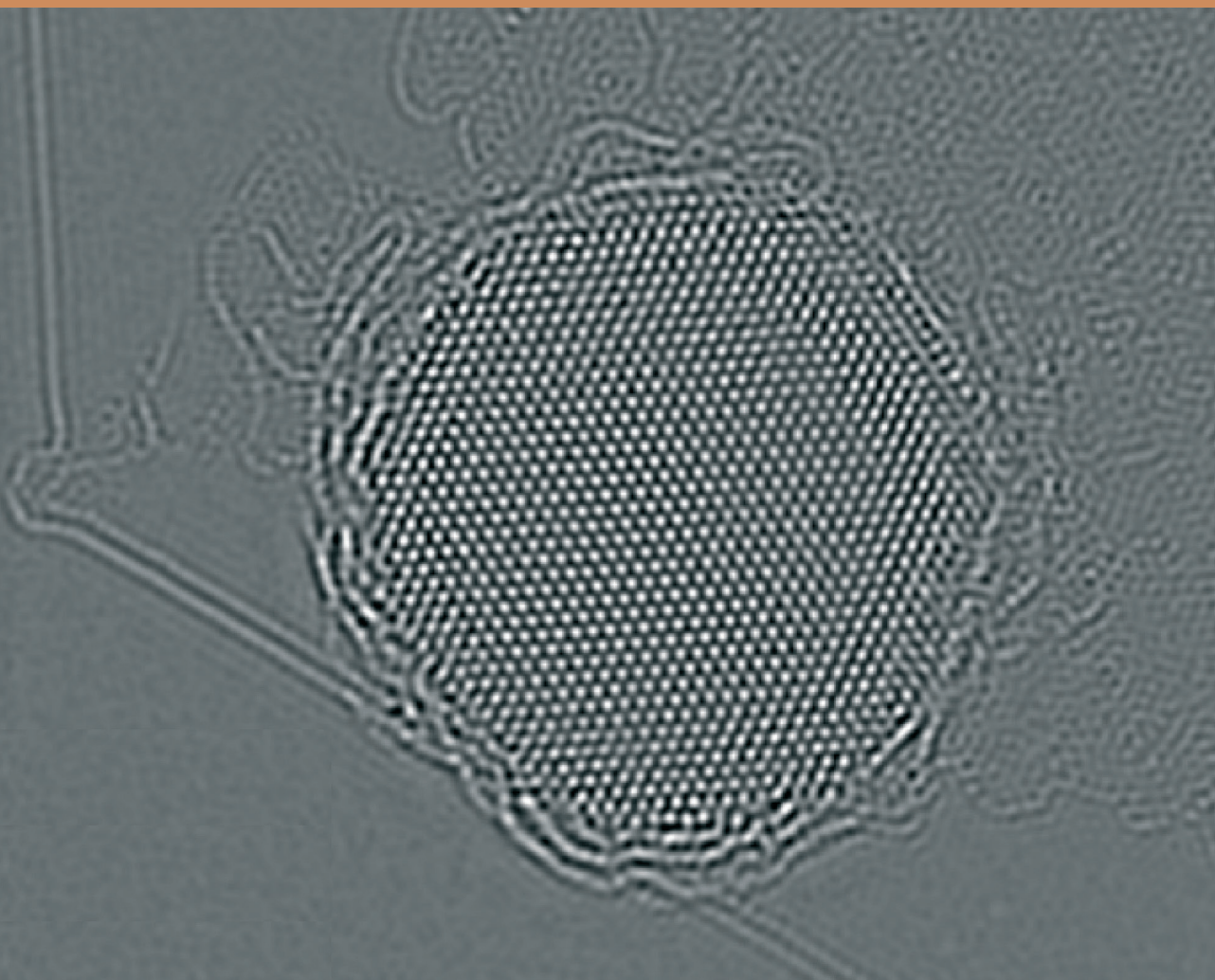


SERBIAN ACADEMY OF SCIENCES AND ARTS
СРПСКА АКАДЕМИЈА НАУКА И УМЕТНОСТИ



FASCINATING WORLD OF NANOSCIENCES
AND NANOTECHNOLOGIES
ФАСЦИНАНТНИ СВЕТ НАНОНАУКА
И НАНОТЕХНОЛОГИЈА

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ЦИКЛУС ПРЕДАВАЊА

Књига 6

ФАСЦИНАНТНИ СВЕТ НАНОНАУКА И НАНОТЕХНОЛОГИЈА

Примљено на I скупу Одељења техничких наука,
одржаном 22. јануара 2020. године

Уредници

ВЕЛИМИР Р. РАДМИЛОВИЋ

Српска академија наука и уметности

и

ЏЕФ Т. М. ДЕХОСОН

Холандска краљевска академија наука и уметности

БЕОГРАД 2020

SERBIAN ACADEMY OF SCIENCES AND ARTS

LECTURE SERIES

Book 6

FASCINATING WORLD OF NANOSCIENCE
AND NANOTECHNOLOGY

Accepted at the 1st meeting of the Department of Technical Sciences
held on January 22, 2020

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BELGRADE 2020

Published by
Serbian Academy of Sciences and Arts
Belgrade, 35 Kneza Mihaila St.

Издаје
Српска академија наука и уметности
Београд, Кнеза Михаила 35

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Technical editor
Nikola Stevanović

Технички уредник
Никола Стевановић

Print run
400 copies

Тираж
400 примерака

Printed by
Planeta print

Штампа
Планета принт

ISBN 978-86-7025-859-4

ISBN 978-86-7025-859-4

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FASCINATING WORLD OF NANOSCIENCE AND NANOTECHNOLOGY

Researchers whose work has led to significant discoveries, looking much further, beyond the immediate resolution of technical problems, are asking themselves important questions such as: why individual phenomena occur, how they develop, and why they work. In order to enhance our knowledge about the world around us, and to see pictures of worlds that elude the human eye, through history many experimental and theoretical methods have been developed and are still being improved, including the development of telescopes and microscopes, which enable us to see "very large" and "very small" things.

Researchers involved in the "big things" (the universe, galaxies, stars and planets) have found that a galaxy of an average size of about 100,000 light-years has, on average, around one quadrillion (10^{15}) stars. Researchers involved in the "little things" (nanostructures, molecules, clusters of atoms, individual atoms, atomic defects, etc.) have discovered that 1 cm³ of aluminum alloys also contains approximately one quadrillion (10^{15}) nanoparticles that strengthen these alloys in order to be utilized as a structural material for aircrafts, without which modern transport is unimaginable. How do we count the number of stars in a galaxy or the number of nanoparticles in an aluminum alloy? Relatively easy, because we can see the nanoparticles in aluminum alloys using electron microscopes, and stars in a galaxy using telescopes. Scientific discoveries form the basis for scientific and technological progress, and one such example are the discoveries in the fields of nanosciences and nanotechnologies.

Why is this monograph dedicated to nanosciences and nanotechnologies?

To answer this question, we must first answer the question: what are nanoscience and nanotechnology? In the inevitable *Wikipedia*, *Encyclopedia Britannica* (and any other encyclