 Characterization of as-synthesized AgNPs

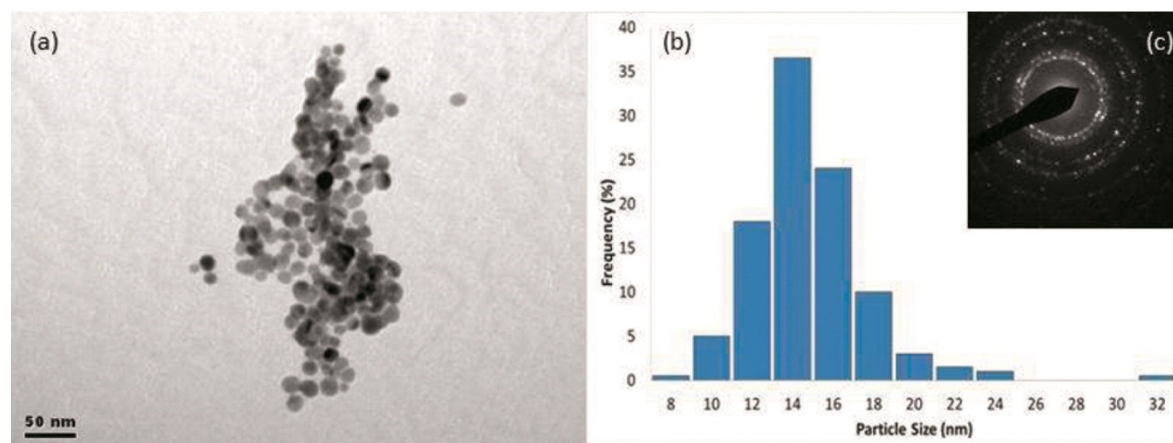


Figure 5.1 Characterization of as-synthesized AgNPs. BFTEM image of as-synthesized AgNPs (a), their size distribution (b) ($n = 200$) and a SAED pattern (c) collected from an area containing multiple AgNPs. Reprinted with permission from ref. ⁷⁹. Copyright 2013 American Chemical Society.

AgNPs were synthesized in-house in order to ensure a full control over their physicochemical properties. Their complete characterization has been reported by Leo *et al.*⁷⁹ In summary, AgNPs had a spherical morphology (**Figure 5.1a**), with an average diameter of 14 ± 1.6 nm (**Figure 5.1b**). Selected area electron diffraction (SAED) patterns (**Figure 5.1c**) revealed that as-synthesized AgNPs had characteristic lattice spacings 0.236 nm, 0.204 nm, and 0.145 nm, corresponding to the (111), (200), and (220) planes of metallic silver (ref. #01–087–0597). TEM-EDX analysis confirmed that sulfidation of AgNPs had not occurred and all impurities had been removed after washing with DI water.⁷⁹ AgNPs had a negative surface charge of -17.9 ± 3.0 mV.⁷⁹

5.3.2 Effect of AgNPs on *M.tb* viability

To evaluate the antibacterial properties of AgNPs against *M.tb*, bacterial growth was assessed by CFU assays. *M.tb* growth was reduced to around 50% after a 24-hour exposure of *M.tb* to 10 $\mu\text{g/mL}$ of AgNPs (**Figure 5.2**). A dose of 25 $\mu\text{g/mL}$ of AgNPs induced a further 10% decrease in *M.tb* growth. AgNO_3 was used as a positive control for *M.tb* killing at equivalent doses as AgNPs, but both doses completely abrogated *M.tb* growth. These findings are in accordance to previous reports in the literature. For instance, Bondarenko *et al.* recently evaluated the toxicity of AgNPs with different surface coatings to Gram-negative and Gram-positive bacterial strains.¹⁹⁵ For AgNPs, the 4-hour half maximal effective concentration (EC_{50}) varied widely between bacterial strains, from 0.35 to 46

$\mu\text{g/mL}$. However, Ag^+ ions were much more toxic to bacteria, with the 4-hour EC_{50} varying only slightly between bacterial strains, from 0.3 to 1.2 $\mu\text{g/mL}$.¹⁹⁵

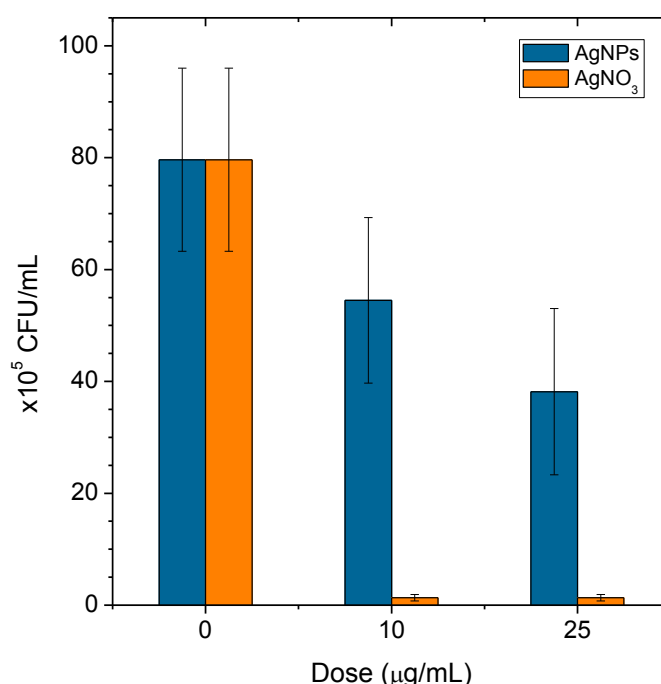


Figure 5.2 Effect of AgNPs and Ag^+ ions on the viability of *M.tb* measured by a CFU assay. Results are represented as mean $\pm$ SD from two independent experiments.

The morphology of non-treated bacteria was examined by BFTEM. **Figure 5.3a** shows a control *M.tb* bacterium, with a diameter of around 700 nm. The *M.tb* bacterium is known to be rod-shaped and measures around 0.5 μm in diameter and 1-4 μm in length.⁴⁷⁴ The cytosol had a nearly uniform electron density, while the nucleoid (**Figure 5.3a**, arrow), which contains the genetic material of the bacterium, may be discerned against the cytosol. Bacterial DNA, which is thought to appear as strands under electron microscopy, seems to be randomly distributed in the nucleoid. **Figure 5.3b** shows the cellular envelope of *M.tb*, revealing the complex structure of its cell wall. Although *M.tb* is classified as a Gram positive bacterium, it does not retain any bacteriological stain (e.g. the crystal violet stain used in the Gram staining method of bacterial differentiation⁴⁷⁵) due to the high lipid content in its wall. The cell wall of *M.tb* (**Figure 5.3c**) has a unique waxy composition, consisting of three covalently linked polymers: the basal peptidoglycan (PG) layer, an intermediate electron-transparent layer (ETL) that is probably composed of the mycolic acid covalently bound to arabinogalactan and an outer electron dense layer formed by slight heavy metal deposits bordering the lipid-rich ETL.⁴⁷⁶ The architectural arrangement of the lipids and glycolipids on the outer surface of the mycobacterial cell envelope confers to the bacilli properties such as a strong resistance to

degradation by host enzymes, impermeability to toxic macromolecules, and inactivation of reactive oxygen and nitrogen derivatives.³⁰³

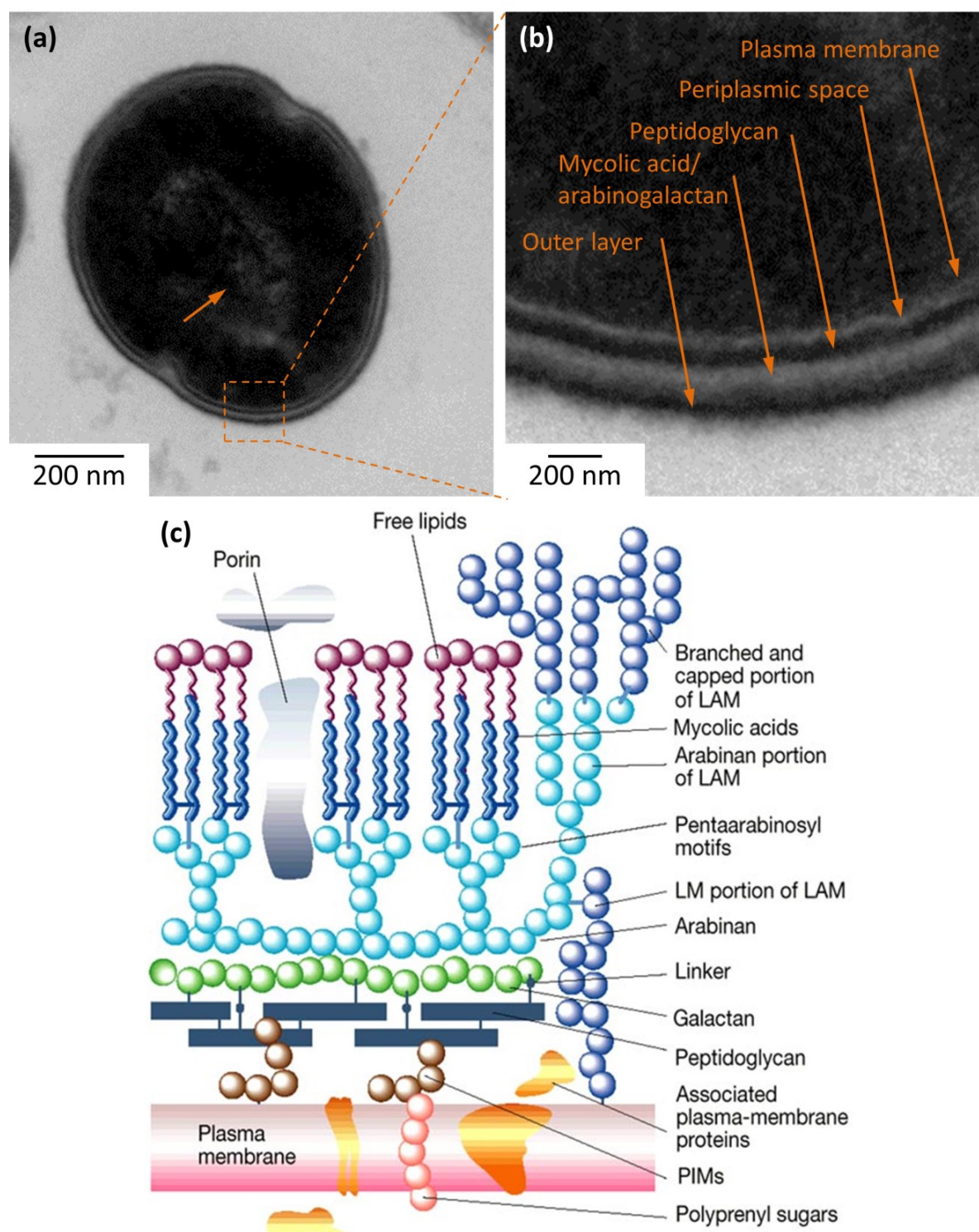


Figure 5.3 (a) Bright field transmission electron microscopy (BFTEM) image of a control *M.tb* bacterial cell. The arrow indicates the bacterial nucleoid. (b) is the magnification of the area squared in (a) showing the structure of the bacterial cell envelope. (c) Schematic representation of the complex *M.tb* cell envelope. LAM: lipoarabinomannans, LM: lipomannans, PIM: phosphatidylinositol mannosides. Reprinted with permission from ref. ⁴⁷⁷. Copyright 2000 Macmillan Magazines Ltd.

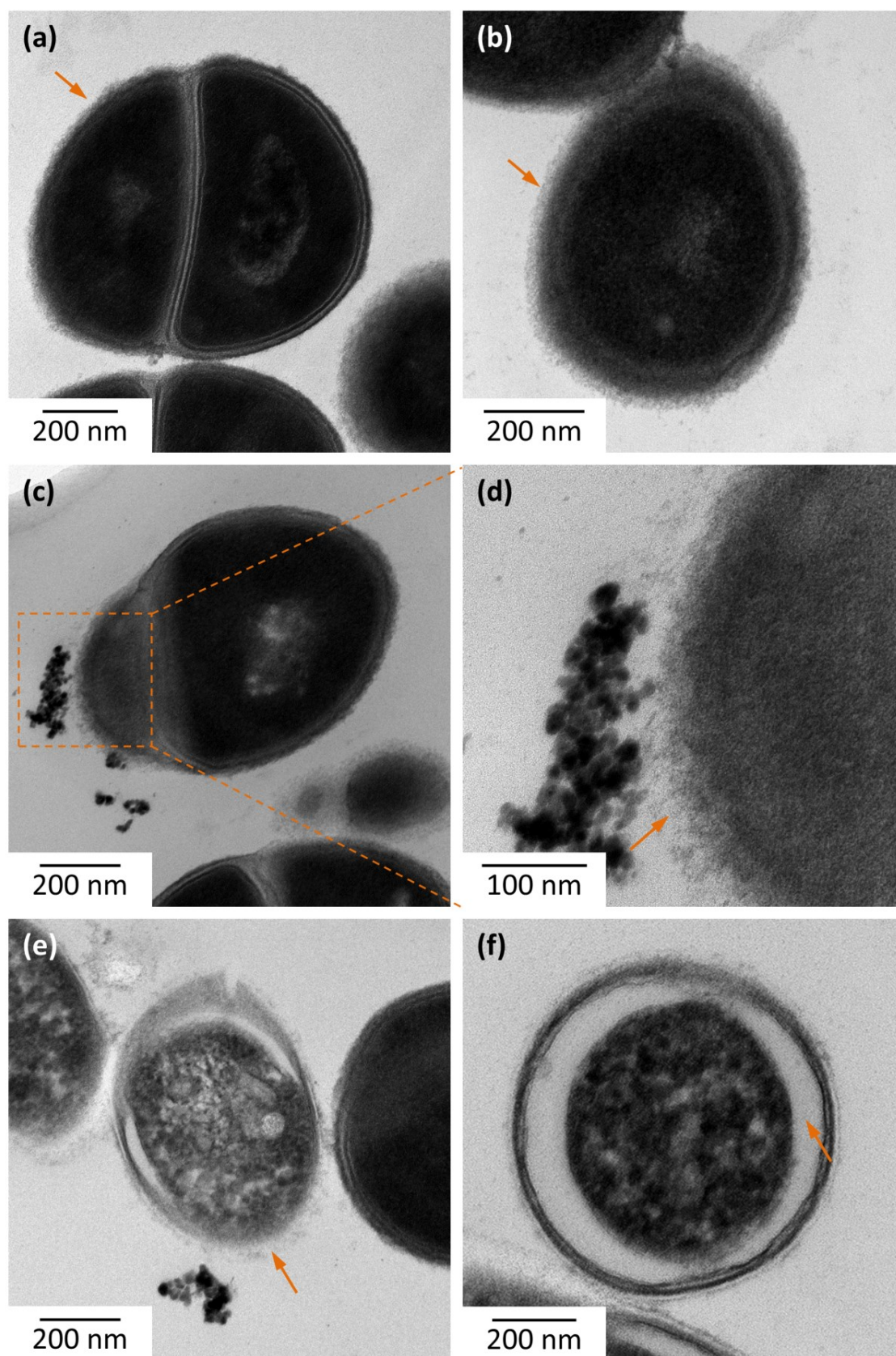


Figure 5.4 (a-f) BFTEM images of ultrastructural damage in *M.tb* bacterial cells treated with 10 µg/mL of AgNPs for 24 hours, showing disorganization of the cell wall structure (a-e, arrows) and detachment of the cell membrane from the cell wall (f, arrow) (d) is the magnification of the area squared in (c).

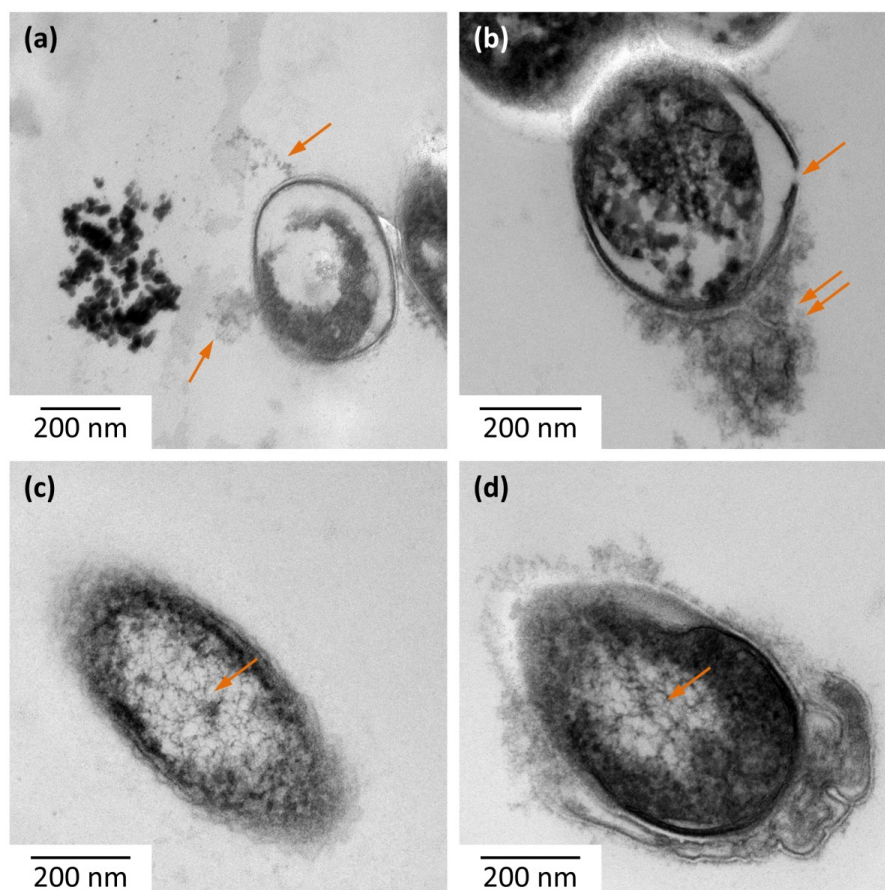


Figure 5.5 (a-d) BFTEM images of ultrastructural damage in *M.tb* bacterial cells treated with 10 µg/mL of AgNPs for 24 hours, showing vesicles emanating from the cell wall (a, arrows), rupture of the cell membrane (b, arrow) and leakage of cytoplasmic content (B, double arrow) and appearance of an electron-light area in the centre of bacteria (c, d, arrows).

The ultrastructural damage in bacterial cells exposed to AgNPs was examined by TEM, using a dose of 10 µg/mL of AgNPs, which represents around 50% reduction from the CFU value of control cells. Following a 24 treatment with AgNPs, several types of ultrastructural damage were observed in bacterial cells by TEM (**Figure 5.4** and **Figure 5.5**). Instead of the smooth cell surface of untreated bacteria (**Figure 5.3a**), damage was observed in the cell envelope of bacteria treated with AgNPs, that ranged from an undulating appearance (**Figure 5.4a**, arrow) to a complete disorganization of the cell surface (**Figure 5.4b**, arrow). This disorganization of the cell wall was particularly evident in areas close to AgNPs (**Figure 5.4c-e**, arrows). In several cases, the cell membrane had detached from the cell wall, leading to the formation of a gap (**Figure 5.4f**, arrow). Furthermore, vesicles were detected emanating from the cell wall (**Figure 5.5a**, arrow), as well as rupture of the cell envelope (**Figure 5.5b**, arrow) and leakage of intracellular content (**Figure 5.5b**, double arrow). Similar mechanisms of cell wall damage have been recently reported by Mei *et al.* in both Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (*S. aureus* and *B. amyloliquefaciens*), using AgNPs functionalized with antimicrobial peptides (AMPs).³³⁸ Finally, an electron light region often appeared

in the centre of *M.tb* treated with AgNPs, with strands of electron dense material visible in this region (Figure 5.5c, e, arrows). This finding resembles those of previous studies, following treatment of *E.coli* with AgNPs or Ag^+ ions.^{296, 322} It has been suggested that the formation of this low density region is a mechanism of defence, by which bacteria conglomerate their DNA to protect it from toxic compounds, when the bacteria sense a disturbance of the cell membrane.^{296, 322}

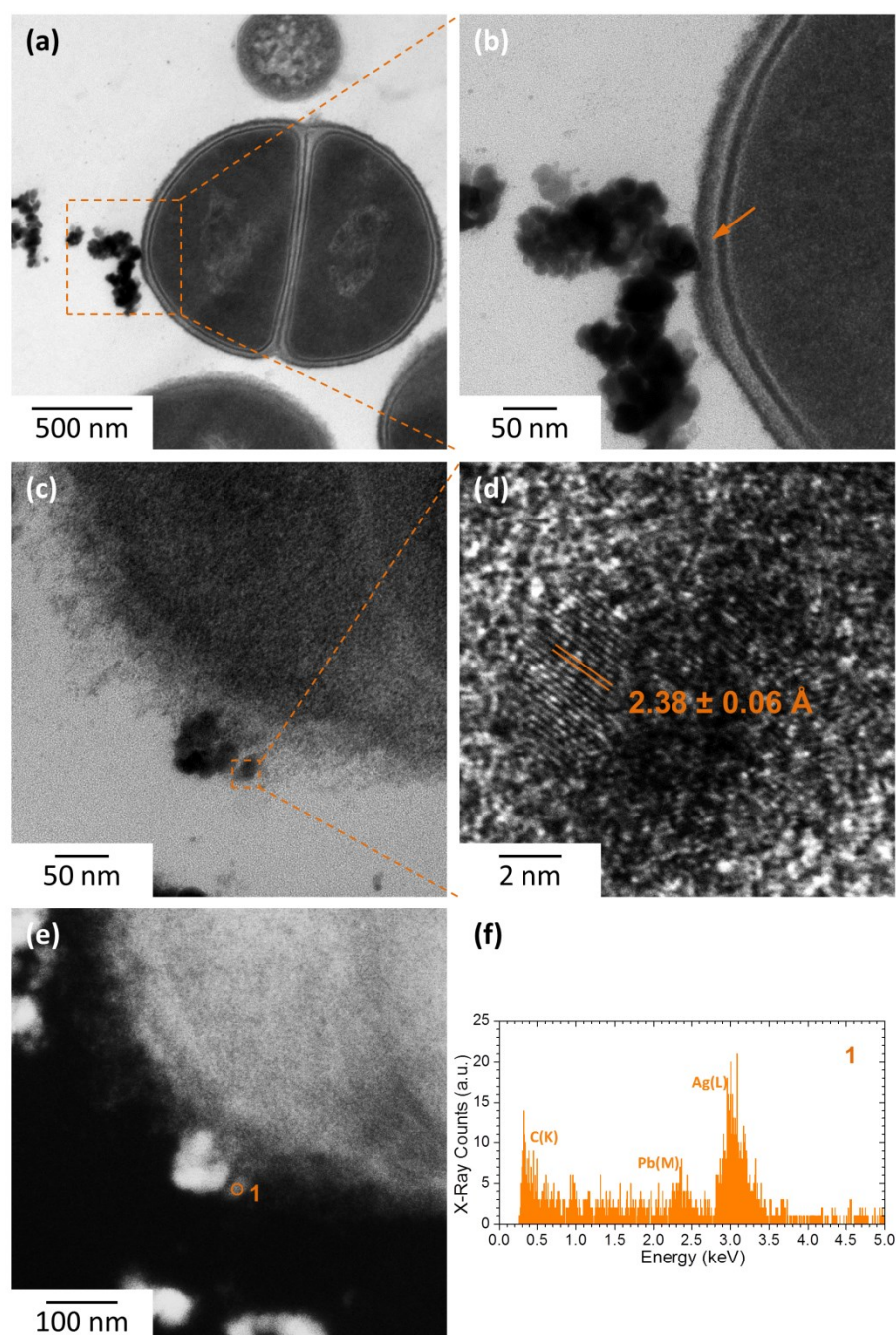


Figure 5.6 (a-c) BFTEM images of *M.tb* bacterial cells treated with 10 $\mu\text{g/mL}$ of AgNPs for 24 hours, showing AgNPs interacting with the cell wall. (b) is the magnification of the area squared in (a). (d) HRTEM image collected from the area squared in (c). (e) HAADF-STEM image collected from the same area as (c). (f) STEM-EDX spectrum collected from the area marked in (e).

Furthermore, analytical TEM techniques were used to investigate the localization of AgNPs with respect to bacterial cells. **Figure 5.6a-b** shows AgNP interacting with the bacterial cell wall, evident by the change in the cell wall outline (**Figure 5.6b**, arrow). AgNPs were found adhering on the cell wall, which appeared severely disorganized (**Figure 5.6c**). The lattice spacing of the particles measured from HRTEM images (**Figure 5.6d**) was 2.38 Å, which is close to the (111) interplanar spacing of metallic silver (ref. #01–087–0597). STEM-EDX analysis revealed only presence of Ag in the EDX spectrum (**Figure 5.6f**), confirming that the particles were metallic AgNPs and that no chemical transformation had occurred. TEM (**Figure 5.6c**) and HAADF-STEM (**Figure 5.6e**) imaging also indicated that aggregation and coarsening of the particles may have taken place, which could be due to the local acidic pH at the plasma membrane.¹⁹⁵ As demonstrated by a previous work using the same AgNPs as this study, a decrease in pH from 7 to 5 and 3 enhanced the dissolution rate of AgNPs, promoting particle aggregation and coarsening. These observations suggest that an increased Ag⁺ release could be expected close the bacterial cell wall, leading to a higher cellular uptake of Ag⁺ ions, as has been hypothesized by recent work.^{194, 195}

Additionally, several small particles were observed translocating across the cell wall and inside the cell cytosol (**Figure 5.7a, b**). HRTEM imaging (**Figure 5.7e**) showed that these particles had a lattice spacing of 0.205 Å, which corresponds to the (200) spacing of bulk Ag (ref. #01–087–0597). Compared to the background spectrum (**Figure 5.7g**), Ag was detected in the STEM-EDX spectra collected from the particles (**Figure 5.7h**), confirming that the particles were metallic AgNPs. Only small individual particles, and not particle aggregates, were found inside the cell cytoplasm. The average size of these particles, measured from several TEM images (n=100), was 8 ± 4 nm. Particle size is considered as one of the main physicochemical properties that may affect the bactericidal behaviour of AgNPs.²⁹¹ Morones *et al.* exposed Gram-negative bacteria to AgNPs with sizes from 1 to 100 nm and showed that only the smallest particles, with diameters of 1–10 nm, were found on the surface of the cell wall and inside the bacteria.²⁹⁶ The authors suggested that the mechanism through which AgNPs were able to penetrate into bacteria was mediated by changes to the structure of the cell wall, which led to an increase in its permeability.²⁹⁷ Moreover, another work demonstrated the effect of particle aggregation by comparing 20-30 nm citrate-coated AgNPs to citrate- and polycyclic aromatic hydrocarbon (PAH)-coated gold nanoparticles (AuNPs). PAH-coated AuNPs, that did not tend to aggregate, bounded more strongly to cell walls and penetrated more readily inside bacteria than citrate-capped AgNPs, which aggregated more readily.³²⁹ Consequently, the aggregation and coarsening of AgNPs observed close to the cell wall could be the reason for the limited uptake of particles inside *M.tb* bacteria.

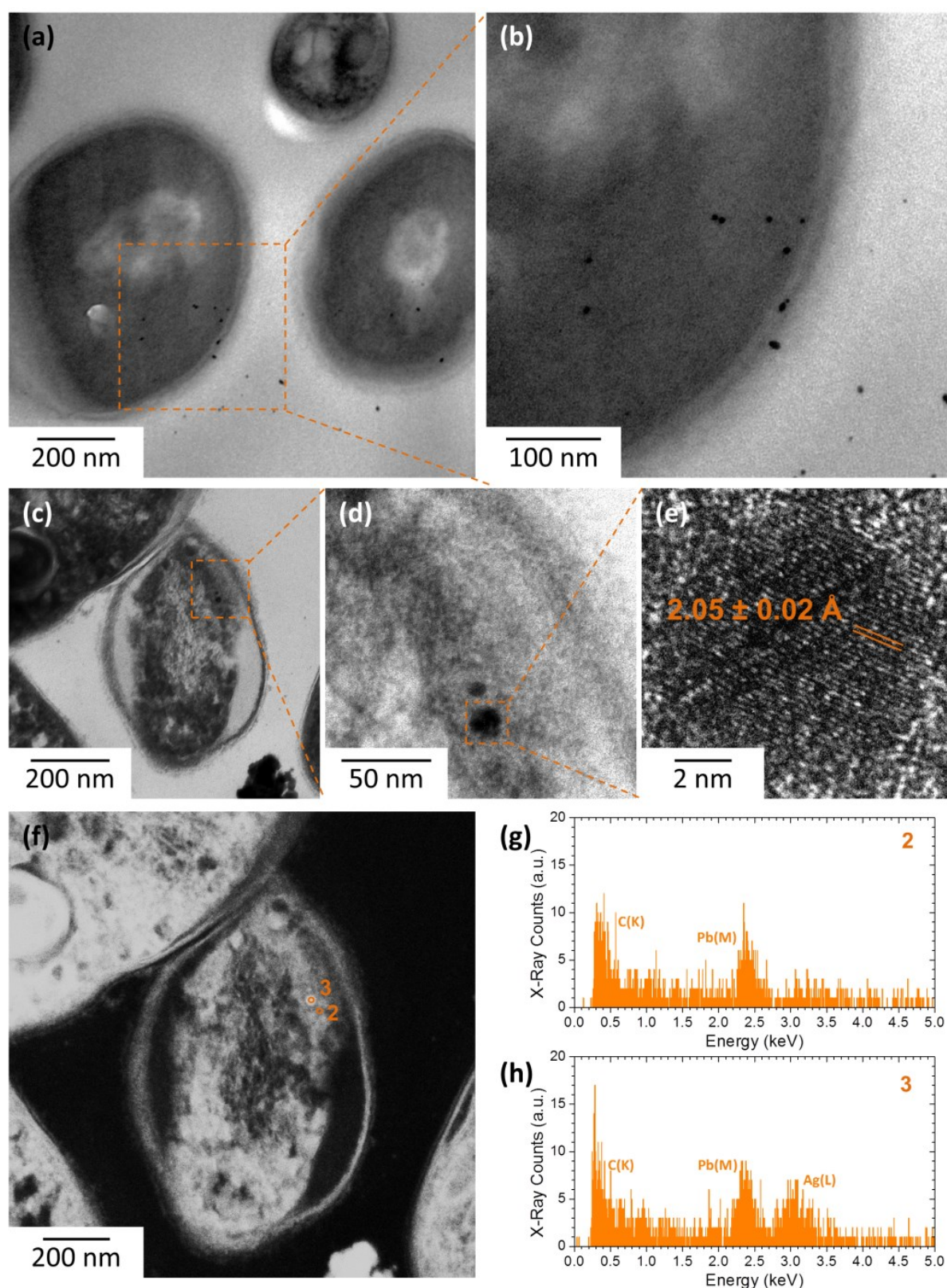


Figure 5.7 (a-d) BFTEM images of *M.tb* bacterial cells treated with 10 $\mu\text{g/mL}$ of AgNPs for 24 hours, showing AgNPs translocating across the cell wall and inside the cell cytosol. (b) and (d) are magnifications of the areas squared in (a) and (c), respectively. (e) HRTEM image collected from the area squared in (d). (f) HAADF-STEM image collected from the same area as (c). (g, h) STEM-EDX spectra collected from the areas marked in (e).

5.3.3 Treatment of THP-1 cells with AgNPs

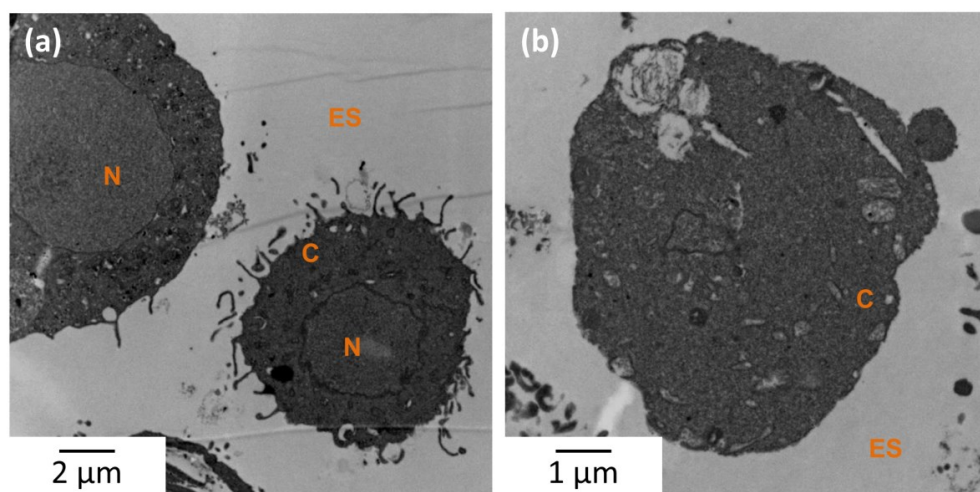


Figure 5.8 (a, b) BFTEM images of control (non-treated) THP-1 cells. N: nucleus, C: cytoplasm and ES: extracellular space.

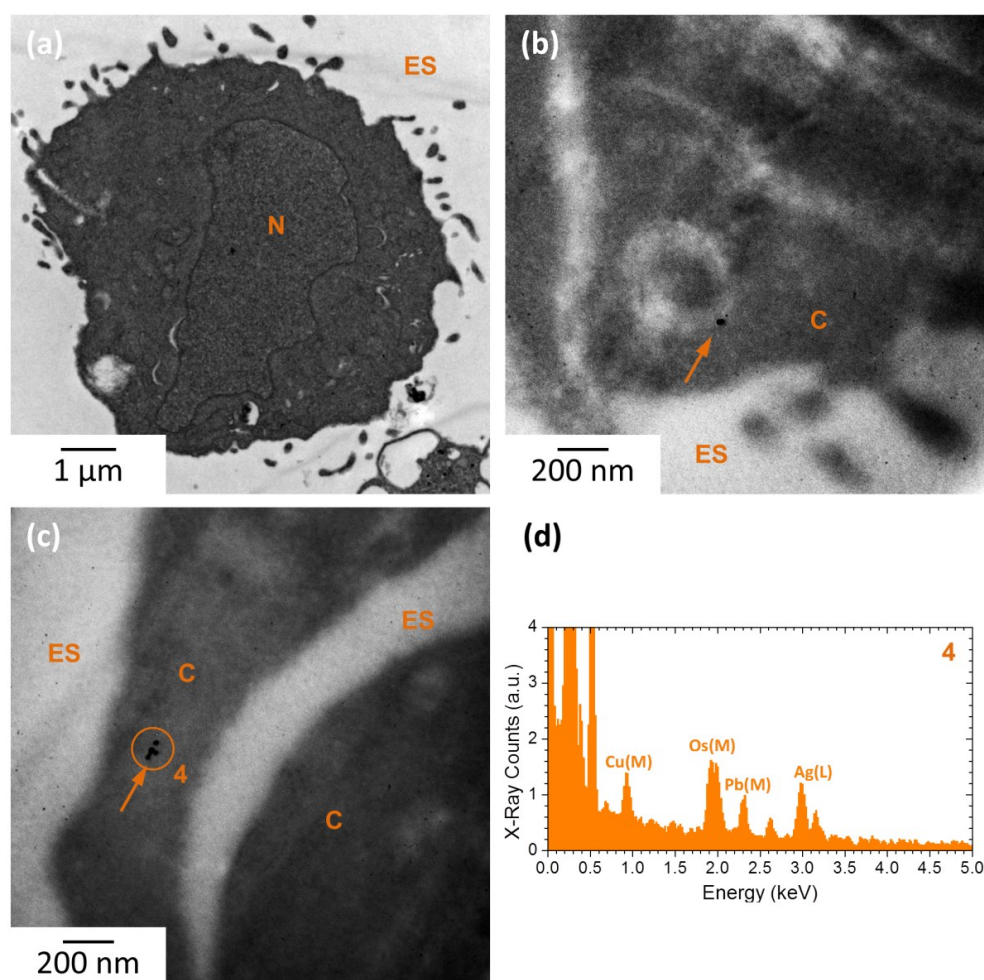


Figure 5.9 (a-c) BFTEM images of THP-1 cells treated with 10 $\mu\text{g/mL}$ of AgNPs for 24 hours. AgNPs were observed in the cytoplasm of THP-1 cells (b and c, arrows) (d) TEM-EDX spectrum collected from the area marked in (c). N: nucleus, C: cytoplasm and ES: extracellular space.

Prior to examining the potential antibacterial effects of AgNPs in *M.tb*-infected cells, THP-1 macrophages were separately treated with AgNPs or *M.tb*. **Figure 5.9a-c** shows BFTEM images of THP-1 macrophages exposed to 10 µg/mL of AgNPs. The viability and cytotoxicity of AgNPs on THP-1 cells were assessed by collaborators at the Rutgers School of Public Health using a water soluble tetrazolium salts (MTS) assay, which measures the metabolic activity of cells, and lactate dehydrogenase (LDH) release assay, which is associated with cellular membrane damage. No significant AgNP-mediated cytotoxicity was observed in THP-1 macrophages exposed to 0-50 µg/mL AgNPs for 4 or 24 hours (data not shown). Since AgNPs were not toxic to the cells at any of the doses tested, a dose of 10 µg/mL was selected in order to correspond to the dose used in AgNP-treated *M.tb*. In accordance to viability measurements, no morphological changes were observed in AgNP-treated THP-1 macrophages (**Figure 5.9a**) compared to control (non-treated) cells (**Figure 5.8**). BFTEM imaging revealed the presence of electron dense material in the cytoplasm of THP-1 macrophages (**Figure 5.9b, c**, arrows). TEM-EDX spectra (**Figure 5.9d**) collected from the areas around this material showed the presence of Ag, indicating that these particles correspond to AgNPs. TEM data showed that a limited amount of AgNPs was present intracellularly in THP-1 macrophages. This observation could be due to the fact that, at the time-point of the experiments (24 hours), AgNPs had already dissolved in the acidic environment of lysosomes.⁷⁹ Alternatively, uptake of the AgNPs by THP-1 cells may be limited. The pathways by which NPs enter cells may vary widely depending on the cell type, chemical composition of the NP surface,²⁰² particle size and agglomeration state.¹⁶⁴ Haase *et al.* suggested that THP-1 macrophages may internalize AgNPs through both phagocytic and non-phagocytic mechanisms.¹⁶⁴ Using TEM imaging, they showed aggregated AgNPs in vesicles but also individual particles distributed throughout the cytoplasm without being surrounded by membrane envelopes. The authors speculated that aggregated AgNPs were incorporated via phagocytosis while single AgNPs entered cells via non-phagocytic routes. Moreover, the surface charge of NPs may largely influence the uptake mechanism by cells. In general, due to the negatively charged character of the cell plasma membrane, anionic NPs, such as the AgNPs used in the present work, are internalized less efficiently than neutral or cationic NPs.²³⁴

5.3.4 Infection of THP-1 cells with *M.tb*

In order to visualize the damage induced by *M.tb* infection in THP-1 macrophages, cells were infected with a MOI of 10 for 24 hours. **Figure 5.10** shows that the cell infection was successful, with several bacilli residing intracellularly (arrows). These bacilli were found in membrane-bound endosomal/lysosomal-like vesicles (**Figure 5.10b-d**). Macrophages are known to be the main reservoir of infection by *M.tb*.³⁰³ After phagocytosis by the macrophage, *M.tb* bacilli are taken up in phagocytic vacuoles. Their ability to manipulate the phagosome where they reside and prevent the

normal maturation of this organelle into an acidic, hydrolytic compartment, enables their survival within cells. As shown in **Figure 5.11a-b** severe damage was observed in THP-1 cells following infection. The cells appeared necrotic, with no visible cell membrane and release of cell organelles in the extracellular space. Mycobacteria are known to be retained within phagocytic vacuoles until the host cell dies through necrosis or apoptosis.³⁰³ These two forms of cell death may have drastically different outcomes for the course of infection.⁴⁷⁸ Apoptosis (programmed cell death) results in the ordered degradation of cellular contents and the formation of apoptotic vesicles. It has been demonstrated that the apoptotic pathway is directly associated with mycobacterial killing.⁴⁷⁹ On the other hand, necrotic cell death is the disordered, energy independent death of the cell. *M.tb* has been suggested to interfere in processes linked to cell apoptosis, with a necrosis-like form of death taking place instead, which allows the release of viable mycobacteria for subsequent re-infection.⁴⁸⁰ This hypothesis is further supported by the observed structure of mitochondria in *M.tb*-infected cells. **Figure 5.11d-e** shows morphological perturbations in the ultrastructure of mitochondria in *M.tb*-infected cells compared to the mitochondria of control cells (**Figure 5.11c**), with increased electron density and clear definition of the cristae. Similar findings have been reported by Jamwal *et al.* who suggested that these structural changes indicate an augmentation in the functional activity of mitochondria, which could be linked to an inhibition of the apoptotic response by *M.tb*.³⁰⁶

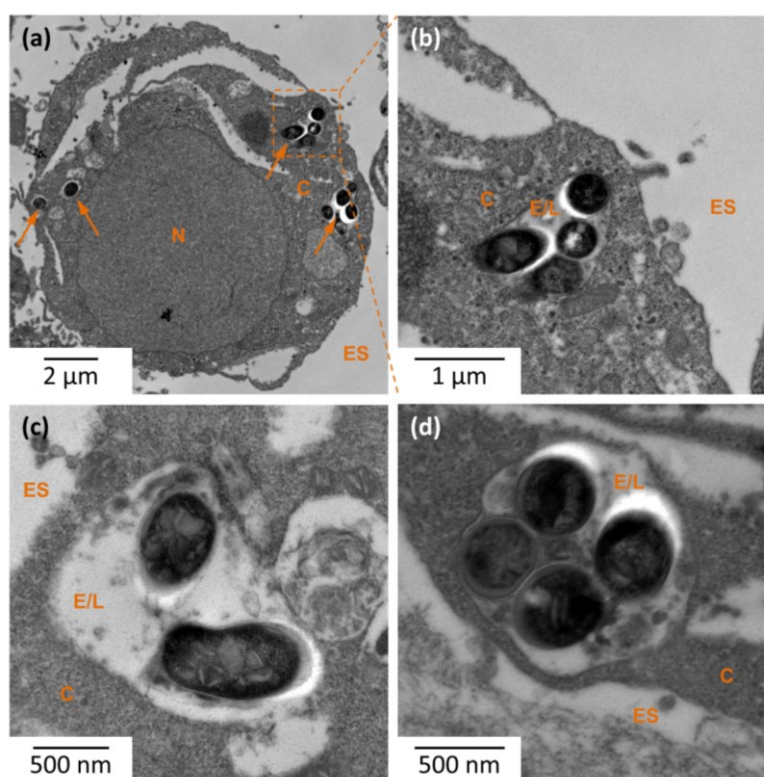


Figure 5.10 (a-d) BFTEM images of THP-1 macrophages after a 24 hour infection with *M.tb* (MOI10). (b) is the magnification of the area squared in (a) The bacilli can be seen contained within membrane-bound vacuoles in the host cell (a-arrows, b, c, d). N: nucleus, C: cytoplasm, E/L: endosome/lysosome and ES: extracellular space.

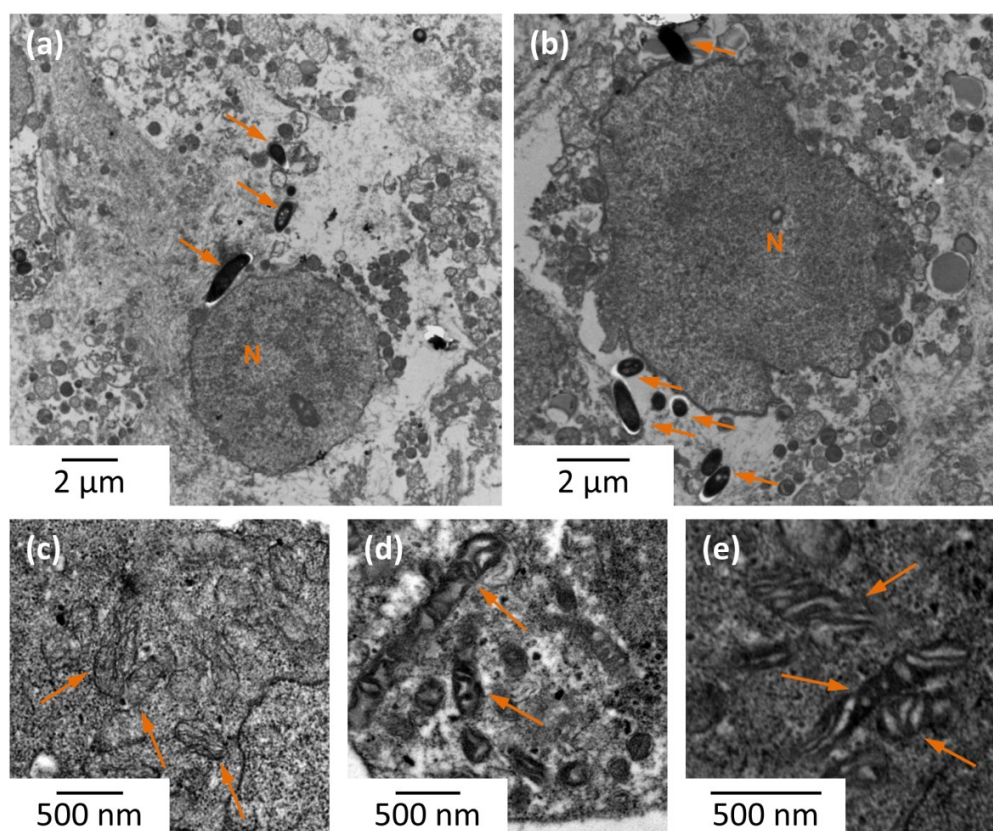


Figure 5.11 (a-b) BFTEM images of THP-1 macrophages after a 24 hour infection with *M.tb* (MOI10), showing a necrotic cell death. N: nucleus. *M.tb* bacilli are indicated by arrows. (c-e) BFTEM images of mitochondria (indicated by arrows) in control (non-treated) THP-1 macrophages (a) and THP-1 macrophages infected for 24 hours with *M.tb* (MOI10).

5.3.5 Simultaneous treatment of THP-1 cells with *M.tb* and AgNPs

Having examined THP-1 macrophages separately treated with AgNPs or *M.tb*, THP-1 cells were simultaneously exposed to both AgNPs and *M.tb* to investigate possible antimicrobial effects of the AgNPs in infected macrophages. Similar to findings described above, THP-1 cells were infected by *M.tb*, which were found in membrane-bound vacuoles (**Figure 5.12a-b**). Severe damage was observed in THP-1 cells, including cell swelling (**Figure 5.12c**) and cell death reminiscent of necrosis (**Figure 5.12d**). Since AgNP treatment alone had not caused any morphological alterations to THP-1 cells (**Figure 5.9a**), these effects may be attributed to *M.tb* infection. However, the structure of *M.tb* bacilli was also compromised. Bacteria found in the extracellular space presented damage similar to what was observed with *M.tb* exposed to AgNPs (**Figure 5.13a**). Damage was particularly evident for bacteria close to AgNPs (**Figure 5.13c, d**), with leakage of bacterial intracellular content (**Figure 5.13d**, arrow). Morphological changes were also observed in bacteria residing in the endosomes of host cells (**Figure 5.13b**). Compared to their morphology in the absence of AgNPs (**Figure 5.10b-d**), an evident disorganization of the cell wall (**Figure 5.13b**, arrow) and the appearance of an electron

light region were observed in the presence of AgNPs. Damage in intracellular bacteria could be due to a simultaneous internalization of both *M.tb* bacilli and AgNPs by THP-1 cells, as shown in **Figure 5.13e**. TEM-EDX (**Figure 5.13g**) confirmed the presence of Ag in the particles close to the bacterial cell, while the observed coarsening of the particles may be indicative of a dissolution process close to the acidic environment of the bacterial cell wall. Alternatively, AgNP uptake may have taken place after infection of THP-1 cells by *M.tb*. **Figure 5.14a, f** shows an infected macrophage, with bacilli in phagocytic vesicles. AgNPs were found close to the plasma membrane of the macrophage, as well as in the cell cytoplasm (**Figure 5.14b**). Moreover, AgNPs were observed close to an *M.tb*-containing vacuole (**Figure 5.14c, g**), with AgNPs possibly interacting with the bacterial cell wall (**Figure 5.14d, h**). HRTEM imaging (**Figure 5.14e**) revealed a lattice spacing of 2.37 Å, which is close to the (111) interplanar spacing of metallic silver (ref. #01-087-0597). STEM-EDX analysis (**Figure 5.14h**) revealed only presence of Ag in the EDX spectrum, confirming that the observed particles correspond to metallic AgNPs.

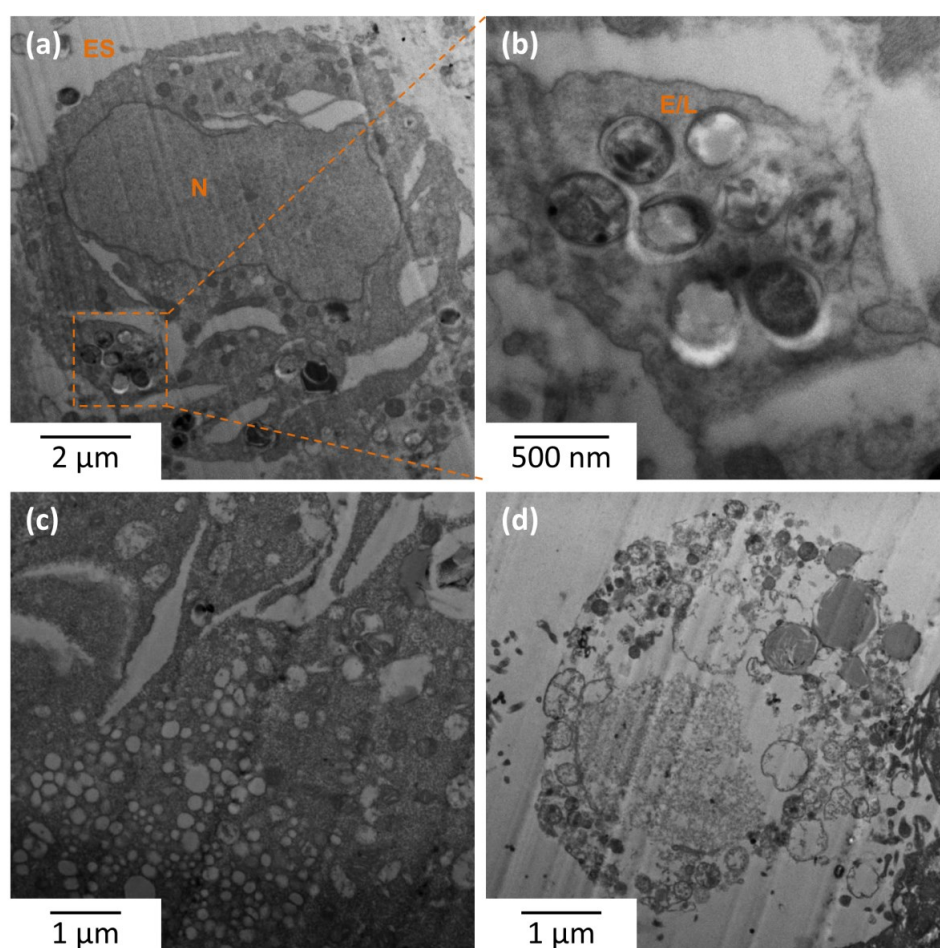


Figure 5.12 (a-d) BFTEM images of THP-1 macrophages after a 24 hour simultaneous infection with *M.tb* (MOI10) and treatment with 10 μg/mL AgNPs. (b) is the magnification of the area squared in (a) The bacilli can be seen contained within membrane-bound vacuoles in the host cell (a, b), while morphological alterations included cell swelling (c) and necrosis (d). N: nucleus, E/L: endosome/lysosome and ES: extracellular space.

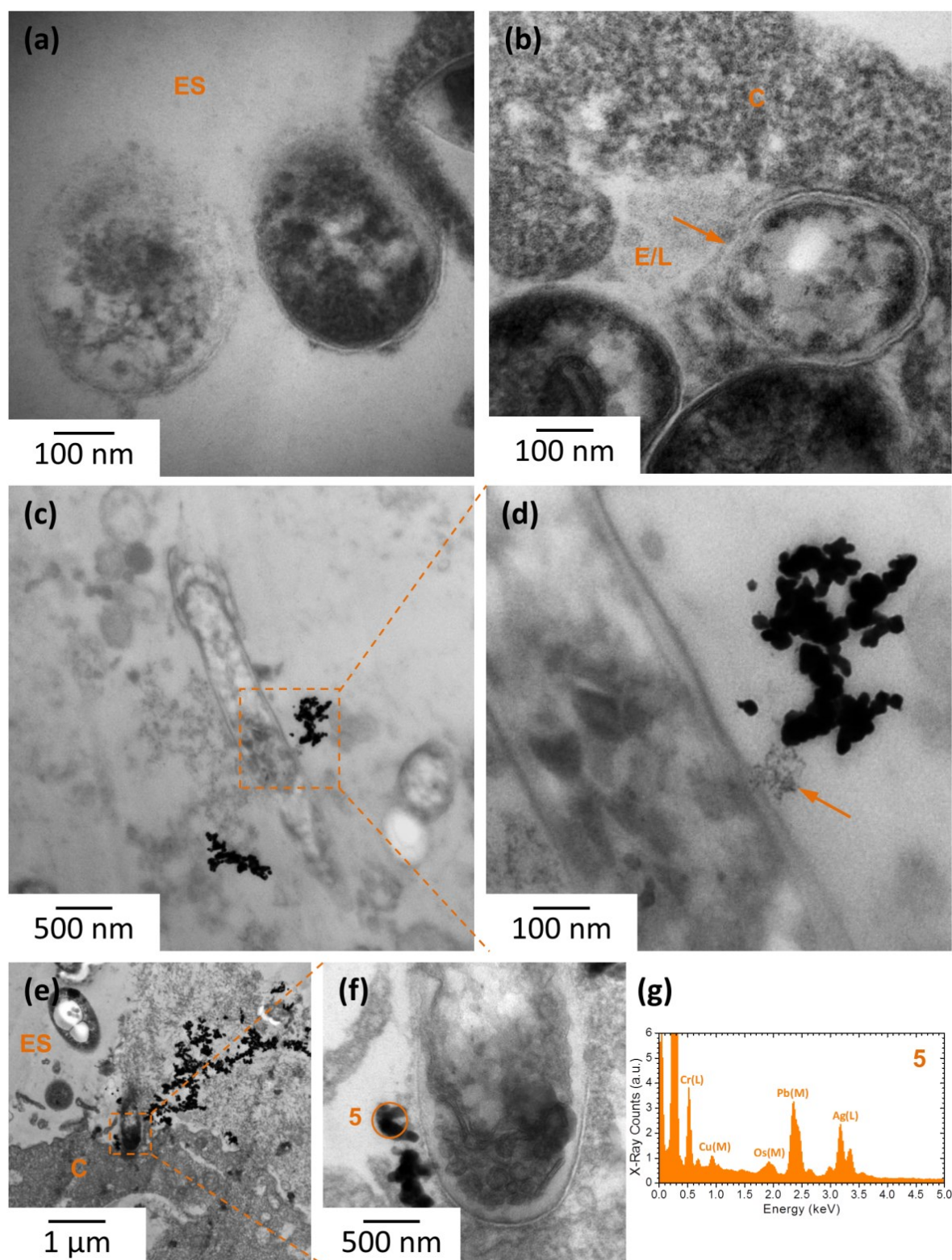


Figure 5.13 (a-f) BFTEM images of *M.tb* bacilli after a 24 hour simultaneous infection with *M.tb* (MOI10) and treatment with 10 μ g/mL AgNPs. (d) is the magnification of the area squared in (c). Morphological alterations in bacilli in the extracellular space (a, c, d) and in phagocytic vacuoles (b). (e, f) Simultaneous uptake of *M.tb* and AgNPs by THP-1 macrophages. (g) TEM-EDX spectrum collected from the area marked in (f). C: cytoplasm, E/L: endosome/lysosome and ES: extracellular space.

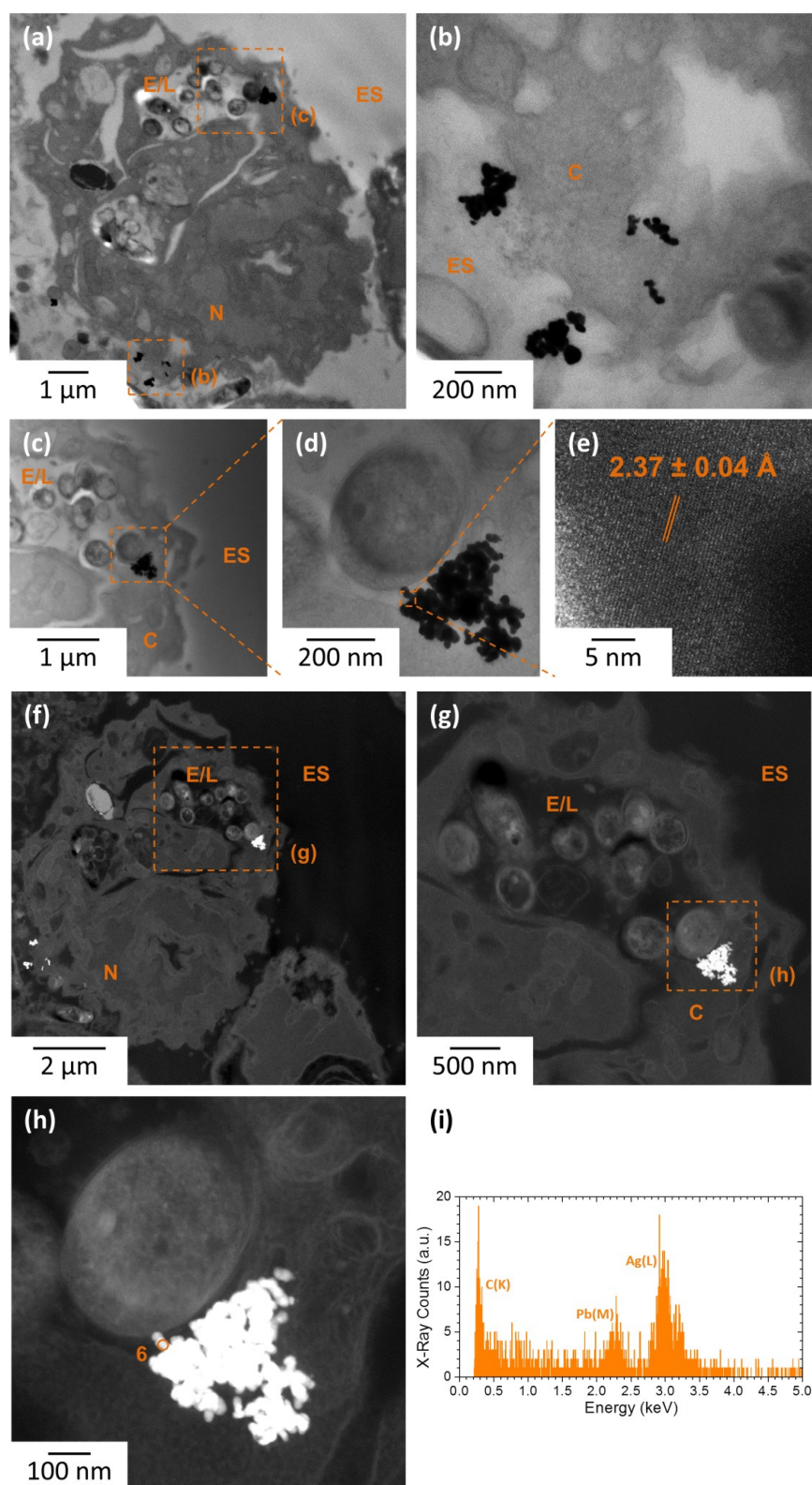


Figure 5.14 (a-d) BFTEM images of THP-1 macrophages after a 24 hour simultaneous infection with *M.tb* (MOI10) and treatment with 10 $\mu\text{g/mL}$ AgNPs, showing bacilli in phagocytic vacuoles (a) and internalization of AgNPs (b-d). (b, c) are magnifications of the corresponding areas squared in (a). (d) is the magnification of the area squared in (c). (e) HRTEM image collected from the area squared in (d). (f-h) HAADF-STEM images collected from the same area imaged in (a). (g, h) are magnifications of the corresponding areas squared in (f). (i) STEM-EDX spectrum collected from the area marked in (h). N: nucleus, C: cytoplasm, E/L: endosome/lysosome and ES: extracellular space.

5.4 Discussion

The data presented in this work has provided evidence for the antibacterial activity of AgNPs against *M.tb*. In terms of anti-TB drug development, this study suggests that AgNPs may represent useful candidates for therapeutic systems. The antibacterial properties of AgNPs against a wide range of Gram-positive and Gram-negative bacterial strains are being investigated.⁴⁸¹ However, these properties have not been previously explored in the case of an *M.tb* strain. Zhou *et al.* reported antibacterial effects of 20-30 nm citrate-coated AgNPs on bacillus Calmette-Guérin (BCG), which is a strain of the attenuated *bovine* tuberculosis bacillus, *Mycobacterium bovis*, by recording fluorescent protein expression levels.³²⁹ Treatment of BCG with 1-10 µg/ml of AgNPs caused a similar reduction in cell viability as an antibiotic positive control (hygromycin). This dose range is close to the dose shown here to induce a 50% reduction in *M.tb* growth. However, no further mechanistic data were provided, while data obtained in other mycobacteria may not be representative of *M.tb*, which has unique structure and functions. In the present work, AgNPs were toxic to *M.tb*, with a wide range of ultrastructural changes shown by TEM, such as cell wall disorganization, leakage of intracellular contents and possible DNA damage. Analytical TEM techniques revealed that AgNPs interacted with the bacterial cell wall, which is considered as one of the first points of attack and important for their antimicrobial activity.⁴⁸¹ This activity is currently thought to rise from a synergistic effect, where Ag⁺ ions are required for their antibacterial effects, but cell-NP contact increases Ag⁺ release and the amount of cellular uptake of Ag⁺ ions.^{194, 195} Recent studies have hypothesised that dissolution at the cell membrane is the primary mechanism of action of AgNPs.²⁹³ Here, the aggregation and coarsening of AgNPs observed close to the cell wall, indicative of a dissolution process,⁷⁹ provides direct evidence to this hypothesis. Several studies have also suggested that a change in cell wall permeability leads to the penetration of particles inside the cytoplasm.⁴⁸¹ In the present work, high-resolution and analytical TEM indicated that AgNPs were internalized by *M.tb*, but only smaller non-aggregated particles were found in the cytoplasm. Therefore, further research is required in order to completely understand the effects of particle size and aggregation in the interaction of AgNPs with the cell wall of *M.tb* and their internalization in the cytoplasm. Determining the optimal particle size for enhanced interaction between AgNPs and bacteria will enable to increase their antibacterial activity with the minimum applied dose.

TEM imaging showed that in THP-1 macrophages exposed to AgNPs, no morphological changes in the cell structure were observed for the dose and time point tested. Moreover, preliminary cell viability data provided no evidence of cytotoxicity of AgNPs against THP-1 cells. In the literature, the toxicity of AgNPs has been reported, with toxicological outcomes including oxidative stress, lipid peroxidation, inhibition of mitochondrial activity, damage of DNA, and cell apoptosis.⁵⁰ Therefore, more detailed studies combining viability assays and imaging are needed in order to

understand the effects of AgNP exposure to alveolar macrophages. The biggest challenge in future studies will be to determine a “therapeutic window”, where AgNPs can be used as antibacterial therapeutics for infected cells with minimum effects on the metabolism of healthy cells.³¹¹ The low toxicity of AgNPs to THP-1 cells observed here may be correlated to the low amount of AgNPs detected intracellularly by TEM. Previous studies have demonstrated that the amount and rate of uptake of AgNPs by cells can differ by several orders of magnitude depending on the size, shape, aggregation state surface charge, and surface functionalization of the particles.⁵⁰ These properties may also affect the pathway through which AgNPs are internalized within cells, with important implications for intracellular trafficking and the activity of particles against bacteria. In TB drug design, it is important to target therapeutics not only to specific cell types but also to the correct sub-cellular compartments. Therefore, in future studies, a more comprehensive investigation on the cellular uptake and localization of AgNPs is required, using particles of different sizes and surface properties. Further research is also necessary in order to identify appropriate molecular targets of the disease, which could lead to the design of AgNPs coated with ligands that target specific receptors. Also, since THP-1 is an immortalized cell line, the extent to which THP-1 cells mimic macrophages *in vivo* is not entirely known. Hence, future work could be extended to other cell types such as primary macrophages.

Finally, TEM of THP-1 cells simultaneously exposed to both AgNPs and *M.tb* provided evidence of bacterial damage, both in the extracellular space as well as inside phagosomal vacuoles. The preliminary data provided here indicate that this damage may be due to interaction between AgNPs and *M.tb* bacilli residing in vacuoles, following internalization of the particles by THP-1 cells. However, as Ag⁺ ions alone are also toxic to bacteria, bacterial degradation may have also occurred due to dissolution of the AgNPs, either extracellularly or intracellularly. Therefore, to elucidate separate effects of particles and ions, further investigation is needed using a Ag ionic control. To build upon the present findings, the cell culture system could also be improved by applying AgNPs to macrophages pre-infected by *M.tb*, instead of simultaneous exposure. Moreover, along with imaging, biological assays may be employed to provide quantitative information on the bacterial ingestion and killing by macrophages in the absence and presence of AgNPs. Nonetheless, the present work shows that the use of analytical TEM techniques can provide invaluable information of the localization, morphology and chemistry of AgNPs. In forthcoming studies, spatially resolved maps of the anatomical distribution and physico-chemistry of AgNPs at the sub-cellular level and observation of changes in the cellular ultrastructure will be pivotal in the development of effective and safe AgNP platforms for therapeutic treatment.

5.5 Conclusions

In summary, AgNPs have been shown to represent useful candidates for the development of TB therapeutic systems. AgNPs induced a wide range of ultrastructural damage to *M.tb*, including cell wall disorganization, leakage of intracellular contents and possible DNA damage. This was probably mediated by interaction of AgNPs with the bacterial cell wall, through a synergistic particulate and ionic effect. Further investigation is however necessary in order to completely elucidate these effects. Bacterial damage was also demonstrated when AgNPs were concurrently applied with *M.tb* infection in THP-1 macrophages. Preliminary TEM data indicated that one possible mechanism could be the interaction of AgNPs with *M.tb* bacilli in vacuoles, following internalization of the AgNPs by THP-1 cells. In conclusion, the findings presented here could be used to guide the design of further studies into the design of AgNP-based therapeutics.

6 Effect of Pulmonary Surfactant on the Dissolution, Stability and Uptake of ZnONWs by Human Respiratory Epithelial Cells

6.1 Introduction

Along with AgNWs, the unique properties of ZnO have placed ZnO nanowires (ZnONWs) at the forefront of application-driven nanotechnology, for optoelectronic devices (*e.g.* light emitting diodes, laser diodes, solar cells), energy harvesting devices, sensors and catalysts.³⁴⁷ The high-sensing capability and high electron mobility of ZnONWs are being explored for various biomedical applications, such as biosensors for intracellular measurements.^{482, 483} In particular, efforts are underway to use ZnONWs for cancer detection and therapy.⁴⁸⁴⁻⁴⁸⁶ Due to the antibacterial properties of ZnO,^{374, 487} ZnONWs also have great potential for the fabrication of medical devices to prevent antimicrobial infections³⁷⁰ including antimicrobial facemasks and tissues.^{368, 371} Therefore, inhalation of ZnONWs represents a key exposure route to investigate, both from the perspective of intentional (diagnosis/therapy) and unintentional (hazard) scenarios.

Although inhalation of 1D-NMs has raised particular concerns for adverse health effects, the effects of ZnONWs on human health upon exposure are currently unclear.³⁷⁶ In the alveoli, where removal is dominated by relatively slow, macrophage-mediated clearance,⁴³⁸ build-up of ZnONWs would potentially result in high alveolar concentrations. Ultrafine ZnO is already known to cause pulmonary inflammation in humans.^{351, 352} *In vivo* inhalation of ZnO nanoparticles (ZnONPs) induced systemic inflammation, as well as cardiac inflammation and fibrosis.³⁶⁰ The toxicity of ZnONPs has been reported for various *in vitro* mammalian systems,³⁷⁶ including human bronchial epithelial cells (BEAS-2B).^{378, 379} Their cytotoxicity was mediated through reactive oxygen species (ROS) generation

and oxidative stress,^{378, 379} apoptosis^{380, 381} and/or genotoxicity.^{382, 383} However, there is a paucity of research regarding the bioreactivity of ZnONWs in the lungs.^{244, 380} In a previous work, ZnONWs were toxic to human monocyte derived macrophages (HDMs) at similar concentrations as ZnCl₂.²⁴⁴ A rise in intracellular Zn²⁺ concentrations was correlated to cell death, showing that ZnONW toxicity was due to a local release of Zn²⁺ inside cells rather than the high-aspect nature of the wires. Therefore, the bioreactivity of ZnONWs with the pulmonary epithelium needs to be further investigated, in order to uncover their toxic potential, and enable the design of efficient systems for biomedical applications.

More importantly, the effects of the lung lining fluid (LLF), which represents a first line of defence of the lungs against inhaled NMs, on the physicochemical properties of ZnONWs and the consequences on their bioreactivity have not been sufficiently studied.^{439, 488} The main component of the LLF is pulmonary surfactant (PS), which forms a monolayer at the air-liquid interface. As has already been demonstrated in **Chapter 4**, AgNWs incubated with PS were coated by a phospholipid corona, which delayed their oxidative dissolution and inhibited aggregation. However, no data exist in the literature on the stability of ZnONWs in the PS, or their subsequent bioreactivity with alveolar epithelial cells.

Given the central role of dissolution in the bioreactivity of ZnONMs and the fact that several factors may affect dissolution (*e.g.* size,⁴²⁷ shape,⁴¹² pH,⁴²⁷ complexing anions^{428, 431}, organic components^{378, 427}), characterization of ZnONWs at the local *point of exposure* in cellular microenvironment is essential in order to draw accurate conclusions about their bioreactivity *in situ*. The purpose of the present work was to examine the impact of PS on the stability of ZnONWs and the subsequent effects on their interaction with alveolar epithelial cells. Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES), dynamic light scattering (DLS), zeta-potential and analytical transmission electron microscopy (TEM) techniques were combined to investigate the dissolution kinetics and colloidal stability of ZnONWs. The effects of PS were compared to those of serum, to investigate whether they are unique to the PS or general to a particle corona. The cellular toxicity of ZnONWs with two lengths (30 nm × 1 and 3 μm) was studied in relation to cell viability of human alveolar epithelial type 1-like cells (TT1).⁴⁵³ Alveolar epithelial type 1 cells (AT1) cover 95% of the total alveolar surface, and are therefore susceptible to inhaled nanosized particles. Alveolar epithelial type 2 cells (AT2) synthesize and secrete PS. As AT1 cells are not commercially available, a unique TT1 cell line was used, generated from immortalised primary human AT2 cells and showing characteristics of AT1 cells.⁴⁵³ Recent findings also suggest that TT1, but not AT2 cells, govern uptake and potential translocation of inhaled NPs across the pulmonary epithelial barrier.⁴⁶⁵ The bioreactivity of ZnONWs was compared to exposure to ZnCl₂, as well as ZnONWs pre-incubated with a model PS. A combination of TEM and ICP-OES were used to monitor and quantify the amount of particle uptake by cells. Finally, the kinetic intracellular dissolution of ZnONWs and its correlation

to dynamic cell death were investigated by confocal microscopy of live TT1 cells exposed to ZnONWs.

6.2 Methods

6.2.1 Materials

Short zinc oxide nanowires (S-ZnONWs, 90 nm×1 µm, dry powder), long zinc oxide nanowires (L-ZnONWs, 300 nm×4-5 µm, dry powder), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, semisynthetic, ≥99%), RPMI-1640 cell culture medium and Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Sigma-Aldrich, UK. Propidium Iodide (PI), Hoechst 33342 and FluoZin™-3, AM were purchased from Invitrogen, UK. Curosurf® was kindly donated by Chiesi Pharmaceuticals, Cheadle, UK. Stock solutions of ZnONWs were prepared by briefly sonicating 10 mg of powders in 1 mL of deionized (DI) water. Fresh stock solutions were prepared at the beginning of each experimental set.

6.2.2 ZnONW stability and dissolution experiments

6.2.2.1 ZnONW incubations

ZnONWs were incubated at a concentration of 25 µg/mL from a 10 mg/mL stock solution in a temperature-controlled dri-block incubator at physiological temperature (37 °C), for 0-48 hours. Accurate dosimetry in laboratory evaluations of the effects of inhaled particles has been a subject of concern, but very few data on ZnONMs are currently available to evaluate realistic occupational and consumer exposures.⁹¹ One study used the multiple-path particle dosimetry (MPPD) model to estimate nanomaterial masses retained in the human lung, as a function of diameter and aspect ratio, for short- and long-term exposure durations (24-hour and 45-year working lifetime).⁴⁸⁹ Based on that study,⁴⁸⁹ the dimensions of the ZnONWs used in the present work and a 5 mg/m³ exposure (corresponding to the average threshold limit value (TLV) for ZnO recommended by the Occupational Safety and Health Administration (OSHA, USA) and the National Institute for Occupational Safety and Health (NIOSH, USA)), the alveolar mass retained per alveolar surface was estimated to be in the order of 1-2.25 µg/cm² for a 24-h exposure and in the order of 100-225 µg/cm² for a working lifetime exposure. Assuming that the NM mass retained at the lung surface can be correlated to NM mass sedimented on the bottom surface of a well in *in vitro* experiments,⁴⁸⁹ these values correspond to doses of 1.25-3.6 µg/mL and 160-360 µg/mL, respectively. Therefore, a

concentration representative of the lower-end of these ranges was used for these experiments. Moreover, the dose of 25 µg/mL was selected in order to provide direct comparisons with the work on the effects of LLF on AgNWs (**Chapter 4**).⁴⁸⁸

The presence of complexing anions such as PO_4^{3-} in cell culture media or simulated body fluids is expected to lead to the precipitation of insoluble zinc compounds, which would confound the measurement of free Zn^{2+} ions in solution.⁴³¹ Therefore, normal saline (NS) solution (0.9% Sodium Chloride (BDH, 99.5%) in DI water) was selected as the dispersion medium. The pH of the buffers was adjusted to 7 or 5, using either Hydrochloric Acid (HCl, Sigma-Aldrich, 37%) or Sodium Hydroxide (NaOH, Sigma-Aldrich, anhydrous, 99.99%). These pH values were selected to simulate characteristic environments found in the lung.

To study the effects of PS on the stability of ZnONWs, two model surfactants were used, DPPC and Curosurf[®]. A stock solution of DPPC (10 mg/mL) in DI water was prepared by magnetic stirring overnight. DPPC and Curosurf[®] were used at amounts that correspond to a total lipid concentration of 100 µg/mL, to correspond to the average values observed in the bronchoalveolar lavage fluid of healthy persons.⁴⁰ To investigate the effect of serum, ZnONWs were incubated in NS solutions (pH 7 and 5) containing 10% newborn calf serum (NCS; Invitrogen, UK).

6.2.2.2 Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES)

To determine the amount of free Zn^{2+} ions released from the incubated ZnONWs from 0 up to 48 hours, aliquots were collected at each time point and centrifuged at 13000×g for 10 minutes through 2 kDa centrifuge membranes (Sartorius Stedim VIVACON 500) to remove the ZnONWs. The amount of Zn^{2+} in the filtrates was measured by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES, Thermo Scientific, UK) with a Zn^{2+} detection limit of 5 ng/mL. Each experiment was repeated three times and the results are reported as the mean ± standard deviation (SD).

6.2.2.3 Transmission Electron Microscopy (TEM) sample preparation

To prepare TEM samples from the incubated ZnONWs, aliquots were removed at each time point and, after washing three times with DI water to remove excess salts or organic PS, they were drop cast on 300 mesh holey carbon film TEM grids (TAAB) in the dark. The grids were blot-dried with filter paper and were immediately placed under vacuum to avoid reactions induced by ambient atmosphere. To enhance phospholipid contrast, samples were positively stained with 2% uranyl acetate in DI water.

For dissolution of ZnONWs at pH 5 a different sample preparation was followed. Aliquots of as-received ZnONWs from a stock solution in DI water were drop cast on 300 mesh holey carbon film TEM grids. The grids were blot-dried with filter paper and aliquots of the pH 5 dissolution buffer, with or without DPPC/Curosurf[®]/NCS, were immediately drop cast on the grids. After 10 seconds, the grids were again blot-dried with filter paper. The same procedure was repeated with DI water 3 times, to remove excess salts or biomolecules. Finally, the grids were placed under vacuum for drying and storage.

6.2.2.4 TEM

Bright field transmission electron microscopy (BFTEM), high resolution transmission electron microscopy (HRTEM) and high angle annular dark field scanning transmission electron microscopy (HAADF-STEM), combined with selected area electron diffraction (SAED) and energy-dispersive X-ray spectroscopy (EDX) were carried out using a JEOL 2000, a JEOL JEM-2100F or an FEI Titan 80/300 TEM/STEM fitted with a Cs (image) corrector. The scattering intensity in HAADF-STEM is proportional to Z^n ($n \sim 2$), therefore this technique is highly sensitive to atomic number (Z) variations within the sample. An accelerating voltage of 200 kV was used in TEM/STEM experiments for characterization of ZnONWs and dissolution experiments, while a voltage of 80 kV was used in TEM/STEM experiments for cell imaging and analysis. For STEM experiments using the JEOL JEM-2100F, the inner and outer HAADF collection angles were 150 and 400 mrad, respectively, and the probe diameter was <1 nm. STEM experiments with the FEI Titan were performed with a convergence semi-angle of 14 mrad and inner and outer HAADF collection angles of 49 and 239 mrad, respectively, and the probe diameter was <0.5 nm. The TEM images presented and the statistics on ZnONWs were obtained by viewing several ZnONWs ($n \geq 100$ for each sample), from multiple areas of 3 samples ($n=3$) prepared under identical conditions.

6.2.2.5 Dynamic Light Scattering (DLS) and ζ -Potential

DLS and ζ -Potential measurements were performed on a Zetasizer Nano ZS (Malvern, UK) at 25 °C. To characterize as-received ZnONWs, samples were prepared in DI water (pH 6.4) or RPMI-1640 (pH 7.2), at a concentration of 25 $\mu\text{g/mL}$, and were bath sonicated for 1 minute immediately before collecting each measurement. To investigate the effect of PS and 10% NCS on the agglomeration of S-ZnONWs over time, samples were prepared at a concentration of 25 $\mu\text{g/mL}$ in DI water (pH 6.4), to exclude effects of ionic salts or other biomolecules present in cell culture media.⁵⁰ For agglomeration measurements, samples were bath sonicated for 1 minute immediately before the

t=0 measurement, but were gently mixed with a pipette for all subsequent measurements. Each measurement consisted of 10 readouts and was repeated in triplicate, with the mean $\pm$ SD reported.

6.2.2.6 Endotoxin analysis

Endotoxin assays were performed using an LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, UK). All the glassware used for the ZnONM-suspension preparation and LAL assays was used after oven sterilization at 120°C for at least 2 hours. Three separate experiments were performed (n=3) and results represent endotoxin units (EU)/mL. Endotoxin analysis was performed by Dr. Pakatip Ruenraroengsak.

6.2.3 Cell Viability and analysis of Zn²⁺ uptake

6.2.3.1 TT1 treatment with ZnONWs

The immortalized AT1-like human alveolar epithelial cell line (TT1) was created from primary human alveolar epithelial type 2 cells (AT2), isolated from lung tissue with normal appearance following surgery for lung cancer as previously described by Kempt *et al.*⁴⁵³ TT1 cells were cultured in complete DCCM-1 medium (Cadama, UK) supplemented with a mixture of 100U/100U/2mM of Penicillin/Streptomycin/L-Glutamin (SPG; Invitrogen, UK) and 10% newborn calf serum (NCS; Invitrogen, UK). Cells were seeded at 10,000 cells/well on 96 well plates (Corning, UK) and maintained at 37 °C with 5% CO₂ to reach confluence. Cells were serum starved by incubating with serum free DCCM-1 for 24 hours before ZnONM exposure. After starving, TT1 cells were rinsed twice with serum-free RPMI-1640 medium (Sigma, UK) before being exposed to ZnCl₂ or ZnONWs for 4 and 24 hours. ZnCl₂ or ZnONWs were prepared in serum-free RPMI-1640 medium, at concentrations ranging from 0-100 µg/ml and bath sonicated at 135W, 42 kHz (VWR, UK) for 1 min immediately before exposure. To assess the effect of serum on the bioreactivity of ZnONWs, S-ZnONWs were prepared in RPMI-1640 medium containing 10% NCS. To investigate the effect of PS, ZnONWs were incubated at room temperature and with agitation for 24 hours with a 1:1000 dilution of Curosurf® in RPMI 1640 culture medium. The incubated ZnONWs were centrifuged at 10000×g for 10 minutes and the pellet was washed twice with PBS by repeated cycles of centrifugation. Following washing, the pre-incubated ZnONWs were suspended in serum-free RPMI 1640 culture medium for cell exposure. Cell culture was performed by Dr. Pakatip Ruenraroengsak.

6.2.3.2 Cell viability assessment (WST assay)

Cell viability of cells exposed to ZnCl_2 , S-ZnONWs and L-ZnONWs was analysed using the cell proliferation reagent 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, (WST-1; Roche, UK). Briefly, cells were exposed to ZnONMs, 0-100 $\mu\text{g/ml}$, for 4 and 24 hours. The cells were rinsed $3\times$ with PBS and incubated with RPMI-1640 medium containing WST-1 tetrazolium salt (dilution 1:10 from stock) for 1 hour. The medium was collected and the absorbance at 450nm was measured. Cell viability was calculated in relation to the control non-treated (NT) cells and data are presented as percent viability in proportion to that of the NT-cells. Three separate experiments were performed ($n=3$) and the data are presented as mean $\pm$ SD. The WST assay was performed in collaboration with Dr. Pakatip Ruenraroengsak.

6.2.3.3 Lactate dehydrogenase assay (LDH)

After 4- and 24-hour cell exposures, the conditioned medium was collected and centrifuged at 14,000 g for 20 min to remove the cell debris and residual ZnONMs. LDH was analyzed using the Cytotoxicity Detection kit PLUS (LDH; Roche, UK). For the control and substance control, a known concentration of LDH standard (0.05 U/ml) was incubated with a sample of RPMI medium, with or without increasing concentrations of the ZnONMs, and the assay performed in an identical manner. There was no significant interference of the ZnONWs, which were removed by centrifugation prior to reading the optical density ($n=3$). The LDH assay was performed in collaboration with Dr. Pakatip Ruenraroengsak.

6.2.3.4 Quantification of intracellular Zn^{2+} by ICP-OES

To quantify the amount of Zn^{2+} taken up by TT1 cells, TT1 cells were treated with ZnCl_2 or S-ZnONWs (bare or pretreated with Curosurf[®]); the total amount of Zn added to TT1 cells for the uptake study was 25 μg (*i.e.* 1 ml of a 25 $\mu\text{g/ml}$ solution of ZnCl_2 or S-ZnONWs, prepared in RPMI-1640 culture medium). TT1 cells were exposed for 24 hours, after which the conditioned medium was removed. The cells were rinsed three times with 1 mL of fresh RPMI-1640 culture medium. The initial treatments and subsequent washes, containing the extracellular Zn, were stored at -21°C until further processing. Cells were scraped into 1 ml of fresh RPMI-1640 medium and stored at -21°C . Each experiment was repeated three times.

The intracellular amounts of Zn^{2+} were determined by ICP-OES, following acid digestion. The cell pellets were digested with 5 mL of HNO_3 (Sigma-Aldrich, 69%) overnight. The samples

were diluted in ultrapure water (Milli-Q) to a final concentration of 5%, yielding clear digest solutions, and analysed by ICP-OES.

6.2.3.5 TEM quantification of S-ZnONW uptake

Following the same treatment as described in “Quantification of intracellular Zn^{2+} by ICP-OES”, cells were rinsed with fresh RPMI 1640 culture medium and then fixed with 2.5% glutaraldehyde in 0.1M HEPES buffer, pH 7.2 for 1 hour at 4 °C.²⁴⁴ The fixatives were removed by washing the cells with 0.1 M HEPES buffer 3 times. Cells were scraped, transferred to 1.5 mL Eppendorf tubes and centrifuged at 1000×g for 20 minutes to obtain cell pellets. The cell pellets were not stained by osmium, since the staining chemicals may cause dissolution of the ZnONWs.³ The cell pellets were dehydrated in graded solutions of ethanol (50%, 70%, 95%, and 100%), for 5 minutes three times in each, and then washed three times, for 10 minutes each, in acetonitrile (Sigma). After dehydration, samples were progressively infiltrated with a Quetol-based resin, produced by combining 8.75 g quetol, 13.75 g nonenyl succinic anhydride, 2.5 g methyl acid anhydride, and 0.62 g benzyl dimethylamine (all from Agar Scientific). Samples were infiltrated in a 50% resin:acetonitrile solution for 2 hours, in a 75% resin:acetonitrile solution overnight and in 100% resin for 4 days, with fresh resin being replaced daily. The embedded samples were cured at 60 °C for 24 hours. The fixation, dehydration and embedding process had no effect on the solubility or chemistry of ZnONWs (**Appendix B, Figure 10.1**). Thin sections (90 nm) were cut from the resin blocks directly into a water bath using an ultramicrotome and a diamond knife with a wedge angle of 35°. Sections were immediately collected on bare, 300 mesh gold TEM grids (Agar Scientific), dried and stored under vacuum until TEM analysis.

6.2.3.6 Cellular dissolution of ZnONMs correlated to cell death assessed using laser scanning confocal microscopy of live cells

TT1 cells were routinely grown on glass-bottom petri dishes (μ -Dish^{35mm}, ibidi GmbH, UK) in complete medium as described above and maintained at 37 °C with 5% CO_2 to reach confluence. Cells were serum starved for 24 hours before exposure to ZnONWs. Cells were rinsed 2× with PBS and incubated with Hoechst 33342 in RPMI1640, to stain cell nuclei, at 37 °C with 5% CO_2 for 30 min. Cells were rinsed 3× with PBS and incubated with 5 μM of Zn^{2+} -selective indicator (FluoZinTM-3, AM) in serum-free RPMI, for 30min at 37 °C with 5% CO_2 . Cells were then rinsed 2× with warm PBS and fresh RPMI medium containing 10 $\mu\text{g}/\text{ml}$ of PI. Live imaging of the TT1 cells started immediately after addition of the PI-containing medium ($t=0\text{min}$), using a Leica SP5 MP (Leica, Germany) inverted confocal microscope, with an environmental chamber set at 37 °C with 5% CO_2 .

RPMI medium containing 25 µg/ml of S-ZnONWs was added immediately and the cells were imaged. FluoZinTM-3 and PI were detected at excitation/emission wavelengths of 488/516nm and 535/617nm, respectively. Images were acquired every 10 min over 3-4 hours, using a 63× objective (oil immersion). The data were analysed using ImageJ software (FIJI), and the fluorescent signals at the time before addition of the ZnONMs were measured for background subtraction of the exposed cells. The data are presented as mean fluorescent intensity (MFI) ± SD. Three separate experiments were performed for each sample. Seven different cells (n=3 with a total of 21 observed cells) were randomly selected from each experiment and their MFI values after background subtraction were analysed and plotted against time of monitoring. Confocal imaging was performed in collaboration with Dr. Pakatip Ruenraroengsak.

6.2.4 Statistical analysis

In vitro cell-free dissolution, DLS and ζ-potential experiments were repeated at least three times. Values are shown as mean ± SD. Statistical analyses were carried out using one-way ANOVA in Origin 9.1. Differences were considered statistically significant at $p < 0.05$.

Toxicity data of ZnCl₂ and ZnONWs (WST and LDH) were analysed using two-way analysis of variance (ANOVA) with a Post-Hoc (Bonferroni) test to confirm the difference between ZnCl₂, S- and L-ZnONWs at different concentrations using GraphPad Prism[®] 5.0. A similar analysis was applied to determine the difference in different treatments (± 10% NCS or Curosurf[®]) at different ZnONWs concentrations. In confocal experiments, differences in MFI values of red and green fluorescent signals over time were also analysed using 2-way ANOVA. Differences were considered significant at ** $p < 0.01$ and *** $p < 0.001$. Values are presented as mean ± SD.

6.3 Results and Discussion

6.3.1 Characterization of ZnO Nanomaterials

The as-received ZnONWs (AR-ZnONWs) were characterized by transmission electron microscopy (TEM) (**Figure 6.1**), high resolution TEM (HRTEM) (**Figure 6.1**), selected area electron diffraction (SAED) (**Appendix B, Figure 10.2**), energy dispersive X-Ray spectroscopy (TEM-EDX) (**Figure 6.1**), dynamic light scattering (DLS) (**Table 6.1**) and zeta (ζ) potential (**Table 6.1**). High-aspect ratio S-ZnONWs (**Figure 6.1a-f**) and L-ZnONWs (**Figure 6.1g-l**) had average lengths of 1.3 ± 1.1 µm (**Figure 6.1d**) and 3.3 ± 2.9 µm (**Figure 6.1j**), respectively. Both types of ZnONWs had similar diameters (**Figure 6.1b, h**), with averages of 28 ± 15 nm (**Figure 6.1e**) and 27 ± 16 nm

(Figure 6.1k), respectively. The ZnONW nominal dimensions provided by the manufacturer (90 nm×1 µm for S-ZnONWs and 300 nm×4-5 µm for L-ZnONWs) were, therefore, not representative of their actual dimensions measured by TEM. Figure 6.1c, i are HRTEM images of S-ZnONWs and L-ZnONWs with the arrows indicating the long axis of the wires. The ZnONWs were crystalline and the lattice fringe spacings were 2.58 ± 0.04 Å and 2.61 ± 0.05 Å, respectively, corresponding to the interplanar spacing of wurtzite ZnO (002) lattice planes (JCPDS #89-1397). This is in accordance to previous findings that showed that the [0001] direction is the preferred growth direction for ZnONWs.⁴⁹⁰ For both types of ZnONWs, the interplanar spacings measured from SAED patterns (Appendix B, Figure 10.2), collected from areas containing several nanowires, corresponded to the (100), (002), (102), (110) and (103) planes of wurtzite ZnO. Finally, the composition of ZnONWs and absence of impurities were confirmed by TEM-EDX (Figure 6.1f, l). Endotoxin assays indicated that ZnONW suspensions contained very low levels of endotoxin, with 0.0014 ± 0.0001 EU/mL for S-ZnONWs and 0.0009 ± 0.0002 EU/mL for L-ZnONWs.

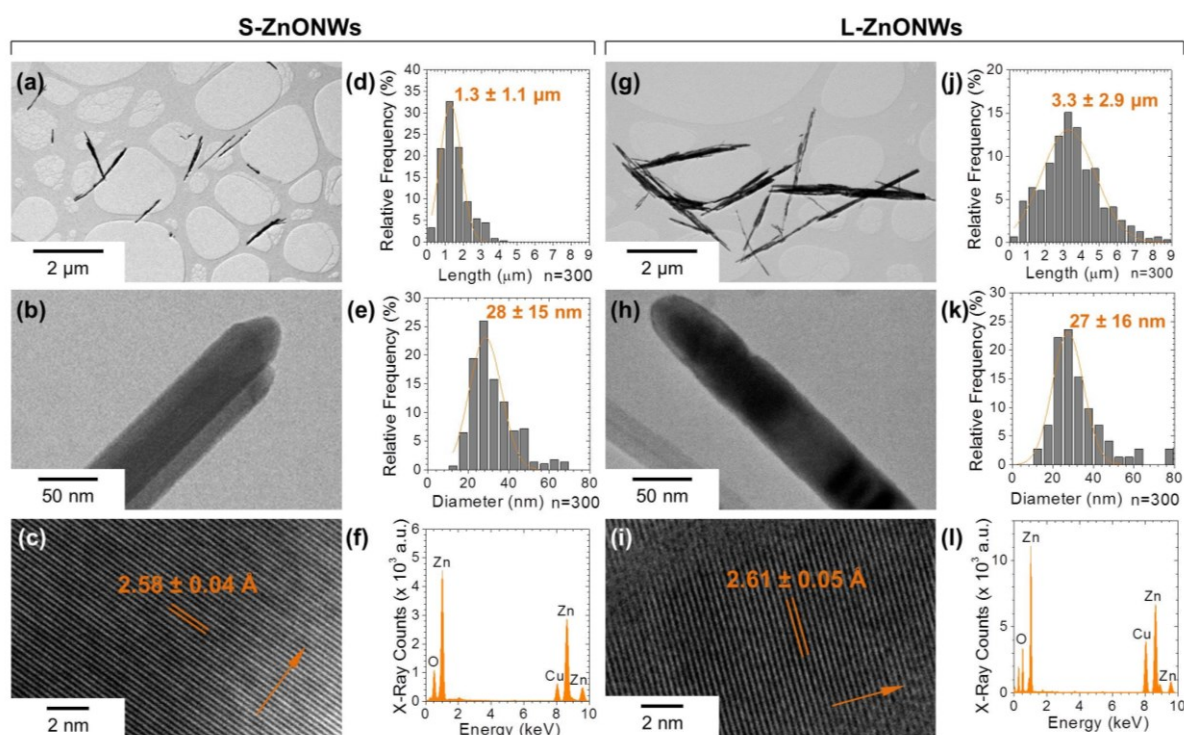


Figure 6.1 Physicochemical characterization of "short" (S-ZnONWs; a-f) and "long" (L-ZnONWs; g-l) ZnO nanowires. Low-magnification bright field transmission electron microscopy (BFTEM; a, g), high-magnification BFTEM (b, h) and high resolution TEM (HRTEM; c, i) images of S-ZnONWs and L-ZnONWs respectively, and their length (d, j) and diameter (e, k) distributions. In (c, i) the arrows represent the direction of the long axis of ZnONWs. (f, l) TEM energy dispersive X-Ray (TEM-EDX) spectra collected from areas containing multiple S-ZnONWs (f) and L-ZnONWs (l). The curves in (d, e, j and k) represent the Gaussian fit to the data.

Table 6.1 shows the hydrodynamic sizes of ZnONWs by DLS and their ζ -potentials, in DI water and RPMI-1640 cell culture medium. Although S-ZnONWs and L-ZnONWs were dispersed by bath sonication immediately before measurement, their hydrodynamic sizes were not representative of the sizes measured by TEM in either medium. This is because NP size information in DLS is obtained by measuring the particle's diffusion coefficient, which, in the case of high-aspect ratio NMs, has both a translational and rotational component. The measured sizes may therefore be interpreted only as the sizes of spherical particles that would have the same diffusion coefficient as S-ZnONWs and L-ZnONWs. The ζ -potential had similar magnitudes for both types of ZnONWs in each medium, with positive values in DI water, but which became negative in RPMI-1640.

Material	DI water (pH 6.4)			RPMI-1640 (pH 7.2)		
	Hydrodynamic size (nm)	PDI	ζ -potential (mV)	Hydrodynamic size (nm)	PDI	ζ -potential (mV)
S-ZnONWs	815 $\pm$ 104	0.35	16.0 $\pm$ 0.9	1164 $\pm$ 130	0.44	-10.4 $\pm$ 0.6
L-ZnONWs	957 $\pm$ 132	0.33	11.9 $\pm$ 0.3	1319 $\pm$ 190	0.44	-10.3 $\pm$ 0.8

Table 6.1 Hydrodynamic sizes and polydispersity indices (PDI) measured by dynamic light scattering (DLS), and ζ -potential of S-ZnONWs and L-ZnONWs in DI-water and RPMI-1640 cell culture medium.

6.3.2 PS alters ZnONW dissolution kinetics and coats ZnONWs with a phospholipid corona

First, the effects of PS on ZnONW dissolution were examined at different pH values, representative of environments found in the lung and that mimic endocytotic conditions. The main paradigm describing the toxicity of ZnONMs is the accumulation of ZnO inside cells due to cellular uptake of the ZnONMs.^{244, 397} In the endocytic pathway, the pH progressively decreases from about 7.1 to lower than 5.5 in lysosomes. Consequently, following uptake, pH-triggered intracellular dissolution may lead to the release of Zn²⁺ ions, which are toxic at elevated levels.^{244, 397} However, the effects of PS on the solubility of ZnONMs have not been previously explored. In the absence of PS, ZnONW dissolution was strongly pH-dependent (**Figure 6.2a**). At pH 7, both types of ZnONWs tested were relatively stable. The amount of Zn²⁺ ions released increased over time and reached equilibrium after 4 hours, with a maximum of about 10% of ZnO solubilized. At pH 5, there was an apparent instantaneous and complete dissolution of ZnONWs. Similarly, previous work has demonstrated an increase in ZnO dissolution at lower pH, for both spherical ZnONPs^{427, 429} and ZnONWs.^{244, 412} These findings indicate that ZnONWs will be fairly stable extracellularly; therefore their toxicity is expected to be triggered by their rapid intracellular dissolution. Furthermore, no

significant differences in the dissolution kinetics were observed between the two types of ZnONWs tested, at pH 5 or pH 7. In principle, an increased surface area, leading to a higher surface free energy and activity of NPs as their size decreases, is expected to lead to an enhanced dissolution for smaller particles.⁴⁹¹ However, a size selective etching of ZnONMs has rarely been measured experimentally, and only for very small particles (< 10 nm diameter).^{428, 492} In accordance with our findings, most previous studies have reported similar dissolution rates and equilibrium Zn^{2+} concentrations between bulk- and nano-ZnO particles^{374, 422, 423} as well as ZnONMs of different sizes and shapes⁴²⁶, which is usually attributed to aggregation of the particles. Agglomeration would lead to the formation of irregularly shaped structures and decrease the rate of particle dissolution.⁴⁹³ Due to aggregation, the reactive surface area of NMs exposed to solution is reduced, while the solution properties within the aggregates may differ from the bulk solution.

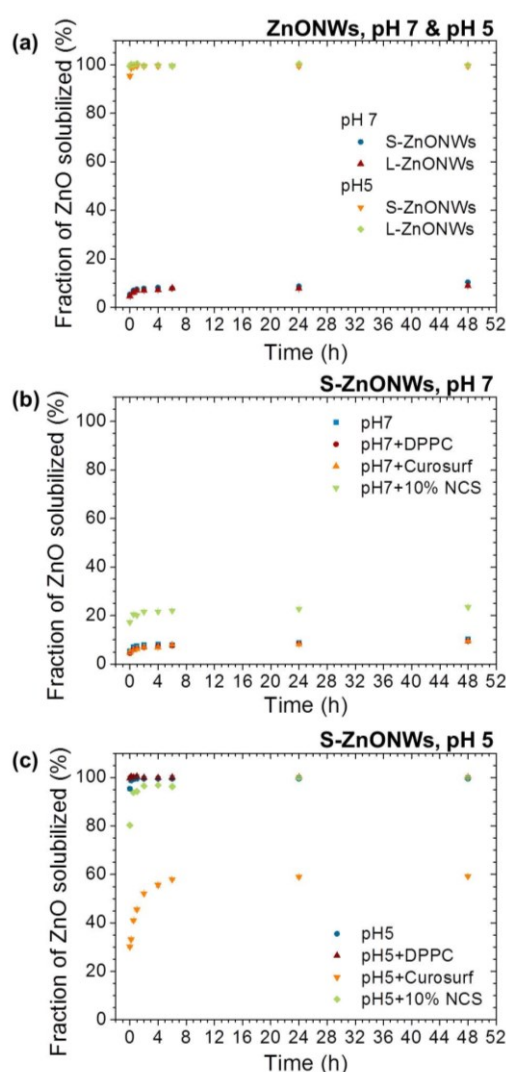


Figure 6.2 PS reduces the dissolution kinetics of S-ZnONWs at lysosomal pH. (a) Inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis of the amount of free Zn^{2+} ions released from S-ZnONWs and L-ZnONWs incubated in normal saline (NS) solutions at pH 7 and pH 5, for 0 up to 48 hours. (b, c) ICP-OES analysis of the amount of free Zn^{2+} ions released from S-ZnONWs incubated in NS at pH 7 (b) or pH 5 (c), in the absence or presence of DPPC, Curosurf[®] or 10% NCS. Each experiment was repeated 3 times (n=3) and data are presented as mean $\pm$ SD.

Since dissolution was the same for S-ZnONWs and L-ZnONWs, the effects of PS and 10% newborn calf serum (NCS) on dissolution kinetics were examined only for S-ZnONWs (**Figure 6.2b, c**). At pH 7 (**Figure 6.2b**), the presence of DPPC or Curosurf[®] had no effect on the dissolution kinetics of S-ZnONWs, as we have also demonstrated in the case of silver nanowires (AgNWs).⁴⁸⁸ In contrast, 10% NCS markedly accelerated the amounts of Zn²⁺ ions released, with the maximum fraction of S-ZnONWs solubilized reaching around 24% after 48 hours ($p < 0.05$). Previous studies have shown that ZnO NP dissolution increased in the presence of foetal bovine serum (FBS),⁴⁹⁴ as well as bovine serum albumin (BSA)⁴³⁰. This increase in dissolution may be due to the adsorption of proteins (e.g. albumin) from NCS on the surface of ZnONWs and the formation of a protein corona.⁴⁹⁵ Serum albumin could act as Zn²⁺ binding ligand, changing the reaction equilibrium and inducing a higher Zn²⁺ liberation from the ZnONWs.⁴²⁹ At pH 5 (**Figure 6.2c**), the addition of DPPC or 10% NCS did not lead to large differences in the dissolution of S-ZnONWs. A prominent decrease in dissolution was, however, observed in the presence of Curosurf[®]. Instead of immediate dissolution, only 35% of S-ZnONWs had solubilized within 1 hour and dissolution reached equilibrium at 60% after 8 hours. This finding supports the work on AgNWs, where Curosurf[®] was more effective than DPPC in delaying the dissolution of AgNWs at pH 5 (**Figure 4.2**).⁴⁹⁶ Similar to AgNWs,⁴⁹⁶ the reason for a reduced S-ZnONW dissolution could be their coating by phospholipids, which blocks direct contact between the NW surface and the aqueous environment.

The morphological alteration of S-ZnONWs in the absence and presence of PS and 10% NCS against time and pH was examined by TEM (**Figure 6.3**). After incubation at pH 7 for 1 hour, no noticeable changes were observed in the morphology of the S-ZnONWs, which still appeared to have smooth surfaces (**Figure 6.3a**), in agreement with the low dissolution measured at this time point by ICP-OES (**Figure 6.2a**). After 24 hours at pH 7, S-ZnONWs had been substantially etched and presented irregular surfaces (**Figure 6.3b**). Similar observations were made for S-ZnONWs incubated at pH 7 for 24 hours in the presence of DPPC (**Figure 6.3c**), Curosurf[®] (**Figure 6.3d**) and 10% NCS (**Figure 6.3e**). In a previous work, the flat surfaces of the hexagonal cross section of ZnO wires also became rough following their interaction with DI water.⁴⁹⁷ Since dissolution of S-ZnONWs was very rapid at pH 5 (**Figure 6.2a**), their morphology was inspected at very short incubation times. Within 10 seconds at pH 5, in the absence (**Figure 6.3f**) or presence of 10% NCS (**Figure 6.3g**) or DPPC (**Figure 6.3h**), S-ZnONWs had been severely etched. They no longer appeared as continuous wires but had dissolved to smaller individual and irregular particles. The incubation media only contained Cl⁻ ions, therefore the precipitation of insoluble Zn compounds was not expected. The lattice spacing measured from HRTEM images (**Figure 6.3i**) was 2.56 ± 0.06 Å, which corresponds to the (002) interplanar spacing of wurtzite ZnO (JCPDS #89-1397). STEM-EDX (**Figure 6.3j, k**) revealed only the presence of Zn and O, confirming that the observed structures correspond to dissolved S-ZnONWs. Similar morphological alterations have been previously reported for ZnONWs in simulated body fluids at pH 7.4 and pH 5.2.²⁴⁴ Zhou *et al.* showed that ZnO wires dissolved in a

range of biofluids.⁴⁹⁷ The etching process usually started at the edges of the hexagonal cross-section of the wires, leaving the flat surfaces initially spared and resulting in a rough appearance of the wires. The number of edges, kinks or defect sites in NMs may create “hot spots” of dissolution.⁴²⁸ Moreover, differences in surface energies of different crystallographic planes may affect dissolution rates. ZnONWs grow along their polar (0001) direction, and the top polar (002) face that is more unstable than the side crystal faces may be etched preferentially; therefore anisotropic dissolution of ZnONWs is expected.⁴¹³ However, such anisotropic dissolution has not been observed here. Illy *et al.* suggested that diffusion limitations on the dissolution process and chloride stabilization of the (002) planes must also be taken into account to explain the selective etching of ZnONWs.⁴¹² In their work, anisotropic dissolution was observed for ZnO nanorods with a diameter >100 nm, whereas nanorods with a diameter <100 nm, like the ZnONWs used in this work, dissolved homogeneously.⁴¹²

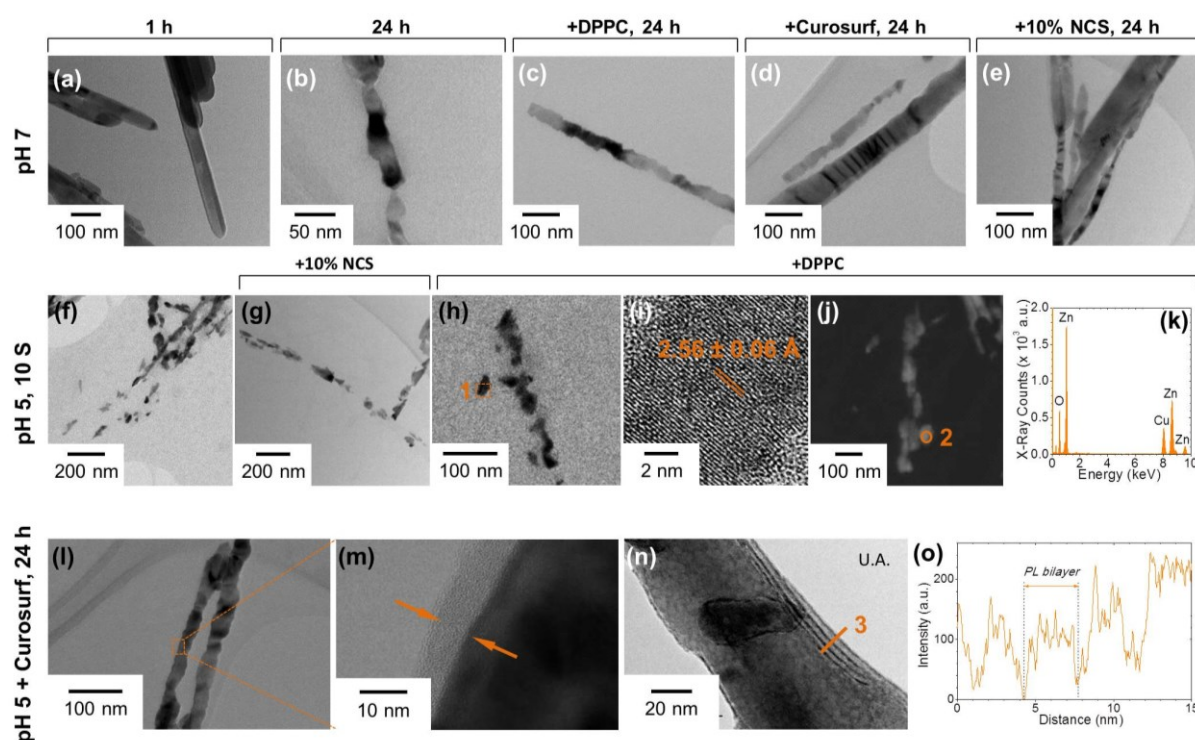


Figure 6.3 PS reduces the etching of S-ZnONWs at lysosomal pH and forms a phospholipid corona. (a-e) BFTEM images of S-ZnONWs incubated in NS at pH7 for 1 hour (a) or 24 hours (b-e), in the absence (a, b) or presence of DPPC (c), Curosurf[®] (d) or 10% NCS (e). (f-h) BFTEM images of S-ZnONWs incubated in NS at pH5 for 10 seconds, in the absence (f) or presence of 10% NCS (g) or DPPC (h). (i) HRTEM image of the area marked 1 in (h). (j) High angle annular dark field scanning transmission electron microscopy (HAADF-STEM) image of S-ZnONWs incubated in NS at pH5 for 10 seconds (k) STEM-EDX spectrum collected from the area marked 2 in (j). (l-n) BFTEM images of S-ZnONWs incubated in NS at pH5 in the presence of Curosurf[®] for 24 hours. (m) is the magnification of the area squared in (l). In (n) samples were positively stained with uranyl acetate (UA) to enhance phospholipid contrast and shows multiple phospholipid bilayers formed on the surface of S-ZnONWs. (o) Intensity line profile collected from the line marked 3 in (n).

In contrast to the rapid etching of S-ZnONWs at pH 5, incubation of S-ZnONWs at pH 5 in the presence of Curosurf[®] for 10 seconds led to no observable changes to their structure (**Appendix B, Figure 10.3**). After 24 hours of incubation, even though considerable etching had taken place at their surface, S-ZnONWs still appeared as continuous wires (**Figure 6.3l**). These observations support the decrease in S-ZnONW dissolution at pH 5 in the presence of Curosurf[®] measured by ICP-OES (**Figure 6.2c**). Imaging at higher magnification revealed the presence of an amorphous layer at the surface of S-ZnONWs (**Figure 6.3m**), which was attributed to the coating of S-ZnONWs by phospholipids. As shown for AgNWs incubated with Curosurf[®], the AgNWs were coated by phospholipid bilayers (**Figure 4.4**) that delayed their dissolution kinetics (**Figure 4.2**).⁴⁹⁶ The same approach was followed to reveal the organization of phospholipids on the surface of S-ZnONWs, by positively staining samples with uranyl acetate. After staining, striations parallel to the long axis of the S-ZnONWs were observed (**Figure 6.3n**). Since the hydrophilic head groups of phospholipid molecules produce dark contrast whereas the hydrophobic fatty acid tails produce bright contrast,⁴³³ the outermost surface of the lipid coating of S-ZnONWs, facing the aqueous environment, corresponds to hydrophilic head groups (similar for more than 100 S-ZnONWs inspected). When strongly hydrophilic groups are able to interact with the surrounding aqueous medium, the sorption of an organic compound on nanoparticles facilitates the stability of the nanoparticle dispersion.⁴⁹⁸ Therefore, the coating of S-ZnONWs by phospholipids is expected to improve their dispersion in aqueous media. The bilayer thickness was measured by collecting several intensity line profiles (**Figure 6.3o**) and had an average of 3.9 ± 0.6 nm. This value is the same as the bilayer thickness measured for AgNWs and close to the estimated maximum hydrocarbon bilayer thickness of 4.16 nm.⁴⁵⁶ In contrast to AgNWs, where phospholipids from various sources (DPPC, Curosurf[®] and *murine* LLF, **Figure 4.4**) coated AgNWs, no evidence was found that DPPC was able to coat S-ZnONWs (**Appendix B, Figure 10.4**).

6.3.3 PS reduces the agglomeration of ZnONWs

Even though the use of DLS to accurately estimate the dimensions of 1-dimensional NMs may not be straightforward, hydrodynamic size measurements could be used as an indication of agglomeration in NM dispersions. The hydrodynamic size of S-ZnONWs in the absence or presence of DPPC quickly increased within 1 hour of incubation in DI water (**Figure 6.4a**). However, for S-ZnONWs incubated with Curosurf[®] or 10% NCS, the hydrodynamic size remained relatively constant from 0 up to 48 hours. These results were corroborated with ζ -potential measurements (**Figure 6.4b**). In the absence or presence of DPPC, S-ZnONWs had a positive ζ -potential immediately after incubation, which dropped close to 0 within 1 or 4 hours of incubation, respectively. Incubation of S-ZnONWs with either Curosurf[®] or 10% NCS led to a negative ζ -potential, with higher magnitudes in

the case of Curosurf[®]. Although in both cases the ζ -potential magnitude slightly decreased over time, it remained lower than -10 mV after 48 hours.

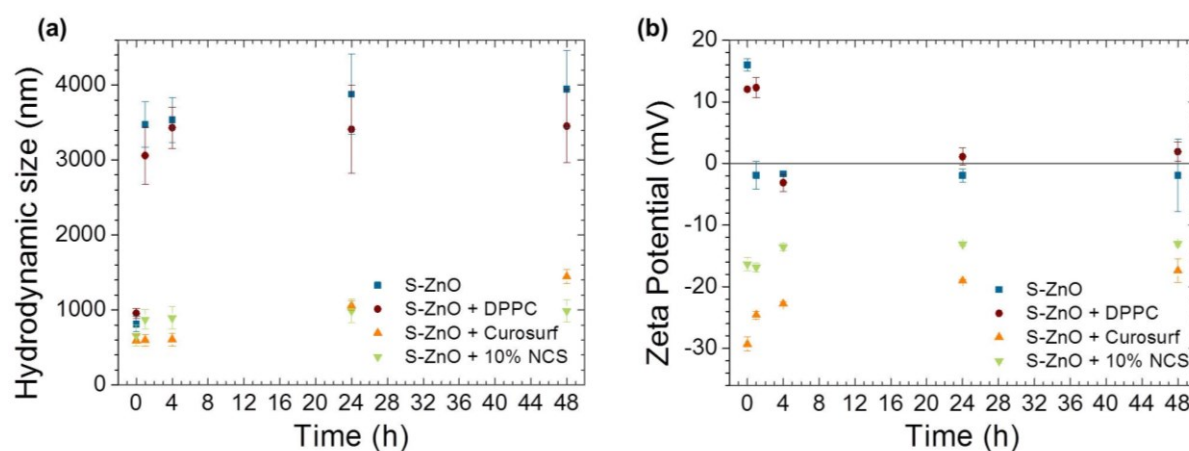


Figure 6.4 PS decreases the agglomeration of S-ZnONWs and imparts a negative surface charge. (a) Hydrodynamic size and (b) ζ -potential of S-ZnONWs incubated in DI water, in the absence or presence of DPPC, Curosurf or 10 % NCS. Each experiment was repeated 3 times (n=3) and data are presented as mean $\pm$ SD.

Taken together, DLS and ζ -potential measurements indicate that S-ZnONWs agglomerate rapidly in DI water, with no effect by the presence of DPPC. In contrast, both Curosurf[®] and 10% NCS prevented the agglomeration of S-ZnONWs. These findings may be correlated with the coating of ZnONWs by a phospholipid corona in the presence of Curosurf[®] but not of DPPC. The principle underlying this effect is possibly that surfactant molecules adsorb and form lipid bilayers on the surface of the particles by virtue of their amphiphilic nature, thus sterically stabilizing the particles.⁴⁹⁸ The absence of similar effects when ZnONWs were incubated with DPPC instead of Curosurf[®] may be due to the more complex composition of Curosurf[®].⁴⁹⁹ Apart from DPPC, which is a zwitterionic phospholipid, Curosurf[®] also contains anionic phospholipids, such as phosphatidylglycerol and phosphatidylserine, as well as the hydrophobic surfactant proteins SP-B and SP-C.⁴⁹⁹ Previous studies have indicated that ionic surfactant molecules are superior in contributing to both charge and steric stabilization of nanoparticles.²⁰⁸ Furthermore, SP-B/C are known to act as a “fusogen” that promotes lipid mixing and increases the adsorption rate of phospholipids.⁴⁰ In the case of 10% NCS, previous studies have shown that serum proteins stabilized ZnONPs against aggregation and sedimentation.^{494, 500} One possible stabilising agent in serum is albumin, the most abundant protein in serum, which may adsorb to the surface of ZnO creating a protein corona.⁴⁹⁵ Albumin carries a negative charge (around -20 elementary charges per molecule) at physiologic pH and may therefore stabilise particles by imparting a net surface charge,⁵⁰¹ as indicated by the ζ -potential measurements.

6.3.4 PS increases ZnONW bioreactivity with TT1 cells

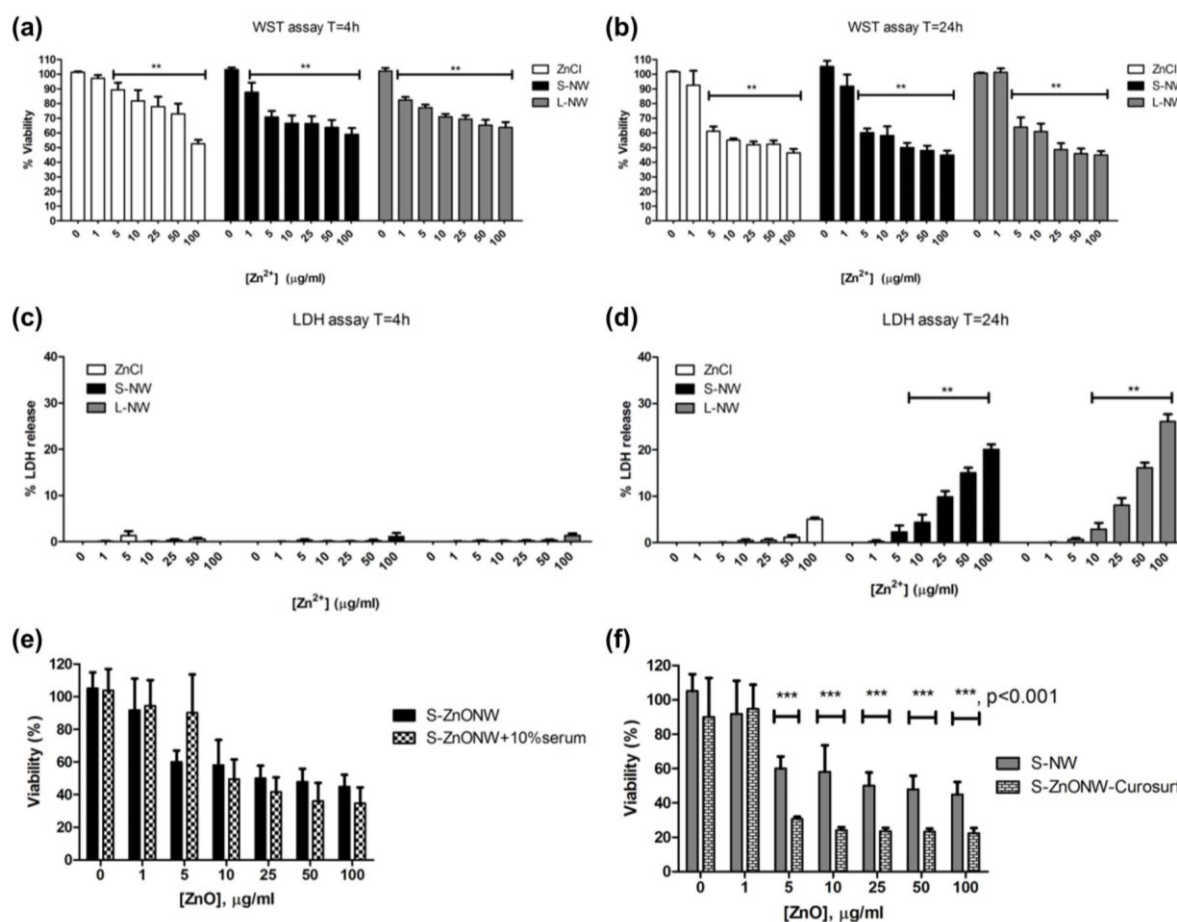


Figure 6.5 PS increases the bioreactivity of S-ZnONWs with type I-like (TT1) human alveolar epithelial cells. (a, b) Cell viability (WST assay; n=3, **p<0.01, ***p<0.001) and (c, d) LDH release (n=3, **p<0.01, ***p<0.001) following treatment of TT1 cells with S-ZnONWs and L-ZnONWs for 4 hours (a, c) and 24 hours (b, d). ZnCl₂ was used as a control for free Zn²⁺ ions. (e, f) Cell viability (WST assay; n=3, **p<0.01, ***p<0.001) following treatment of TT1 cells with S-ZnONWs for 24 hours, in the absence or presence of 10% NCS (e) or Curosurf® (f).

Having examined how the properties of high-aspect ratio ZnONWs change over time at different pH values and in the presence of PS, their bioreactivity with human alveolar epithelial type I-like (TT1) cells was tested. Their bioreactivity was assessed by a water soluble tetrazolium salts (WST) assay and lactate dehydrogenase (LDH) release assay, which measure the metabolic activity of cells and necrotic cell death, respectively. To compare with the effects of Zn²⁺ ions, ZnCl₂ was used as a source of soluble ionic zinc. The WST assay demonstrated a time- and concentration-dependent decrease in cell viability following exposure of TT1 cells to the Zn compounds (**Figure 6.5a, b**). Compared to the non-treated (NT) control, both S-ZnONWs and L-ZnONWs significantly reduced the viability of TT1 cells at doses of 1 μg/mL and above after 4 hours (**Figure 6.5a**), whereas the threshold of ZnCl₂-induced cell death was at 5 μg/mL. This threshold, between 1 and 5mg/mL of

ZnONWs, was a consistent feature of the cell death studies. Thus, after 24 hours, significant cell death occurred following exposure to 5 $\mu\text{g/mL}$ and above of all materials tested, the degree and profile of which was similar (where cell death plateaued with increasing ZnONW concentration; **Figure 6.5b**), and, with the exception of the 1 $\mu\text{g/mL}$ dose, was significantly greater than that observed at 4 hours. The median lethal doses (LD_{50}) after 24 hours were estimated to be 10.13 $\mu\text{g/mL}$ for ZnCl_2 , 10.90 $\mu\text{g/mL}$ for S-ZnONWs and 11.18 $\mu\text{g/mL}$ for L-ZnONWs, in terms of Zn^{2+} ion concentration. Therefore, both lengths of ZnONWs exhibited similar effects on the viability of TT1 cells, in accordance to the similar dissolution kinetics in cell-free *in vitro* measurements (**Figure 6.2a**). Their LD_{50} values are close to the LD_{50} doses previously measured for human MDMs exposed to ZnCl_2 and high aspect ratio ZnONWs.²⁴⁴ Similarly, ZnO nanorods (20-70 nm $\times$ 100-200 nm) were toxic to human aortic endothelial cells at doses above 10 $\mu\text{g/mL}$,²³ while 70 and 420 nm ZnONPs had similar toxicities in the human lung carcinoma cell line A549.³⁸⁷ Moreover, both types of ZnONWs had similar effects to those observed with ZnCl_2 . Along with the pH-dependent profile of ZnONM dissolution (**Figure 6.3a**), this finding suggests that ZnONW bioreactivity is dominated by intracellular dissolution and release of toxic Zn^{2+} ions. Previous studies have also shown that the toxicity of spherical and rod-shaped ZnONPs (10-200 nm) to mouse neuronal stem cells was independent of particle shape and size, and similar to ZnCl_2 .³⁸⁹

LDH release after 4 hours was not significant compared to the NT control for any of the materials at the dose range tested (**Figure 6.5c**). Relative to the NT control, exposure to up to 100 $\mu\text{g/mL}$ of ZnCl_2 for 24 hours did not result in significant LDH release (**Figure 6.5d**). Addition of ZnCl_2 to the cell culture medium may have caused supersaturation of Zn^{2+} with respect to the zinc carbonate and/or phosphate equilibrium solubility of the medium, leading to the formation of particulates with unknown effects on membrane integrity.⁵⁰² In contrast, both S-ZnONWs and L-ZnONWs caused a similar degree of cellular LDH leakage after 24-hour exposures to doses of 10 $\mu\text{g/mL}$ and above (**Figure 6.5d**). Normally, early-stage plasma membrane permeabilization (within 3-6 hours) is associated with necrosis whereas its delayed occurrence is the signature of apoptosis and secondary necrosis at a later stage. Therefore, our findings indicate that ZnONWs induce early-stage programmed TT1 cell death without disrupting the plasma membrane, and, in the absence of macrophage efferocytosis and other cell death clearance mechanisms, induction of late stage cellular necrosis. Non-necrotic cell death has also been suggested to occur after application of 5 and 200 nm ZnONPs to a breast adenocarcinoma cell line (MCF-7) and a nasopharyngeal carcinoma cell line (KB).⁵⁰³ Ahamed *et al.* suggested that ZnO nanorods (50 $\times$ 150 nm) caused apoptosis in A549 cells by inducing the expression of Hsp70, a biomarker of cell damage, upregulating the cell cycle checkpoint protein p53 and downregulating the antiapoptotic protein, survivin.³⁸⁰ Using TEM, Muller *et al.* demonstrated that human MDMs treated with ZnONWs or ZnCl_2 showed signs of both apoptosis, such as chromatin condensation and mitochondrial pyknosis, as well as necrosis, such as plasma membrane rupture and leaching of cytoplasmic contents.²⁴⁴ This heterogeneity in cellular responses

could be due to the fact that, in the case of ZnONWs, cells are exposed to highly variable local concentrations of zinc.

Since both types of ZnONWs and ZnCl₂ had similar effects on the viability of TT1 cells, the effects of PS and 10% NCS were assessed only for S-ZnONWs. Exposure of TT1 cells to S-ZnONWs for 24 hours with the addition of 10% NCS led to a similar decrease in cell viability to that observed in the absence of serum, regardless of exposure dose (**Figure 6.5e**). Even though 10% NCS treatment caused a slightly increased dissolution of ZnONWs at pH 7 (**Figure 6.2b**), this had no effect on the viability of TT1 cells compared to that observed with bare ZnONWs. In accordance to this finding, Hsiao *et al.* showed that the enhanced extracellular Zn²⁺ release by micro- and nano-ZnO particles in serum-containing tissue culture medium did not contribute as a main factor in the cytotoxicity of the ZnO particles.⁴⁹⁴ On the other hand, despite the decrease in dissolution at pH 5 (**Figure 6.2c**), exposure to S-ZnONWs pre-incubated with Curosurf[®] led to a significantly higher decrease (from 40-50% cell death to 70-80% cell death; $p < 0.001$, $n = 3$) in TT1 viability compared to bare S-ZnONWs, for doses of 5 µg/mL and above (**Figure 5f**).

6.3.5 PS increases the uptake of S-ZnONWs by TT1 cells

In order to test whether the differences in cell viability in the presence of Curosurf[®] arise due to differences in the amounts of S-ZnONWs internalized by the cells, uptake in TT1 cells was quantified by ICP-OES analysis (**Figure 6.6a**). TT1 cells were incubated with S-ZnONWs, in the absence and presence of Curosurf[®] or 10% NCS, while ZnCl₂ was used as an ionic control. After 4 hours, TT1 cells had taken up similar amounts of ZnCl₂, S-ZnONWs in serum-free medium and S-ZnONWs in medium containing NCS, which was approximately 10% of that applied to the cells (**Figure 6.6a**). In contrast, pre-incubation of the wires with Curosurf[®] led to about a 4-fold increase in intracellular zinc (in serum-free medium; **Figure 6.6a**). The percentage of cells positive for S-ZnONW uptake estimated by BF-TEM imaging indicated that a similar higher degree of internalization had taken place for S-ZnONWs pre-incubated with Curosurf[®] compared to bare S-ZnONWs (**Figure 6.6b**). Therefore, the higher toxicity of S-ZnONWs in the presence of Curosurf[®] (**Figure 6.5e**) can be correlated with the higher extent of intracellular uptake (**Figure 6.6a, b**) of S-ZnONWs, which may be attributed to the formation of a phospholipid corona (**Figure 6.3n**). Previous findings have shown that human lung lining fluid increases the uptake of polystyrene NPs, regardless of NP size or surface modification.⁴⁶⁵ It has also been demonstrated that Curosurf[®] slightly increased the uptake of AgNWs by TT1 cells after 24 hours (**Figure 4.8**). Konduru *et al.* suggested that the phospholipid coating on single walled carbon nanotubes represented an “eat me” signal on their surface that facilitated their recognition and internalization by macrophages.²¹⁰ In the presence of 10% NCS, however, no significant effect on S-ZnONW uptake in TT1 cells was observed. The exact role

of adsorbed protein in NP uptake is not clear, with some studies suggesting that proteins promote NP uptake via receptor-mediated endocytosis while others have demonstrated a reduction or no effect in uptake.⁵⁰⁴ It is likely that differential adsorption of components of serum proteins by different nanomaterials impacts on the bioreactivity and mode of cellular uptake, if any. In one study, the addition of 5-10% FBS to the cell culture medium prevented agglomeration and sedimentation of ZnONPs and lowered their toxicity against A549 cells.⁴⁹⁴ The authors suggested that the formation of a protein corona around the ZnONPs protected the cells from direct interaction with the particles, decreasing their toxicity.

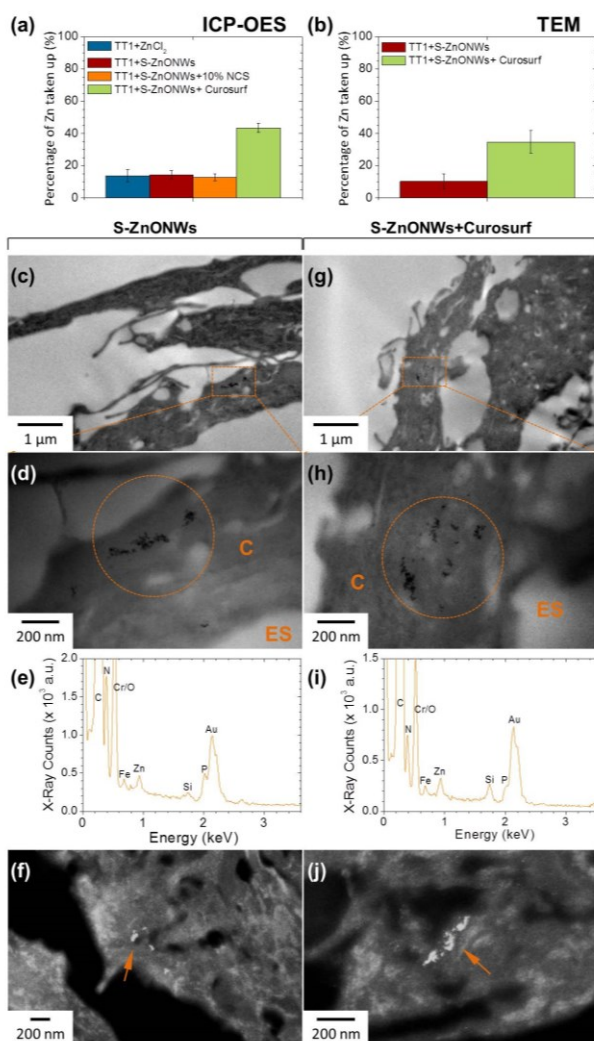


Figure 6.6 PS increases the S-ZnONW uptake by human alveolar epithelial cells. (a) ICP-OES quantification of the amount of intracellular Zn²⁺ in TT1 cells, following treatment for 4 hours with ZnCl₂ or S-ZnONWs, in the absence or presence of 10% NCS or Curosurf[®]. Experiments were repeated 3 times (n=3) and data are presented as mean ± SD. (b) BFTEM quantification of intracellular Zn in TT1 cells treated for 4 hours with S-ZnONWs, in the absence or presence of Curosurf[®]. Experiments were repeated 3 times (n=3) and data are presented as mean ± SD. BFTEM images (c, d, g, h) of TT1 cells exposed to S-ZnONWs in the absence (c, d) or presence (g, h) of Curosurf[®] (n=3). (d) and (h) are magnifications of the areas squared in (c) and (g) respectively. (e, i) TEM-EDX spectra collected from the areas circled in (d) and (h) respectively. (f, j) HAADF-STEM images of TT1 cells exposed to S-ZnONWs in the absence (f) or presence (j) of Curosurf[®] (n=3). (C: cytoplasm; ES: extracellular space)

The morphology of S-ZnONWs taken up by TT1 cells in the absence and presence of Curosurf[®] was investigated by TEM. BF-TEM imaging of TT1 cells exposed to S-ZnONWs for 4 hours revealed that there were no intact S-ZnONWs intracellularly, either in the absence (**Figure 6.6c, d**) or presence (**Figure 6.6g, h**) of Curosurf[®]. Instead, electron dense material was observed inside TT1 cells, and TEM-EDX (**Figure 6.6e, i**) showed the presence of Zn in the areas around this material, both in the absence (**Figure 6.6e**) and presence of Curosurf[®] (**Figure 6.6i**). Zn peaks were not detected in TEM-EDX spectra collected from areas in the extracellular space (**Appendix B, Figure 10.5**). HAADF-STEM imaging, a technique sensitive to local atomic number variations in samples, confirmed the presence of material with high atomic number inside TT1 cells (**Figure 6.6f, j**, arrows). This material was attributed to remnants of S-ZnONWs following intracellular dissolution. In most of the studies where TEM imaging was employed to examine cells exposed to ZnONPs, no particles could be detected intracellularly, possibly due to their rapid dissolution.⁵⁰⁵ For instance, within 1 hour of exposure of human MDMs to ZnONWs, no intact wires were observed intracellularly, but cells instead contained “ghost” structures of electron dense material.²⁴⁴

6.3.6 PS delays the intracellular dissolution of S-ZnONWs within TT1 cells and the onset of cellular damage

To further investigate the correlation between intracellular ZnONW dissolution and cell death, the cellular distribution and dissolution of S-ZnONWs were dynamically analysed in live TT1 cells by laser scanning confocal microscopy. TT1 cells were preloaded with a Zn²⁺ indicator (FluoZinTM-3, AM), which is a cell-permeant acetoxymethyl (AM) ester that displays green fluorescence upon binding of ionic Zn²⁺. Propidium iodide (PI), a red fluorescent dye that is membrane impermeable and generally excluded from viable cells, was added to stain the nuclei of necrotic cells. Following introduction of S-ZnONWs, the cells were imaged at regular intervals up to 3 hours (**Figure 6.7**). In the absence of Curosurf[®], green fluorescence was observed within 20min following exposure (**Figure 6.7b**), indicating a rise in intracellular Zn²⁺ concentrations. This was confirmed by an increase in the mean fluorescence intensity (MFI) of FluoZinTM-3 (**Figure 6.7k**). Within 1 hour, the FluoZinTM-3 MFI had reached its maximum value, followed by a drop in FluoZinTM-3 fluorescence, possibly indicating leakage of the Zn²⁺-dye complex from the cells. Concomitantly, an increase in PI fluorescence was observed, indicating necrotic cell death. TT1 cells exhibited a heterogeneous response to S-ZnONWs, with some cells dying within 40 minutes (**Figure 6.7c**), while others more than 2.5 hours (**Figure 6.7i**) after exposure. This heterogeneity may be due to the tendency of S-ZnONWs to agglomerate, leading to exposure of TT1 cells to highly variable concentrations of S-ZnONWs in different areas of the TT1 cell monolayer. Together with the effects of pH on dissolution shown in **Figure 6.3**, these findings suggest that the toxicity of S-ZnONWs is

due to an intracellular increase in ionic Zn^{2+} following uptake and dissolution of the ZnONWs inside cells, as has been also demonstrated for human MDMs.²⁴⁴

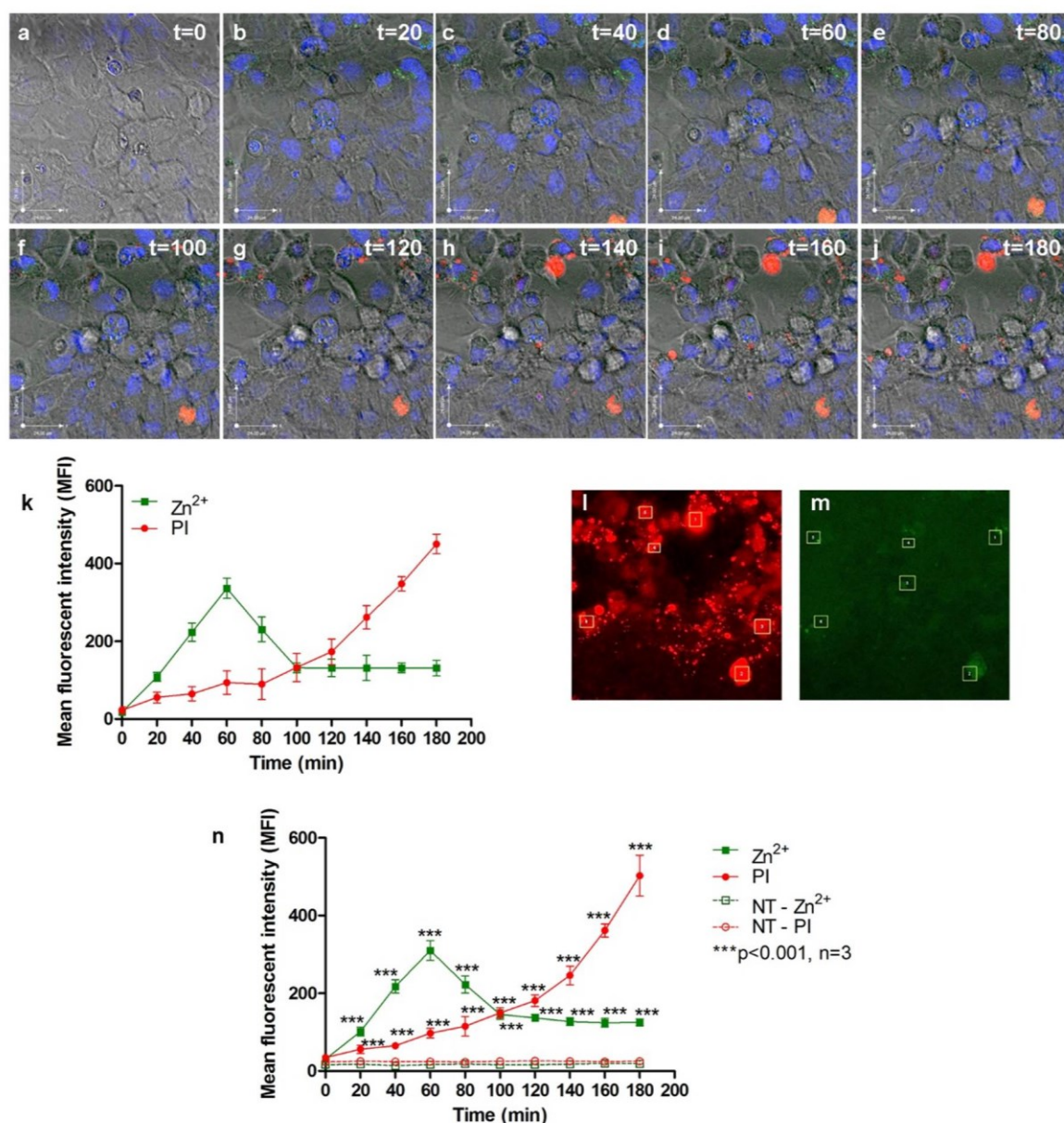


Figure 6.7 Intracellular dissolution of S-ZnONWs within live TT1 cells correlates to cell viability. (a-j) Live TT1 cells were incubated with S-ZnONWs at 37°C with 5% CO₂ and imaged by laser scanning confocal microscopy every 20 minutes (a-j) up to 3 hours. Cells were stained with Hoechst 33342 (blue, nuclei) and FluoZinTM-3, AM (green, Zn²⁺) prior to exposure to S-ZnONWs in PI-containing (red, necrotic cells) serum free RPMI cell culture medium. (k) The dynamic intracellular dissolution of S-ZnONWs (green line) and their effect on cell viability (red line; PI), were quantified by measuring the mean fluorescent intensity (MFI) of FluoZinTM-3, AM (l; green in a-j) and PI (m; red in a-j) against time in six cells from one single experiment. Region of interests (ROI 1-6, squared in l, m) indicate the areas where the fluorescent signals were measured and plotted in k. (n) Three experiments were performed with a total of 21 cells observed, and the signals of Zn²⁺ and PI were plotted against the time in comparison to the background signals obtained from non-treated cells (NT-Zn²⁺ and NT-PI; n=3 with a total of 21 cells observed, ***p<0.001).

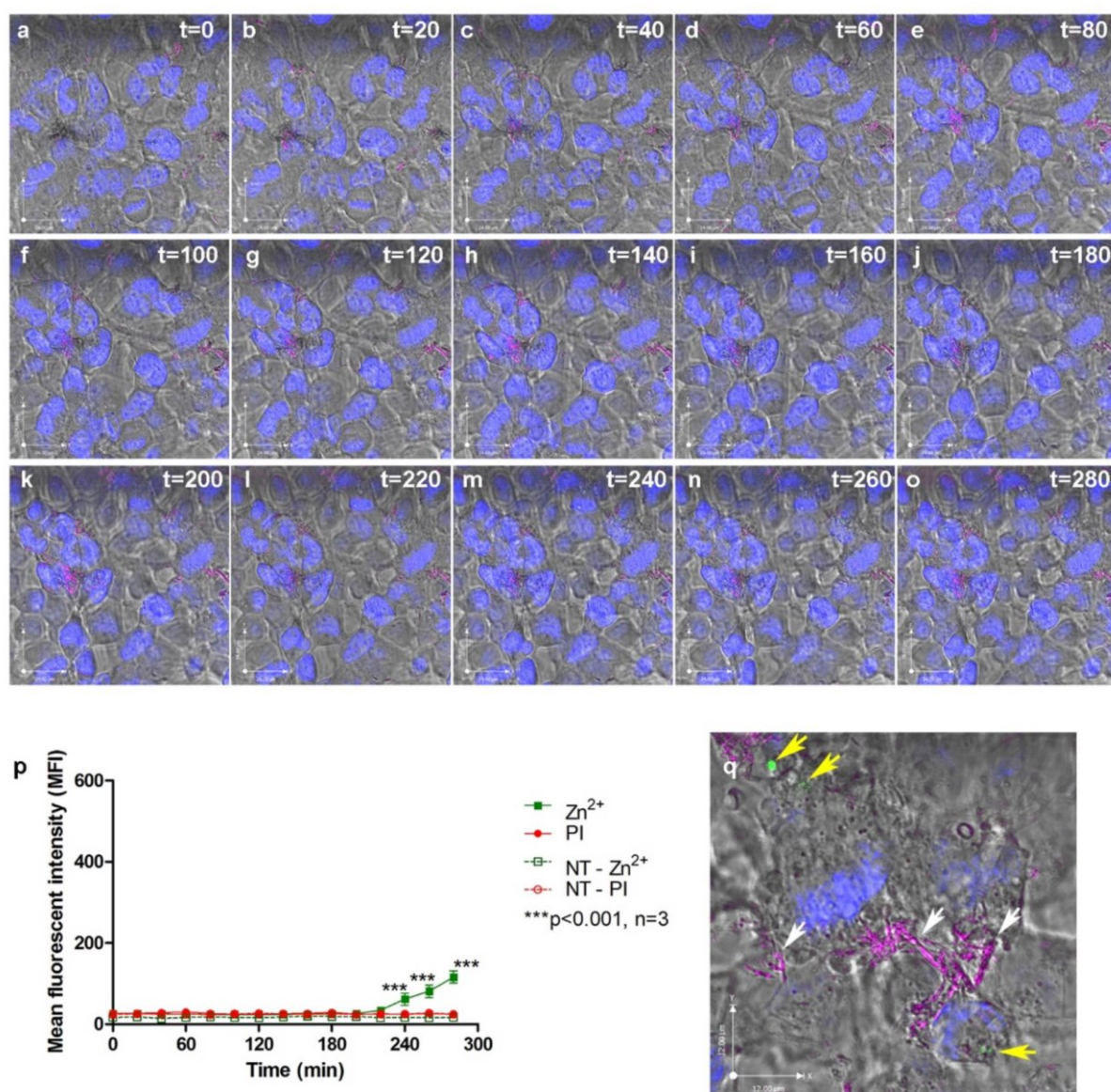


Figure 6.8 PS delays intracellular dissolution of Curosurf®-preincubated S-ZnONWs within live TT1 cells, and the onset of cell damage. (a-o) Live TT1 cells were incubated with S-ZnONWs pre-incubated with Curosurf® and imaged by laser scanning confocal microscopy every 20 minutes up to 5 hours. Cells were stained with Hoechst 33342 (blue, nuclei) and FluoZin™-3, AM (green, Zn²⁺) prior to exposure to S-ZnONWs in PI-containing (red, necrotic cells) serum free RPMI cell culture medium. (p) There was no significant Zn²⁺ release from the Curosurf®-preincubated S-ZnONWs (green line) and no necrotic cell death (red line) was observed within 3 hours of exposure. A significant release of Zn²⁺ ($p < 0.001$; $n = 3$ with a total of 21 cells observed) was observed after 4 hours of exposure (green line in p, yellow arrows in q indicate areas of Zn²⁺ release), with no significant necrotic cell death (red line in p). S-ZnONWs, imaged in reflectance mode (magenta in q, white arrows) remained in the system close to areas of Zn²⁺ release.

When TT1 cells were exposed to S-ZnONWs pre-incubated with Curosurf®, no release of Zn²⁺ was observed within cells over the first 3 hours after the addition of S-ZnONWs (**Figure 6.8a-o**). This finding indicates that Curosurf® may delay the intracellular release of Zn²⁺ from S-ZnONWs, by coating their surface with a phospholipid corona, as previously observed in cell-free *in vitro*

measurements (**Figure 6.3n**). Significant intracellular release of Zn^{2+} from ZnONWs was observed 4 hours after exposure; however no significant PI fluorescence was measured within the timeframe of the experiment (**Figure 6.8p-q**). Since pre-incubation of S-ZnONWs with Curosurf[®] resulted to a higher decrease of TT1 viability at 24 hours compared to bare S-ZnONWs (**Figure 6.5f**), these results suggest that, by delaying intracellular dissolution, the phospholipid corona also delayed cellular damage. The intracellular processing of nanoparticle coronas following uptake, and the consequences on the cytotoxic effects of the nanoparticles are not well understood. Recent findings suggest that biomolecular coronas are retained by nanoparticles as they are internalized by cells and are trafficked to the lysosomes on the nanoparticle's surface.⁵⁰⁶ In the lysosomes, the coronas may protect the cells from damage induced by the bare nanoparticle, until the biomeolecules are enzymatically cleared and the nanoparticle's surface is re-exposed.^{506, 507} Ma *et al.* have recently demonstrated that the rate of proteolytic degradation of protein coronas on gold nanoparticles (AuNPs) varied between different proteins, with half times (time required for degradation of 50% of the corona) ranging from around 4 to 70 hours, and this variation correlated with the degree of cellular damage.⁵⁰⁸ Similarly, in our experiments, the delayed toxicity of the Curosurf[®] pre-incubated ZnONWs may be a result of the time needed for the enzymatic degradation of the phospholipid corona, possibly by phospholipases, which are known to be present in lung tissue.⁵⁰⁹ In type 2 alveolar epithelial cells, the degradation half time of internalized DPPC has been measured at around 2 hours.⁵¹⁰ Therefore, further investigation is required in order to fully understand the role of phospholipid coronas on the internalization of nanoparticles, the intracellular fate of the corona and its impact on the accompanying toxicity of nanoparticles.

6.4 Conclusions

Since the first interaction of any nanoparticle that reaches the alveoli will be with the pulmonary surfactant (PS), its effects on the physicochemical properties of nanoparticles could have a significant impact on their bioreactivity. In this work, by performing detailed characterization we were able to provide new insights on the effects of PS on the dissolution and colloidal stability of ZnONWs, as well as their bioreactivity toward TT1 human alveolar epithelial cells. In summary, *in vitro* adsorption of PS lipids on ZnONWs has been demonstrated. This lipid corona delayed the kinetics of Zn^{2+} ion release from ZnONWs at acidic pH. TEM imaging indicated that the adsorbed phospholipids formed a coating on the surface of ZnONWs and that the polar headgroups of phospholipids faced away from the ZnONWs toward the aqueous phase, which could improve their dispersion in solution. DLS and ζ -potential measurements confirmed that PS prevented the agglomeration of ZnONWs, possibly through contributions of both steric and charge stabilization. Scanning confocal microscopy imaging of live TT1 cells showed that cell damage correlated with an

increase in intracellular ionic Zn^{2+} following uptake and dissolution of ZnONWs inside cells. Moreover, confocal imaging suggested that the coating of ZnONWs by a phospholipid corona slowed down their dissolution kinetics intracellularly, which also delayed the onset of cellular damage. However, pre-incubation of ZnONWs with PS led to uptake of the ZnONWs by a higher percentage of TT1 cells; this increase in cellular uptake dominated over their delayed intracellular dissolution and resulted to a higher reduction in cell viability after 24 hours. On the other hand, serum had no significant effect on ZnONW uptake or cell viability, despite also preventing the agglomeration of ZnONWs, probably through a protein corona. These findings illustrate that the nature of nanoparticle corona, its effect on particle uptake and the intracellular fate of the nanoparticles have interconnected roles on the interaction between nanoparticles and alveolar epithelial cells, and require further investigation. Furthermore, our data indicate that, in the case of ZnONWs, their bioreactivity may not be strongly dependent on their aspect ratio, which did not significantly change their dissolution kinetics or toxicity against TT1 cells. In conclusion, our findings show that the PS can significantly alter the dissolution kinetics, aggregation state and surface chemistry of ZnONWs, with important consequences on how they are internalized and processed by the underlying epithelial cells. This work highlights that combinations of spatially resolved static and dynamic techniques are required to develop a holistic understanding of the parameters that govern ZnONW bioreactivity at the point of exposure and to accurately predict their risks on human health and the environment. More generally, our observations illustrate that the role of PS is central in understanding interactions at the bio-nano interface of the alveoli, and their impact on subsequent epithelial–endothelial nanoparticle translocation.

7 Synthesis of ZnONWs

7.1 Introduction

Various techniques have been used for the synthesis of ZnO nanowires (ZnONWs), with final products of different dimensions or capping agents, leading to differences in dissolution rates, agglomeration kinetics and ultimately toxicological responses. For commercially available ZnONWs, details about their fabrication procedures are rarely disclosed by manufacturers and few characterization data of the products are provided. For instance, as demonstrated in **Chapter 6**, the sizes of two different types of commercially available ZnONWs measured by TEM were different than those stated by the manufacturer. Therefore, in-house synthesis of ZnONWs with well-controlled physicochemical properties is desirable for bioreactivity studies.

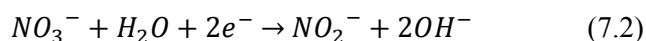
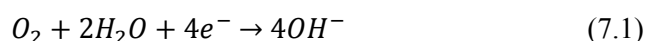
ZnO is a versatile functional material, with a rich family of 1D nanostructures such as nanotubes, nanorods, nanowires, nanobelts, nanorings, and nanosprings. ZnO crystallizes with the Wurtzite structure, which is a hexagonal crystal system that consists of tetrahedrally coordinated Zn and O atoms with alternating layers of oxygen and zinc ions along the major c-axis. The (001) planes, which are perpendicular to the c-axis are composed uniquely of either Zn^{2+} or O^{2-} ions and are therefore polar. The (1 $\bar{1}$ 0), (100), and (010) faces, which are parallel to the c-axis, are composed of Zn–O dimers with overall neutral charge and are non-polar.⁵¹¹

Several techniques have been developed for the fabrication of 1D ZnO nanostructures, such as sol-gel, physical vapour deposition, vapour-liquid-solid growth, metal-organic chemical vapour deposition and pulsed laser deposition.⁵¹² These methods, however, present various limitations, such as low yield or poor control over the shape and dimensionality of the nanostructures. Some of them operate at high vacuum or temperature or are costly, which may limit their potential implementation. Electrochemical deposition in solutions has emerged as a promising technique, due to its simplicity, operation at low temperatures, low cost and scalability.⁵¹³

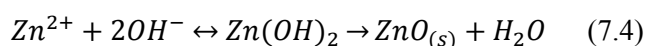
The electrodeposition of ZnO films was first reported independently by Peulon *et al.* and Izaki *et al.*^{514, 515} Izaki *et al.* cathodically deposited transparent ZnO films from aqueous zinc nitrate ($\text{Zn}(\text{NO}_3)_2$) solutions on glass substrates coated with tin oxide.⁵¹⁵ Peulon *et al.* employed a similar

method, using zinc chloride (ZnCl_2) solutions.⁵¹⁴ They suggested that film growth was a heterogeneous process, taking place under quasi-thermodynamic equilibrium conditions at the interface.⁵¹⁶ Deposition temperature and bath composition were also shown to be key parameters in the final quality of the deposited film. At temperatures lower than 50 °C, amorphous insulating hydrated ZnO films (ZnO-Zn(OH)_2 mixtures) were obtained, whereas at higher temperatures, conductive and crystalline ZnO films were formed. Typically, deposition temperatures did not exceed 100 °C.

The principle of ZnO electrodeposition is quite simple and is based on the chemical precipitation of ZnO. The application of a potential in the electrochemical cell leads to the reduction of a hydroxide precursor and the formation of hydroxide ions. Molecular oxygen (O_2) is the most widely used precursor, but the use of hydrogen peroxide (H_2O_2) or nitrate (NO_3^-) has also been reported:⁵¹⁷



The production of hydroxide ions increases the local pH at the vicinity of the substrate (cathode) surface, leading to supersaturation and precipitation of zinc hydroxide on the cathode. Finally, zinc hydroxide spontaneously dehydrates into ZnO:



The formation of ZnO nuclei onto the substrate by heteronucleation is followed by their growth into a film.⁵¹⁸ The growth mode can change from planar film growth to nano-island growth by varying the growth conditions. The effects of electrodeposition parameters on film morphology, thickness, transparency, roughness and crystallographic orientation have been extensively studied. Parameters that may influence the film morphology include the substrate lattice parameter, the pH and chemical composition of the electrolyte, the type and concentration of oxygen precursor, the applied potential and whether the electrode surface has undergone a pre-activation treatment to assist nucleation.⁵¹⁸ By controlling these parameters, 1D nanostructures, such as nanowires, can be obtained.

The growth mechanism for the formation of ZnONWs remains controversial. Elias *et al.* carried out a systematic study of the influence of Zn^{2+} ions at low concentrations.⁵¹⁹ They explained

the formation of 1D nanostructures by a limited diffusion of Zn^{2+} ions, which preferentially react at the top of the rods and thus lead to a growth along the c-axis.⁵¹⁹ Belghiti *et al.*, on the other hand, argued that such a limitation would lead to the formation of conical structures.⁵²⁰ They suggested that preferential growth takes place due to the higher reactivity of the (002) polar faces, while the nonpolar side faces are not reactive enough for growth.⁵²⁰ Illy *et al.* studied a wide range of Zn^{2+} concentrations and showed that neither of the mechanisms proposed could fully explain the formation of structures observed.⁵¹¹

Several studies have reported the synthesis of ZnO nanowires and nanorods by electrochemical deposition. For instance, ZnO nanorods were grown on polycrystalline Zn foil by electrodeposition in an aqueous solution of ZnCl_2 and calcium chloride (CaCl_2) at 80 °C.⁵²¹ Their diameters ranged from 60 to 140 nm and their length was 1.8 μm . ZnO nanorods with diameters 100-300 nm and a length of 2 μm were also fabricated on indium-doped tin oxide covered glass from $\text{Zn}(\text{NO}_3)_2$ solutions.⁵²² Finally, Illy *et al.* demonstrated the formation of ZnO nanorods on Mylar covered with a 20 nm-thick sputtered gold film.⁵¹¹ For low $\text{Zn}(\text{NO}_3)_2$ concentrations (< 2 mM), the nanorods were shown to grow along screw dislocations. Pyramidal structures were observed on the top of the rods, and over time the rods maintained a constant diameter and did not coalesce. The mean rod diameter could be simply controlled by varying the concentration of $\text{Zn}(\text{NO}_3)_2$ independently of other deposition parameters.

Electrochemical deposition may also be used in template-assisted synthesis, where a template is used to direct 1D growth.⁵²³ The obtained shape, direction and size of the nanostructure are determined by the architecture of the porous template. ZnONWs have been electrodeposited into two types of templates with cylindrical channels, anodic alumina membranes (AAM) and track-etched polycarbonate membranes (PCM). Polycarbonate membranes are commercially available with a wide range of pore diameters (10-2000 nm), but their pore density is relatively low (10^9 cm^{-2}), an order of magnitude lower than that of AAM. Additionally, some channels may intersect within the membranes due to the pore-production process.⁵²³ In template-assisted synthesis, one side of the porous template is first coated with a thin layer of metal, such as gold, to act as the working electrode in the three-electrode electrochemical cell. Deposition of the NWs occurs inside the pores of the template, starting from the deposited metal side, while the duration of deposition determines the length of the prepared NWs. Finally, the deposited NWs are obtained by removing the conductive layer and the template. The templates can be removed by dissolving them in a solvent in which the NWs are insoluble.^{523, 524}

ZnONWs have been synthesized by template-assisted electrodeposition within PCM, in an aqueous solution of KCl and ZnCl_2 using H_2O_2 as the oxygen precursor.⁵²⁵ Lai and Riley showed that monocrystalline ZnONWs were obtained through the reduction of nitrate ions (0.01 M $\text{Zn}(\text{NO}_3)_2$) at a constant potential of -0.7 V at 90 °C, while polycrystalline NWs were deposited by reduction of peroxide ions at a fixed potential of -0.2 V at 22 °C.⁵²⁶ Therefore, increasing the temperature of deposition increased the structural quality of the obtained wires.^{527, 528} Sharma *et al.* deposited

ZnONWs using 0.1 M $\text{Zn}(\text{NO}_3)_2$ at 75 °C, under different potentials (-0.8, -1.0 and -1.2 V) and showed that ZnONWs grown at higher negative potentials had a higher fraction of large size crystallites.⁵²⁴ Recently, high aspect ratio ZnONWs were prepared by ECD into PCM in an aqueous solution of 0.1 M $\text{Zn}(\text{NO}_3)_2$ solution under a potential of -0.8 V, at 65 °C.²⁴⁴

The purpose of this work is to synthesize and characterize ZnONWs with well-controlled dimensions. Template-free electrochemical deposition was employed to synthesize films of ZnONWs on substrates, while a protocol was developed for the removal of the NWs from the substrates. Template-assisted electrochemical deposition within track-etched PCM was also used for ZnONW synthesis. The morphology and size of the obtained products were characterized by scanning electron microscopy (SEM).

7.2 Methods

7.2.1 Template-Free Synthesis of ZnONWs

7.2.1.1 *Preparation of the working electrode*

The working electrode for the cathodic deposition of ZnONWs was a 12 nm gold (Au) film sputtered on glass (Emitech k575X). Prior to sputtering, microscope glass slides (VWR, UK) were successively washed in Acetone (VWR, UK), Methanol (VWR, UK), 1.0 M Sodium Hydroxide (NaOH, VWR, UK), and deionized (DI) water. The electrical connection between the Au film and an electrical wire was achieved by a drop of silver paint (Agar, UK), covered by copper tape. The contact was then covered with insulating paint (Micro Super XP200 Lacquer, Hi-Tek Products Ltd, UK) and dried in ambient atmosphere overnight. Before deposition, the films were cleaned with compressed air.

7.2.1.2 *Electrochemical deposition*

Potassium chloride (KCl, 0.1 M, VWR, UK) was used as the supporting electrolyte and zinc chloride (ZnCl_2 , 0.1 mM, Sigma-Aldrich, UK) was used as the zinc precursor. All the chemicals were of analytical grade and were used without further purification. Oxygen gas (O_2 , BOC, UK) was bubbled for 30 minutes prior to electrodeposition, to saturate the solution with O_2 , and maintained throughout the deposition. The temperature was kept constant at 85 °C using a hot plate equipped with a temperature sensor. The electrochemical cell employed a three-electrode setup. The Au-coated glass slide served as the working electrode, while a platinum (Pt) mesh and a silver/silver chloride

(Ag/AgCl)/KCl electrode were used as the counter and reference electrode, respectively. Electrodeposition was performed under the potentiostatic transient mode, with a potential of -0.9 V vs. Ag/AgCl. A potentiostat (VersaSTAT 3, Princeton Applied Research, USA) was used to control the potential and measure the current. The deposition was terminated when the desired amount of charge had passed through the system. The deposited films were washed by briefly immersing in DI water, dried in ambient atmosphere and stored in a desiccator.

7.2.1.3 Liberation of ZnONWs from the substrate

Gold is known to become unstable and dissolve in the positive potential region, especially in solutions containing chloride ions.⁵²⁹ To liberate the nanowires from the substrate, the Au film was oxidized by the application of an anodic potential. Potassium Chloride (KCl, 0.05 M, VWR, UK) was used as the electrolyte in a similar setup as for electrodeposition. A potential of 1.4 V was applied and maintained until all of the Au was oxidized (*i.e.* the measured current reduced to 0). The substrate was left to dry in ambient atmosphere. The electrical connections were carefully removed and the substrates were sonicated in ethanol (VWR, UK) for 2 minutes. The ethanol dispersions of the NWs were mixed with hexane at a 2:1 hexane:ethanol ratio and the NWs were collected by centrifugation at 4500 rpm for 15 min. The resulting pellets were redispersed in ethanol.

7.2.2 Template- Assisted Synthesis of ZnONWs

Whatman™ Nuclepore™ Polycarbonate Track-Etched Membranes (Sigma Aldrich, UK) were used as templates for ZnONW growth by electrochemical deposition. The membranes had a thickness of 6 μm , a diameter of 25 mm and a pore density of $6 \times 10^8 \text{ cm}^{-2}$. Membranes with two different nominal pore sizes were used, 50 nm (PCM50) and 30 nm (PCM30). One side of the membranes was sputter coated with a 100 nm layer of Au, to serve as a working electrode. A Pt mesh and a Ag/AgCl electrode were used as the counter and reference electrodes, respectively. Based on previous work,²⁴⁴ electrodeposition was conducted using a zinc nitrate ($\text{Zn}(\text{NO}_3)_2$, 0.1 M, VWR, UK) aqueous solution under a constant potential of -0.8 V vs. Ag/AgCl at 70 °C. The ECD was carried out for 30 min. Prior to electrodeposition, the membrane was placed in the cell with $\text{Zn}(\text{NO}_3)_2$ solution for 30 min, under stirring, to equilibrate the temperature and allow wetting of the pores.

After electrodeposition, the polycarbonate templates were dissolved by incubation in 2 mL dichloromethane (VWR, UK) for 1 hour. The solution was gently agitated to dislodge the NWs and the gold foil was removed with tweezers. The ZnONWs were collected by centrifugation at 4500 rpm for 15 min and the resulting pellets were washed 6 times with 2 mL dichloromethane to remove

undissolved polycarbonate residue. Finally, the dichloromethane was evaporated using a stream of nitrogen and the NWs were suspended in ethanol.

7.2.3 Scanning Electron Microscopy (SEM)

The morphology and size distribution of the ZnONWs were characterized using a LEO 1525 Field Emission Gun Scanning Electron Microscope (FEG-SEM, Carl Zeiss Microscopy GmbH, UK). The SEM was operated in secondary electron mode at an accelerating voltage of 5 kV, using the InLens detector. The size distribution of the Zn NWs was characterized using SEM images and ImageJ software (<http://rsb.info.nih.gov/ij/>).

7.3 Results and Discussion

7.3.1 Template-Free Synthesis of ZnONWs

Films of ZnO nanowires on gold-sputtered glass substrates were successfully fabricated by cathodic electrodeposition, according to protocols previously developed in Ryan's group (**Figure 7.1**).^{511, 518, 530} By controlling the deposition parameters, ZnO films with distinct morphologies may be obtained.⁵¹⁸ The concentration of ZnCl₂ used here corresponds to a low supersaturation level of the solution, which has been associated with the formation of isolated ZnO nanorods.⁵¹¹ SEM imaging of the top view of the deposited films showed that they consisted of isolated ZnONWs with a hexagonal cross section (**Figure 7.1a**). SEM imaging of the side view of the films showed that the NWs were perpendicular to the substrate and that no coalescence was observed (**Figure 7.1b**). The diameter of the ZnONWs, measured from several top-view SEM images, was 37 ± 13 nm (**Figure 7.1e**). The diameter of the ZnONWs may be tuned by increasing either the Zn²⁺ concentration in solution or the applied potential.⁵¹¹

To detach the ZnONWs from the glass substrates, the Au layer was first dissolved by application of an anodic potential. Immediately after Au electro-dissolution, the NWs were no longer perpendicular to the glass substrate but had detached from their point of attachment with the Au layer, while they still remained on the glass surface (**Figure 7.1c**). Their length, immediately after the removal of Au, was 670 ± 250 nm. (**Figure 7.1f**) ZnONWs of the desired length can be easily obtained by controlling the total amount of charge passed through the system during electrodeposition.

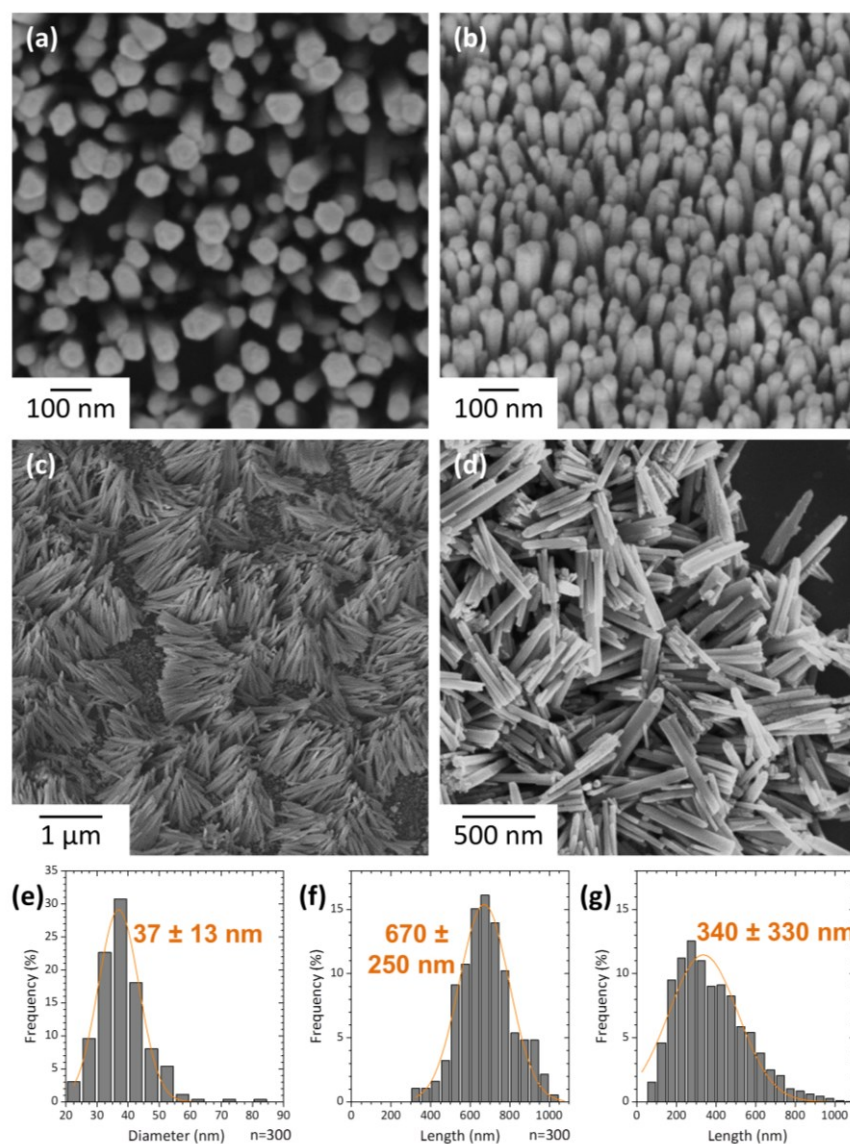


Figure 7.1 (a-d) SEM images of ZnONWs synthesized by template-free electrodeposition. Top-view (a), diameter distribution (e) and 30° side view (b) of ZnONWs films grown on Au-coated glass substrates. Top-view (c) and length distribution (f) of ZnONWs after dissolution of the Au layer. SEM image (d) and length distribution (d) of ZnONWs after their removal from the substrates.

To obtain suspensions of ZnONWs, the glass substrates were sonicated in ethanol briefly, to minimize breakage of the wires, and the NWs were collected by centrifugation. **Figure 7.1d** shows an SEM image of the collected NWs. The NWs were successfully liberated from the glass substrates and appeared clean. Their diameter was uniform along their length, with some exhibiting conical tips. The length of the wires after removal from the substrates was 340 ± 330 nm (**Figure 7.1g**). Compared with the length distribution of the NWs immediately after Au dissolution (**Figure 7.1f**), the length of the collected NWs had a wider distribution, while its average value had significantly decreased.

7.3.2 Template-Assisted Synthesis of ZnONWs

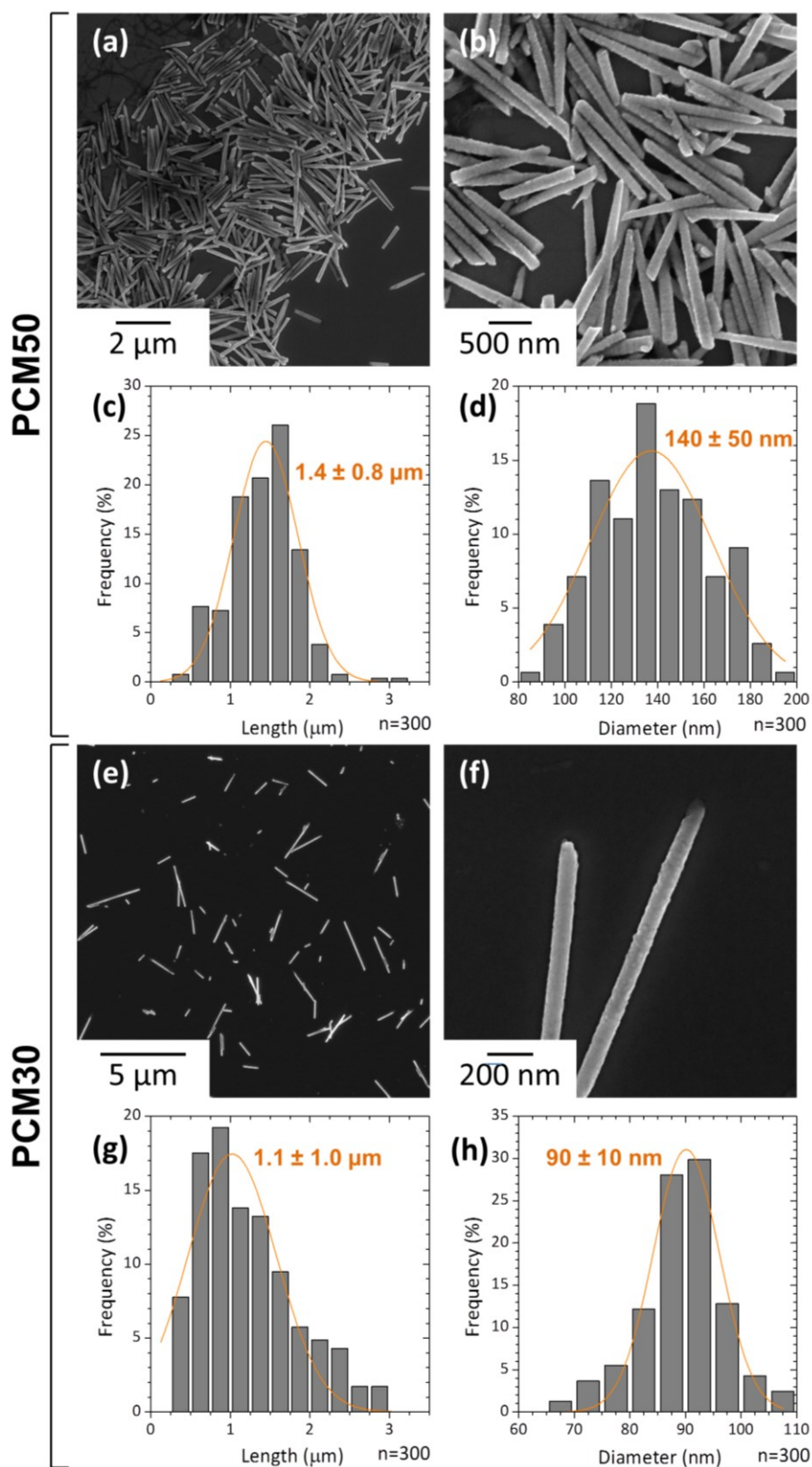


Figure 7.2 SEM images (a, b, e, f), length distributions (c, g) and diameter distributions (d, h) of ZnONWs synthesized by template-assisted electrodeposition, using track-etched polycarbonate membranes with nominal pore sizes of 50 nm (PCM50, a-e) and 30 nm (PCM30, e-h).

High aspect ratio ZnONWs were also synthesized by electrodeposition into polycarbonate templates with two different pore sizes (**Figure 7.2**). ZnONWs obtained with PCM50 had a slightly tapered shape with narrower tips (**Figure 7.2a, b**), while those obtained with PCM30 had a uniform diameter throughout their length (**Figure 7.2e, f**). The length distributions of the NWs were 1.4 ± 0.8 μm for PCM50 (**Figure 7.2c**) and 1.1 ± 1.0 μm for PCM30 (**Figure 7.2g**). Purification and separation of the ZnONWs from the templates possibly caused some breakages, leading to wide variations in the length of the wires. Although the nominal pore size was 50 nm, ZnONWs grown in PCM50 had a diameter of 140 ± 50 nm, possibly due to pore swelling during electrodeposition (**Figure 7.2e, f**). These NWs may therefore not be suitable for bio-reactivity studies, since the cut-off for cellular uptake of nanoparticles is usually considered to be less than 100 nm.⁵⁰ ZnONWs synthesized in PCM30 had a diameter of 90 ± 10 nm (**Figure 7.2h**).

7.4 Conclusions

In summary, ZnONWs were synthesized by template-free and template-assisted electrodeposition and successfully collected and purified. Using both methods, the ZnONW diameters could be tuned to the desired range, with relatively well-controlled lengths. However, the amounts of ZnONWs obtained by either method were not enough for use in *in vitro* toxicity studies. In future studies, further optimization of the protocols to minimize damage during removal of the ZnONWs from the substrates/templates, and up-scaling of the procedures to increase their yield could allow obtaining ZnONWs with controlled physicochemical properties for bioreactivity studies.

8 Conclusions and Future Work

The present thesis has explored the *in vitro* bioreactivity of Ag and ZnONWs with immortalised type 1 human alveolar epithelial (TT1) cells. The effects of the lung lining fluid (LLF), which is expected to be the pulmonary system's first line of defence for inhaled NMs, have not been previously investigated. In order to accurately predict the mechanisms of biological action of Ag and ZnONWs with the underlying epithelium, however, transformations of their physicochemical properties in the LLF must be thoroughly examined. In the present work, detailed characterization was performed, using correlative techniques that provided both dynamic information about the colloidal stability of the NWs and spatially resolved high resolution elemental analysis. This characterization allowed to deconvolute the system parameters (such as pH, phospholipids and surfactant proteins), and provide new insights on the effects of each parameter on the stability of the NWs. These effects were shown to significantly impact the reactivity of Ag and ZnONWs and the way they are internalized and processed by alveolar epithelial cells.

AgNWs have been recognised as potential occupational and environmental hazards; therefore their human health effects need to be assessed before their production and use are spread more widely. Several papers have shown that AgNWs are able to access the alveoli in the deep lung *in vivo*,^{75 345} but few data exist on their interaction with the pulmonary epithelium using immortalised type 1 human alveolar epithelial cells.³ In the present work, AgNWs were successfully synthesized by a modified polyol process, which ensured a full control over the initial physicochemistry of the material tested. The Ag⁺ ion release kinetics from AgNWs were pH-dependent, which could lead to different extents of AgNW dissolution at different stages of the endocytic pathway. However, the amounts of free Ag⁺ ions measured, were not high enough to expect a significant contribution to the overall toxicity of AgNWs, based on previous measurements on the toxicity of AgNO₃.²⁴¹ By incubating AgNWs with phospholipids, in either simple (DPPC) or more complex mixtures (Curosurf[®], LA), the *ex vivo* adsorption of surfactant lipids on AgNWs was shown for the first time. The formation of a lipid corona resulted to a delay in the kinetics of Ag⁺ ion release from AgNWs. Using TEM combined with uranyl acetate staining and EDX elemental analysis, the organization of adsorbed phospholipids on the AgNW surface was revealed. The polar headgroups of the phospholipids faced away from the AgNWs toward the aqueous phase, supporting the improved

dispersion of AgNMs in the presence of phospholipids observed in previous studies.⁴⁶⁴ Pre-incubation of AgNWs with Curosurf[®] did not significantly increase the internalization of AgNWs by human alveolar type-1-like (TT1) epithelial cells, as previous findings would suggest.²¹⁰ This finding, however, could be due to the fact that uptake by TT1 cells was already quite high for bare AgNWs (about 53% within 1 hour and 66% within 24 hours). Incubation of the AgNWs in complete *murine* LLF resulted to their agglomeration and a decreased release of Ag⁺ ions. Using combinations of separate LLF components, agglomeration of the AgNWs was ascribed to the small aggregate (SA) fraction of the LLF. This result was correlated with the specific binding of SP-A and SP-D on AgNWs, measured in a recent study by Sweeney *et al.*, where AgNWs synthesized for the present thesis were used.⁴³⁹ As a consequence of SA-induced agglomeration, pre-incubation of the AgNWs with complete LLF, in the form of alveolar surfactant harvested from human alveolar type-2 (AT2) cells, resulted in a dramatic decrease in AgNW internalization in TT1 cells. In accordance to this finding, Sweeney *et al.* showed that, pre-incubation of AgNWs with LLF, almost completely abolished the AgNW-induced inflammation and generation of reactive oxygen species (ROS) in TT1 cells. Finally, interaction of AgNWs with TT1 cells, either in the absence or presence of LLF, led to transformation of the AgNW morphology and precipitation of Ag₂S. This process could occur through the action of sulfide species that exist inside cells, such as H₂S, HS⁻ and S²⁻.³⁴⁴ As previously suggested,³ Ag₂S formation could act as a “trap” for free Ag⁺, thus significantly limiting the short-term toxicological effects of AgNWs. These findings suggest that, in contrast to other bio-persistent materials, such as asbestos, AgNWs may be degraded within the cellular environment and transform to insoluble Ag₂S.

To expand on these findings and improve our understanding on the mechanisms of interaction between inhaled AgNWs and the pulmonary epithelium, further investigation of AgNW effects on pulmonary surfactant (PS) function would be valuable. Recently, the Langmuir-Blodgett trough was used to evaluate the impact of multi-walled carbon nanotubes (MWCNTs) on PS monolayers, in their near-native state at the air-water interface.⁵³¹ MWCNTs influenced the function of PS, such as compression resistance and maximum obtainable pressure. These effects were found to be MWCNT length-dependent.⁵³¹ In another similar work, atomic force microscopy (AFM) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) revealed the topography and chemical composition of PS monolayers following interaction with polyorganosiloxane NPs.⁵⁵ TOF-SIMS, a technique for high-resolution surface and thin film analysis that enables label-free detection of chemical composition, allowed the detection of individual PS components (*e.g.* DPPC and SP-C). These techniques could therefore be applied in the case of AgNWs. Since MWCNT effects were length-dependent,⁵³¹ the AgNW synthesis protocol described here could be modified to provide samples with larger lengths (*e.g.* 10 and 20 μ m). The aspect ratio of AgNWs has also been stated to define the onset of frustrated macrophage phagocytosis, an important factor in the post-exposure inflammatory response.^{75, 532} Therefore, the effects of AgNW length on their bioreactivity with alveolar epithelial

cells could be explored, and expanded to other cell types like alveolar macrophages. These investigations could include quantitative analysis of dissolution and changes to the AgNW chemistry and structure following cellular uptake, which lacked from previous studies. Analytical and high-resolution TEM could be combined with other techniques to monitor the uptake and processing of AgNWs inside cells, such as confocal and SIMS imaging. *In vitro* studies could be performed using co-cultures of type-1 and type-2 cells, and alveolar macrophages, to provide a more physiologically relevant model. Finally, *in vivo* inhalation studies using these AgNWs would allow for further investigation of the pro-inflammatory potential of AgNWs in the lung and would allow performing a systematic assessment of the AgNW properties that contribute to lung injury, which is currently missing from the literature.

The present work has also provided new data on the impact of PS on the stability of ZnONWs, and the subsequent effects on their interaction with alveolar epithelial cells. Dissolution of ZnONWs was strongly pH-dependent, suggesting that their toxicity is likely triggered by rapid intracellular dissolution. Dissolution kinetics was similar for both lengths of ZnONWs tested, possibly due to rapid agglomeration of the wires. In accordance to this finding, both lengths of ZnONWs had the same effects on the viability of TT1 cells, which were comparable to that of Zn^{2+} ions. Using similar techniques as with AgNWs, the formation of a phospholipid corona was demonstrated for ZnONWs incubated with Curosurf[®]. This lipid corona decreased the dissolution kinetics of ZnONWs at pH 5 and prevented their agglomeration, possibly through contributions of both steric and charge stabilization. Moreover, confocal imaging suggested that the coating of ZnONWs by a phospholipid corona slowed down their dissolution kinetics intracellularly, which also delayed the onset of cellular damage. However, pre-incubation of ZnONWs with PS led to uptake of the ZnONWs by a higher percentage of TT1 cells; this increase in cellular uptake dominated over their delayed intracellular dissolution and resulted to a higher reduction in cell viability after 24 hours. Incubation of ZnONWs with serum also prevented their agglomeration, likely through stabilization by albumin, which adsorbs to the surface of the wires creating a protein corona.⁴⁹⁵ However, in contrast to Curosurf[®], serum had no effect on the uptake or toxicity of ZnONWs to TT1 cells.

The contrasting effects between Curosurf[®] and serum on the toxicity of ZnONWs with alveolar epithelial cells indicate that the nature of NP corona may have varied effects on ZnONW bioreactivity, and therefore require further investigation. In the case of AgNWs, the small aggregate (SA) fraction of the LLF was the component that had the most pronounced impact on their uptake and toxicity, but these effects have not been investigated in the case of ZnONWs. Therefore, the experiments presented here could be extended to include the complete LLF and its separate fractions, as well as other cell types like alveolar macrophages. Approaches relying on multiple cell co-cultures and experimental setups that may be used at the air-liquid interface could also be explored, to understand how the full LLF influences cell uptake, and to represent more realistic exposure scenarios of ZnONW inhalation. Furthermore, the electrochemical deposition methods presented here for the

synthesis of ZnONWs could be further optimized and scaled-up, to provide ZnONWs with well-defined physicochemical properties for these studies. Ultimately, ZnONWs should be tested by *in vivo* inhalation studies, to explore their effects on lung function and inflammation, which have not been previously investigated. Iron doping³⁵⁷ and silica coating³⁹⁶ of ZnONPs have been previously shown to reduce the dissolution of the particles and consequently their toxicity. Similar strategies could be investigated to synthesize safer-by-design ZnONWs. Recently, ZnONWs synthesized by vapour deposition exhibited intrinsic fluorescence, which was used for targeted imaging of cancer cells.⁴⁸⁵ The fluorescence properties of ZnONWs could thus be employed to dynamically track their uptake and dissolution within cells, *e.g.* by confocal microscopy. Finally, the use of ZnONWs as optical signal enhancing platforms for *in vitro* biodetection could be explored, as recent developments suggest that 1D ZnO NMs have the potential to contribute a significant impact to this field.⁴⁸²

Apart from concerns about the potential toxic effects of Ag NMs, their well-known antibacterial properties could lead the way for the development of novel therapeutic agents for bacterial infections of the pulmonary system. In the present work, exposure of *M.tb* to AgNPs led to a wide range of ultrastructural damage of the bacterial cells, such as cell wall disorganization, leakage of intracellular contents and possibly DNA damage. Bacterial damage was also observed when AgNPs were concurrently applied with *M.tb* infection in alveolar macrophages. These observations could be due to interaction between AgNPs and the bacterial cell wall, through a synergistic particulate and ionic effect. Although further investigation is required to completely elucidate these effects, this work demonstrates that AgNPs represent useful candidates for the treatment of tuberculosis, and could be used to inform the design of future experiments.

Future work in this area could focus on improving our understanding on the stability of AgNPs and the kinetics of Ag⁺ ion release at the nano-bio interface. Environmental SEM using hydrated cells may be applied for dynamic “*in-situ*” characterization of the particle-cell interface, providing valuable information about the interactions of AgNPs with cellular components. SIMS, which has the potential to examine the functional segregation of lipids in cell membranes, could be used to investigate the mechanisms of bacterial cell wall damage induced by AgNPs. The experiments on *M.tb*-infected cells described in the present work were performed with the THP-1 cell line, but the extent to which THP-1 cells mimic macrophages *in vivo* is not fully understood. Hence, future work could be extended to other cell types such as primary alveolar macrophages. Moreover, a more comprehensive investigation of the cellular uptake and localization of AgNPs in macrophages is required, using particles of different sizes and surface coatings or charges. As the work on Ag and ZnONWs demonstrated, the LLF could have significant effects on their physicochemical properties and their subsequent toxicity. These effects should therefore be investigated for AgNPs, to guide the design of effective and safe therapeutics. Along with TEM, imaging techniques with a higher field of view (*e.g.* confocal microscopy) or biological assays could provide quantitative information on the bacterial ingestion and killing by macrophages in the absence and presence of AgNPs. Once *in vitro*

systems have been optimized, animal models, which mimic the whole spectrum of susceptibility and resistance of human populations to tuberculosis, would be useful for testing AgNPs as prospective therapeutic agents.

Ultimately, the data generated through this study could be used to inform regulators or government agencies on how to establish accurate risk assessment procedures for Ag and ZnO NMs. These procedures would allow appropriate regulation of these classes of NMs, to ensure that safe products are produced and the hazards associated with their handling are minimized. Finally, this work may also be useful in the area of nanomedicine, informing the design of efficient and safe nanodrugs.

9 Appendix A

9.1 Optimization of AgNW synthesis protocol

For the synthesis of silver nanowires, the polyol process developed by Xia's group was used as a basis⁴⁴⁰ and optimized to obtain pure AgNWs with controlled size distributions. The protocol for the standard synthesis is described in **Chapter 4.2.1**. To investigate the effect of Cl^- in the formation of AgNWs, syntheses were performed in ambient atmosphere, in the absence and presence of a trace amount of NaCl in the reaction mixture (72 μM in final reaction volume). In both cases, PVP with a molecular weight of 55k was used and the molar ratio of PVP (in terms of monomeric units) to AgNO_3 was 1.5. The reaction was carried out for 22 h and aliquots were removed from the reaction solution at several time points in order to study the morphological evolution of the particles. **Figure 9.1** shows SEM images of the samples collected from the reaction mixture 10 min, 1 h, 1.5 h and 22 h after PVP and AgNO_3 were added.

In the absence of Cl^- , both spherical particles and nanorods were formed after 10 minutes (**Figure 9.1a**). The observation that some particles are formed even after 10 minutes of reaction indicates that the reducing reaction of silver ions is very rapid. EG has been shown to have a high reducing power at 160 °C, with more than 80% of silver being reduced after heating with PVP for 10 minutes.⁵³³ As the reaction proceeds (**Figure 9.1b-d**), the number and size of particles increased. However, there are practically no high-aspect ratio AgNWs observed, only a few rods with small lengths. These findings illustrate that Cl^- has an important role in the polyol synthesis of AgNWs. Cl^- has been suggested to adsorb on seeds and provide electrostatic stabilization of the seeds formed at the initial stage of Ag reduction.⁵³⁴ When no Cl^- is present, Ag seeds quickly aggregate and form large irregular particles (**Figure 9.1e**), which grow at the expense of nanorods.

In the presence of Cl^- , after 10 min (**Figure 9.1e**) the sample was composed of a mixture of spherical and rod-shaped particles. The formation of rod-shaped particles depends on the slow addition rate of the reactants and the use of PVP as a coordination agent. A slow addition rate ensures that multiply twinned particles formed at the initial stage of the reduction process are thermodynamically stable.¹⁷⁶ Preferential adsorption of PVP on different crystallographic planes of

these particles leads to the formation of nanorods.¹⁷⁶ After 1 h, rod-shaped particles increased in length and formed silver nanowires (**Figure 9.1f**). The size of spherical nanoparticles also increased but a lower population was observed compared to nanowires. After 1.5 h, the product consisted of high-aspect ratio AgNWs and only a very small number of particles, possibly due to Ostwald ripening, which causes the larger-sized nanowires to grow at the expense of smaller spherical particles (**Figure 9.1g**)

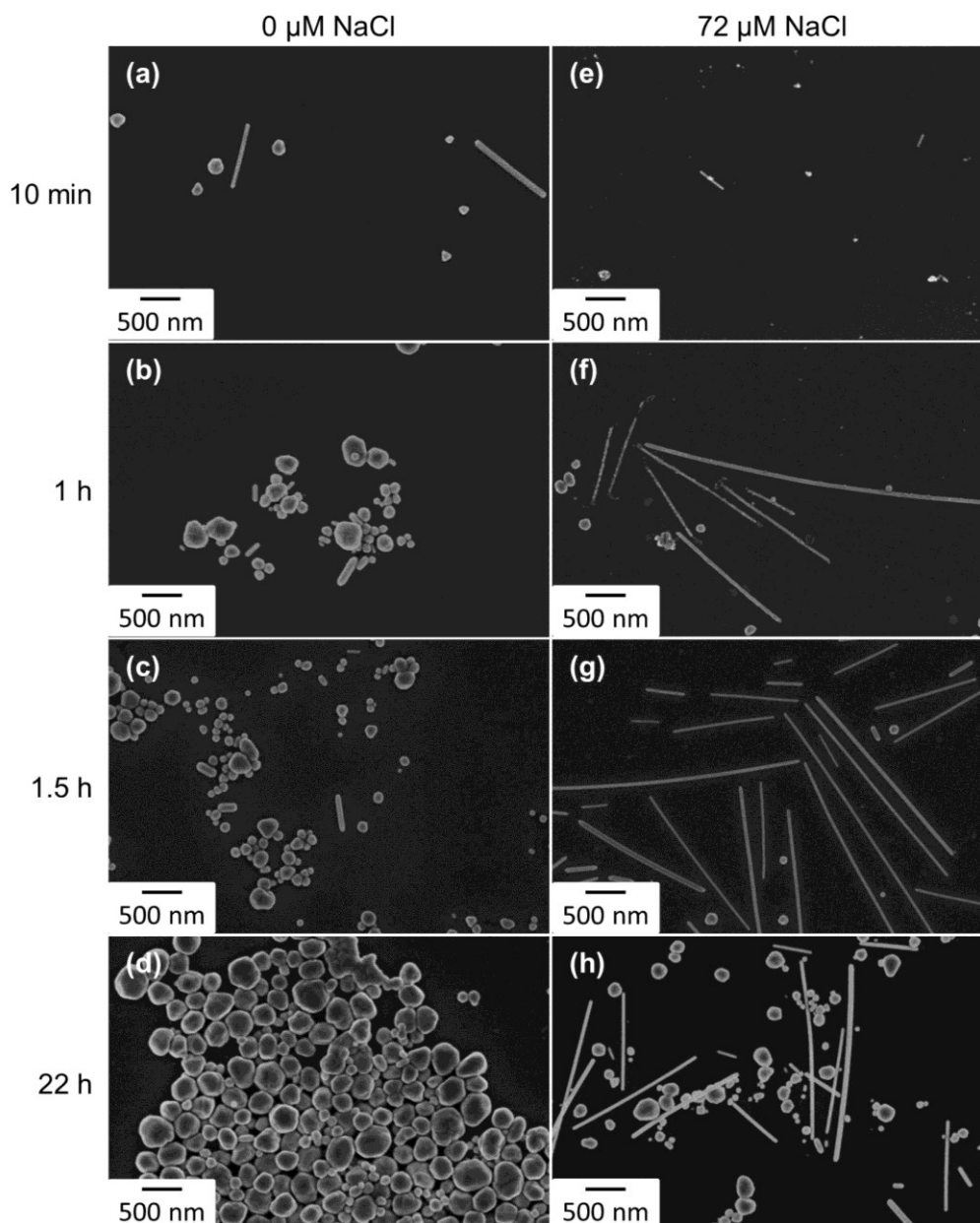


Figure 9.1 (a-h) SEM images of Ag nanostructures obtained by a polyol process at 160 °C, using PVP with $M_w \approx 55k$, a $[PVP]/[AgNO_3]$ ratio equal to 1.5, in the absence (a-d) and presence of 72 μM NaCl (e-h). Reaction times were 10 min (a, e), 1 h (b, f), 1.5 h (c, g) and 22 h (d, h).

It was hypothesized that after prolonged reaction times, Ostwald ripening would cause an increase in the yield of nanowires and eliminate the number of nanoparticles. However, after 22 h, the number and size of spherical particles increased, whereas the yield of nanowires decreased (**Figure 9.1g**). This observation may be due to the fact that the reaction was performed in ambient atmosphere. The presence of chloride together with O₂ has been suggested to enhance the oxidative dissolution of silver.⁵³⁵ Continuous dissolution and reprecipitation of Ag may lead to the formation of nanoparticle morphologies without the multiply twinned structure, which grow to irregular particles through Ostwald ripening. When higher concentrations of NaCl were used (360 or 1440 μ M in final reaction volume), very few AgNWs had been formed after 1.5 h in the case of 360 μ M NaCl (**Figure 9.2a**), and only small AgNPs in the case of 1440 μ M NaCl (**Figure 9.2b**). The increase in the amount of Cl⁻ led to higher oxidative etching and the formation of irregular particles. The above results suggest that the optimum reaction time for the formation of AgNWs was 1.5 hours, which is consistent with the literature, where reaction times between 1 and 1.5 hours have been reported.

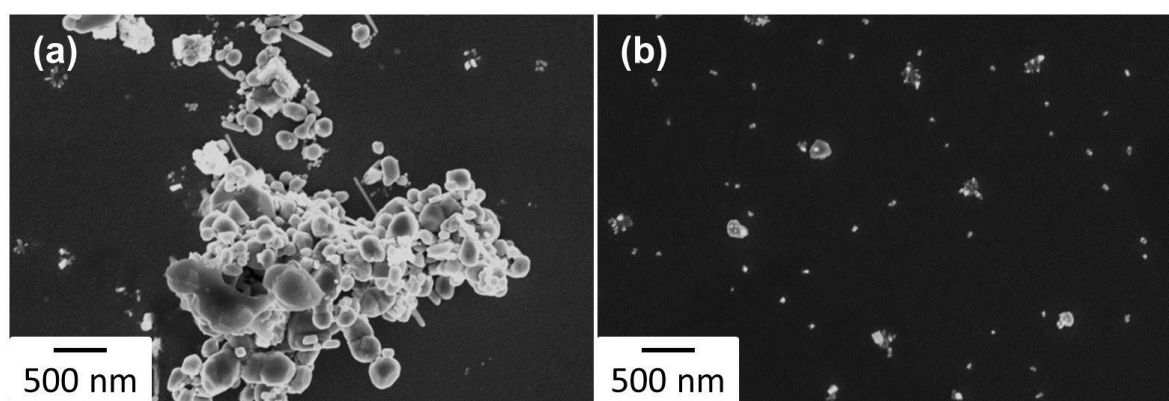


Figure 9.2 (a-b) SEM images of Ag nanostructures obtained by a polyol process at 160 °C, using PVP with $M_w \approx 55k$, a [PVP]/[AgNO₃] ratio equal to 1.5, in the presence of 360 μ M (a) or 1440 μ M (b) NaCl.

The role of the average molecular weight of PVP on the yield and quality of AgNWs was also investigated. PVP with three different molecular weights was used: $M_w \approx 55k$, 360k and 1300k (indicated 55PVP, 360PVP and 1300PVP respectively). In all cases, 72 μ M of NaCl was included in the reaction and the molar ratio of PVP to AgNO₃ was 1.5. SEM imaging of the synthesis products showed that AgNWs were formed in all cases. With 55PVP, the product was a mixture of NPs and NWs (**Figure 9.3a**). When 360PVP was used, the ratio of NWs to NPs increased and the NWs obtained had a higher aspect ratio, compared to 55PVP (**Figure 9.3b**). PVP is known to preferentially adsorb on different faces of the Ag seeds formed at the initial stages of the reaction.¹⁷⁶ With 55PVP, the PVP molecules may not be long enough to sufficiently passivate different faces on the Ag seeds, which could then experience isotropic growth and evolve into spherical NPs. On the other hand, the

much higher length of 1300PVP molecules possibly hinders the addition of silver atoms on crystal seeds. Consequently, aggregated particles and nanorods with irregular shapes were formed (**Figure 9.3c**). Since 360PVP produced the highest yield of AgNWs as well as a smaller number of AgNPs particles, all further studies were carried out using 360PVP.

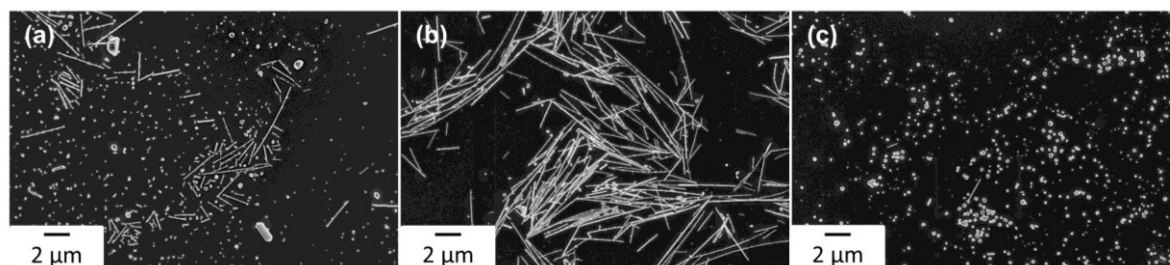


Figure 9.3 (a-c) SEM images of Ag nanostructures obtained by a polyol process at 160 °C, using PVP with $M_w \approx 55k$ (a), $M_w \approx 360k$ (b), and $M_w \approx 1300k$ (c). The [PVP]/[AgNO₃] ratio was 1.5 and 72 μM NaCl were used.

The effect of molar ratio of PVP to AgNO₃ to Ag nanostructures was also examined. Silver nanostructures were synthesized with 360PVP and 72 μM NaCl, with a [PVP]/[AgNO₃] ratio of 1.5 and 3.0 and 6.0. Since a low precursor concentration is important in the polyol process,⁴⁴⁰ the concentration of AgNO₃ was kept the same as in previous syntheses and the ratio was varied by changing the concentration of PVP. For a [PVP]/[AgNO₃] ratio of 1.5 and 3.0, a high yield of AgNWs was obtained (**Figure 9.4a-d**). Characterization of the AgNW dimensions showed that, with increasing PVP concentration, the aspect ratio of the AgNWs also increased (**Table 2.1**). These findings indicate that the aspect ratio of AgNWs can be controlled by changing the concentration of PVP. However, a large increase in PVP concentration led to a product consisting of AgNWs as well as several other AgNP morphologies (**Figure 9.4e, f**). Therefore, the presence of PVP to a large excess may lead to the tight coverage and stabilization of many types of the Ag seeds formed during nucleation, leading to the growth mode of several particle morphologies.

Although AgNWs were successfully prepared using the polyol process, the synthesized products were a mixture of AgNWs with AgNPs. The bioreactivity of AgNPs largely depends on their physicochemical properties; therefore samples consisting of pure AgNWs were required. Since the above experiments were performed in air, an enhanced oxidative etching of the multiply twinned seeds at the presence of O₂ and Cl⁻ may have taken place. A decrease in the population of multiply twinned seeds at the initial stages of the reaction leads to a decrease in the population of AgNWs, while the population of particles with other morphologies increases. To eliminate other morphologies from the final product, all the reactants were purged with argon prior to following syntheses and the reaction was carried out under argon atmosphere. Complete physicochemical characterization of the obtained AgNWs is described in **Chapter 4.3.1**.

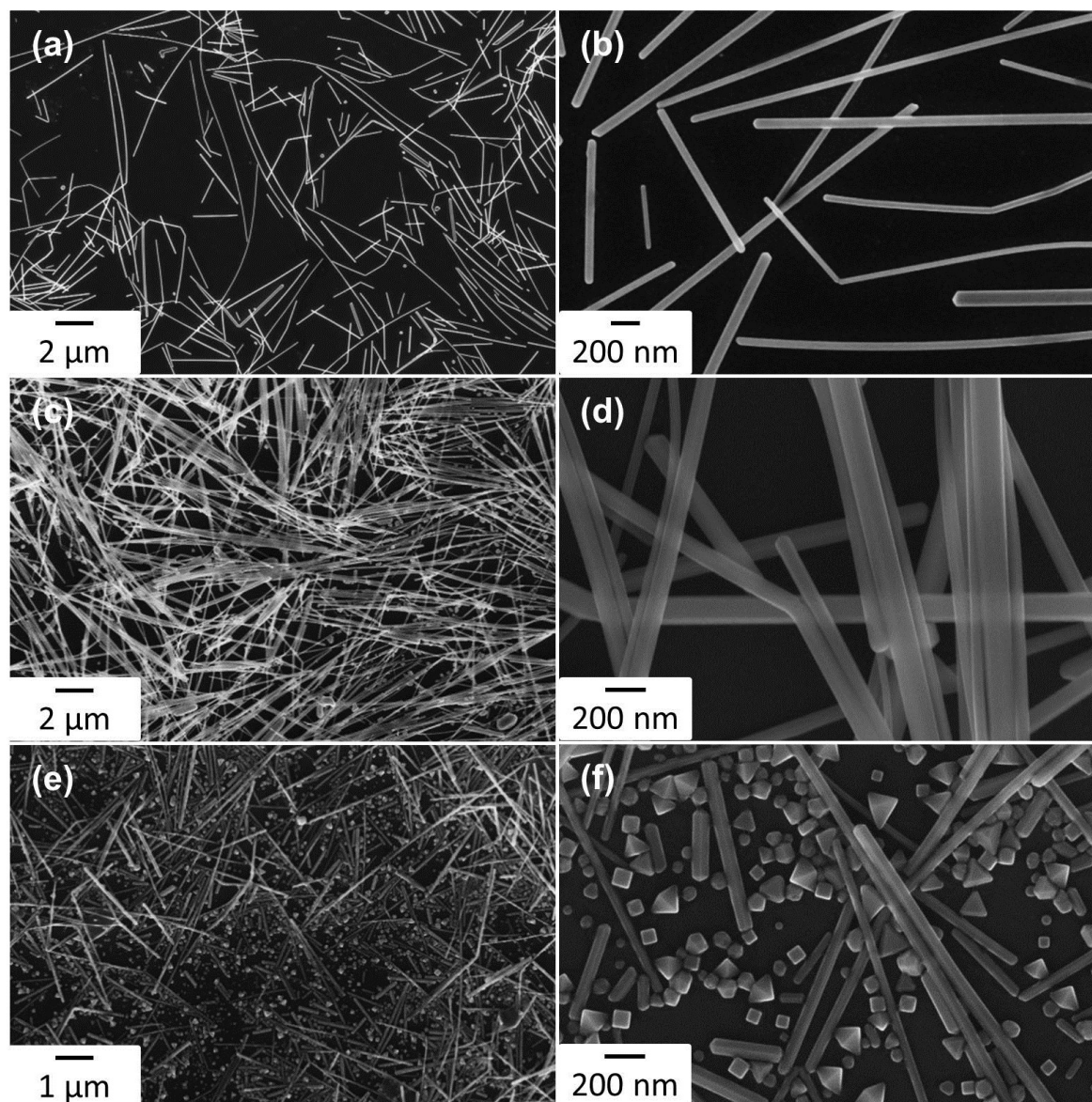


Figure 9.4 SEM images of Ag nanostructures obtained by a polyol process at 160 °C, using PVP with $M_w \approx 360k$, 72 μM NaCl, and a [PVP]/[AgNO₃] ratio equal to 1.5 (a, b), 3.0 (c, d) and 6.0 (e, f).

[PVP]/[AgNO ₃]	Length (μm)	Diameter (nm)	Aspect Ratio
1.5	1.5 ± 1.4	79 ± 21	18.99
3.0	10.5 ± 8.5	73 ± 37	143.8

Table 9.1 Dimensions of AgNWs synthesized by a polyol process at 160 °C, using PVP with $M_w \approx 360k$, 72 μM NaCl, and a [PVP]/[AgNO₃] ratio equal to 1.5 or 3.0.

9.2 AgNW TEM sample storage

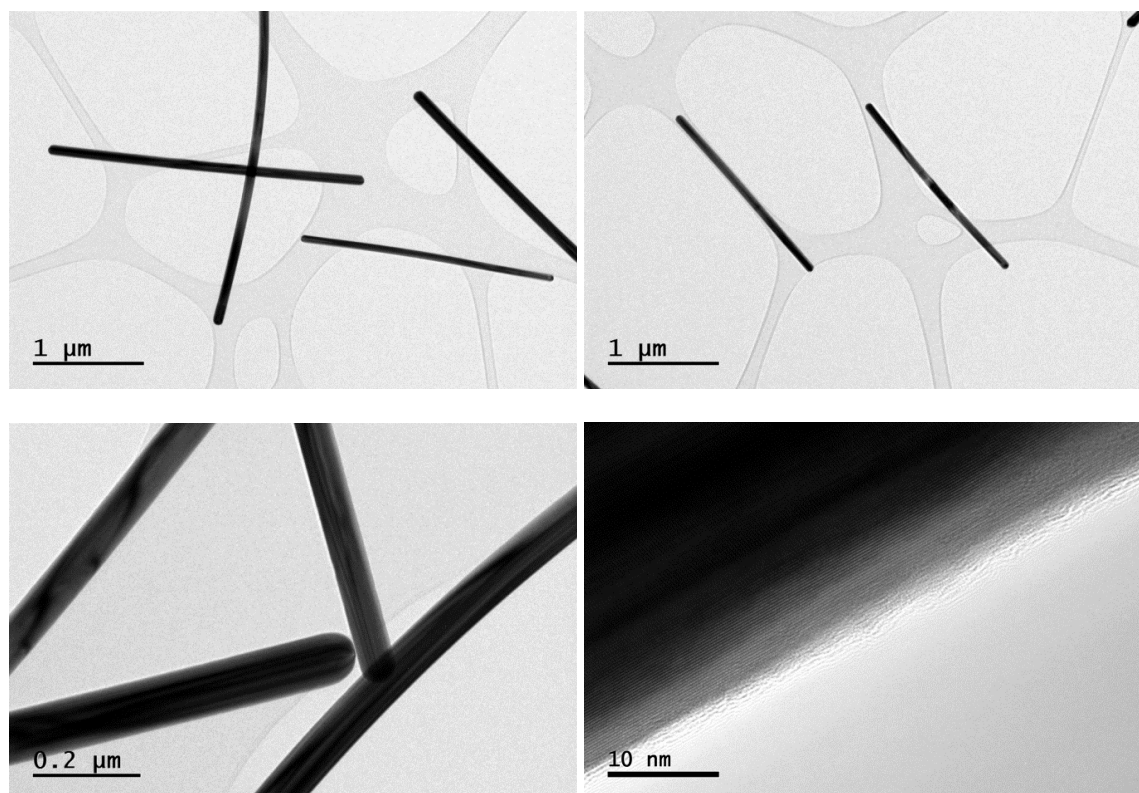


Figure 9.5 TEM images of a sample prepared from as-synthesized AgNWs, imaged after a 3-month storage period under vacuum and in the dark.

9.3 AgNW sample filtration

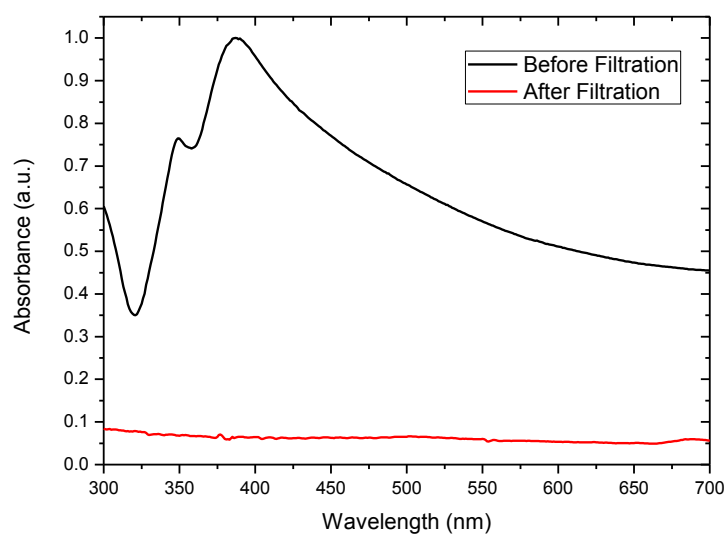


Figure 9.6 UV-Vis spectra of AgNWs incubated at pH 7 for 24 hours, before and after centrifugation at 13000×g for 10 minutes through 2 kDa centrifuge membranes.

9.4 AgNW incubation with DPPC

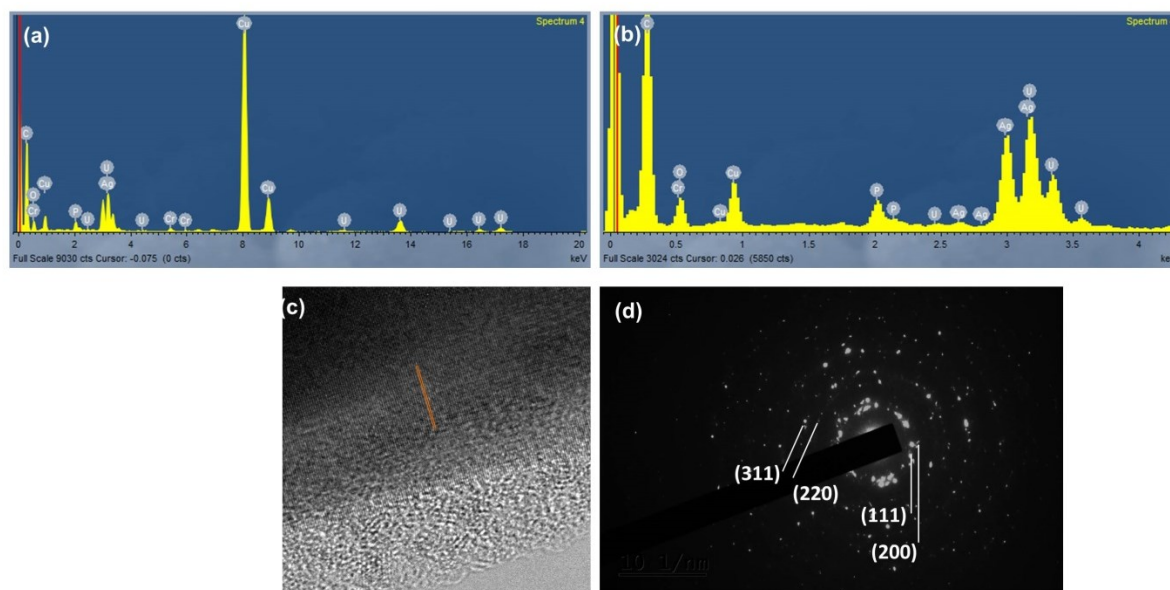


Figure 9.7 (a-b) STEM-EDX spectrum collected from the circled area imaged in **Figure 4.5a**. (c) HRTEM image and (d) SAED pattern collected from AgNWs incubated with DPPC at pH 7 (same sample as **Figure 4.5a-e**). The interplanar spacings in the SAED pattern correspond to the (111), (200), (220) and (311) planes of the bcc form of bulk Ag (ref. # 01-087-0597), confirming that the crystallography of AgNWs incubated with DPPC has not changed.

9.5 The crystal structure of silver and various silver compounds

h	k	l	d (Å)	2Theta (deg)	Intensity (%)
<i>Ag : Cubic, ICSD ref. (01-087-0597)</i>					
1	1	1	2.35917	38.115	100
2	0	0	2.0431	44.299	45.7
2	2	0	1.44469	64.443	22.5
3	1	1	1.23204	77.397	22.2
<i>Ag₂O : Cubic, ICSD ref. (01-041-1104)</i>					
1	1	1	2.7290	32.791	100
2	0	0	2.3620	38.067	28
<i>AgCl : Cubic, ICSD ref. (01-031-1238)</i>					
1	1	1	3.20300	27.831	50
2	0	0	2.77400	32.244	100
2	2	0	1.96200	46.234	50
<i>Ag₃PO₄ : Cubic, ICSD ref. (00-006-0505)</i>					
2	1	0	2.68900	33.293	100
2	1	1	2.45400	36.588	35
3	2	0	1.66760	55.022	20
<i>Ag₂S: Monoclinic, ICSD ref. (00-014-0072)</i>					
-1	1	1	3.437	25.902	35
0	1	2	3.383	26.323	20
1	1	1	3.08	28.967	60
-1	1	2	2.836	31.521	70
1	2	0	2.664	33.614	45
-1	2	1	2.606	34.385	100
0	2	2	2.583	34.701	70
1	1	2	2.456	36.557	70
1	2	1	2.44	36.806	80
0	1	3	2.421	37.105	60
-1	0	3	2.383	37.719	75
0	3	1	2.213	40.74	45
2	0	0	2.083	43.407	45
-1	2	3	1.963	46.209	20

Table 9.2 The crystal structure of silver and various silver compounds based on the Inorganic Crystal Structure Database. Only the main peaks, with intensity ≥ 20 % are listed.

10 Appendix B

10.1 Effect of embedding procedure on ZnONW stability

A 50 μL mixture of ZnONWs (25 $\mu\text{g/mL}$) in 1 % Agarose (low gelling temperature, BioReagent, for molecular biology, Sigma) was prepared at 40 $^{\circ}\text{C}$ and allowed to gel at 4 $^{\circ}\text{C}$. Gel pieces were subsequently fixed, dehydrated and embedded in Quetol-based resin (without treatment with osmium tetroxide or bulk staining with uranyl acetate) as described in the section *TEM statistical analysis of S-ZnONW uptake* of the main manuscript. Thin sections (90 nm) were cut from the resin blocks, collected on bare 300 mesh gold TEM grids (Agar Scientific) and analysed with a JEOL 2000 TEM

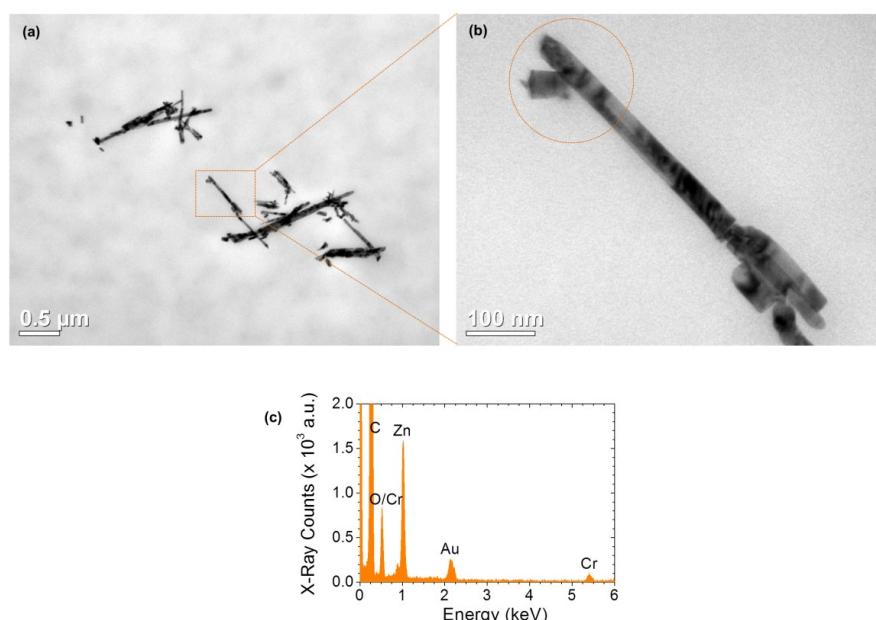


Figure 10.1 Electron microscopy characterization illustrates the effect of the fixation, dehydration and embedding procedure on the stability of ZnONWs. No heavy metal staining was used. ZnONWs were embedded with Quetol and thin sections (90 nm) were collected for TEM imaging and analysis. (a, b) Bright field transmission electron microscopy (BFTEM) images and energy dispersive X-Ray (EDX) spectrum (c) collected from the area circled in (b). This analysis demonstrated that the TEM sample preparation processes did not alter the morphology or chemistry of the ZnONWs.

10.2 ZnONW SAED patterns

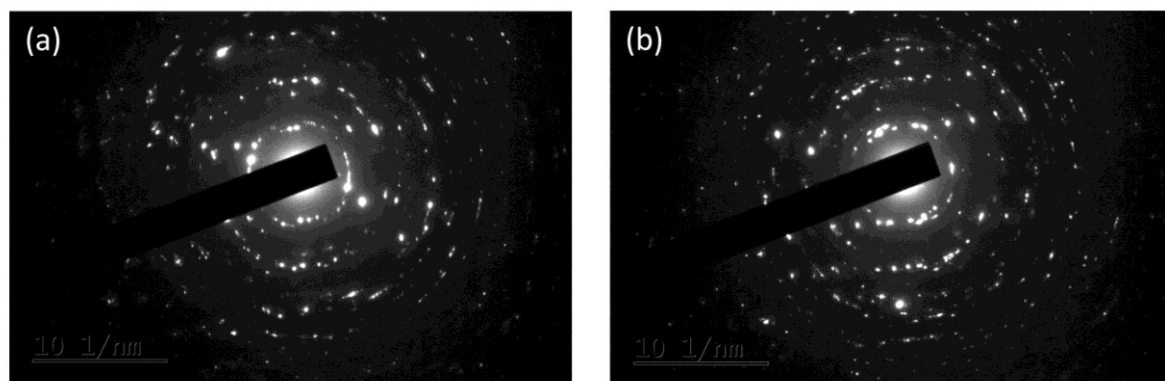


Figure 10.2 (a-b) Selected area electron diffraction (SAED) patterns collected from areas containing several S-ZnONWs (a) and L-ZnONWs (b). The interplanar spacings measured from SAED patterns were $2.83 \pm 0.02 \text{ \AA}$, $2.59 \pm 0.06 \text{ \AA}$, $1.95 \pm 0.04 \text{ \AA}$, $1.61 \pm 0.06 \text{ \AA}$ and $1.45 \pm 0.03 \text{ \AA}$ for S-ZnONWs and $2.82 \pm 0.04 \text{ \AA}$, $2.57 \pm 0.05 \text{ \AA}$, $1.92 \pm 0.05 \text{ \AA}$, $1.59 \pm 0.05 \text{ \AA}$ and $1.47 \pm 0.04 \text{ \AA}$ for L-ZnONWs. These values correspond to the (100), (002), (102), (110) and (103) planes of bulk wurtzite ZnO (JCPDS #89-1397).

10.3 ZnONW incubations with PS

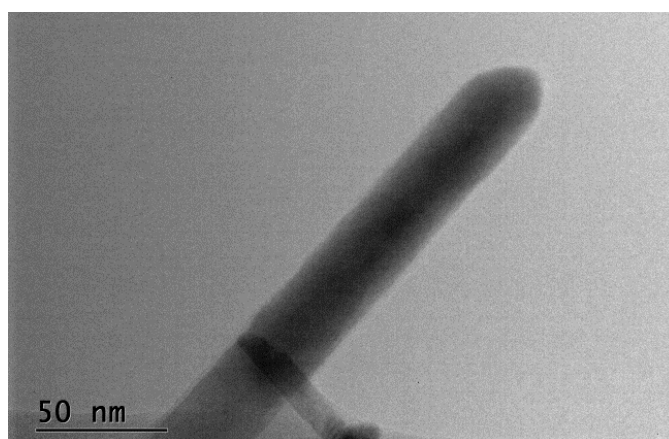


Figure 10.3 BF-TEM image of S-ZnONWs incubated in NS with Curosurf, at pH5 for 10 seconds.

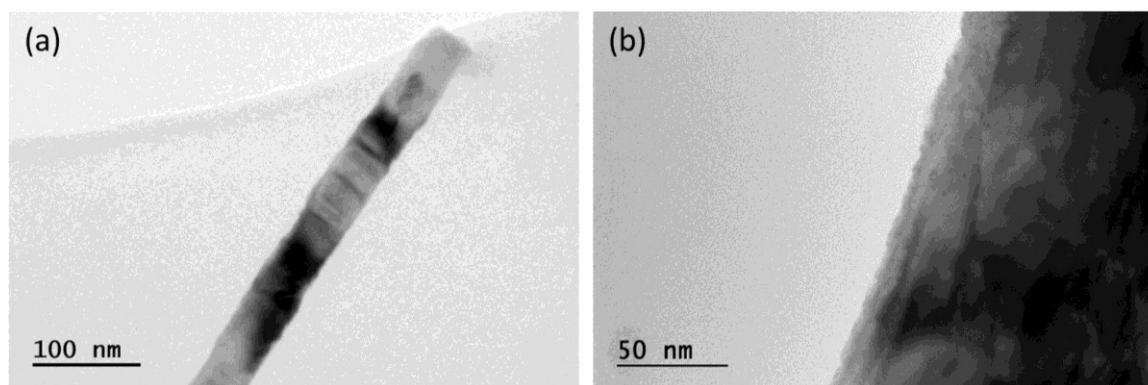


Figure 10.4 (a-b) BF-TEM images of S-ZnONWs incubated in NS with DPPC, at pH5 for 24 hours, stained with uranyl acetate to enhance phospholipid contrast.

10.4 TEM uptake of ZnONWs

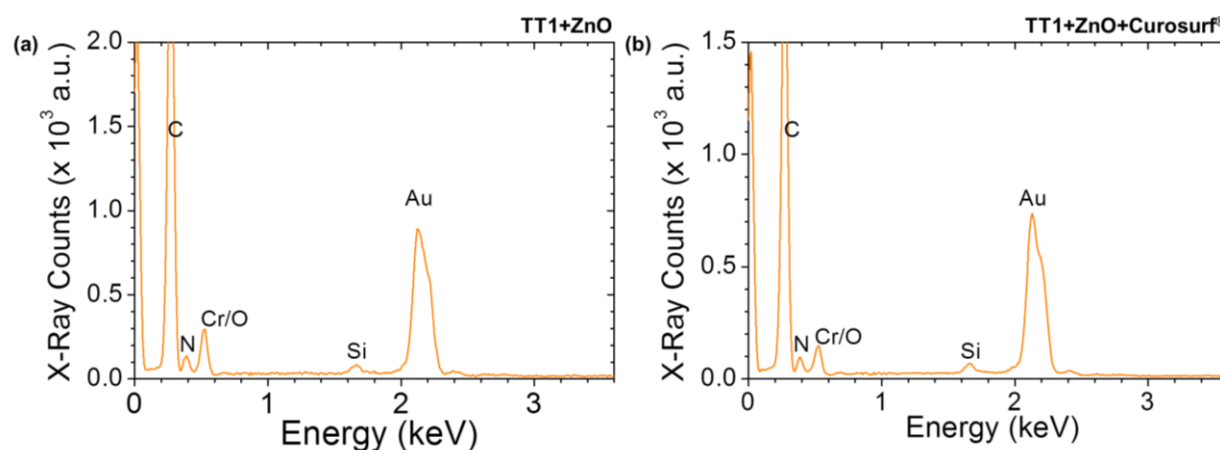


Figure 10.5 (a-b) TEM-EDX spectra collected from areas in the extracellular space of the samples shown in Figure 6d, h, of TT1 cells exposed to S-ZnONWs in the absence (a) or presence (b) of Curosurf[®], showing that Zn peaks were not detected extracellularly.

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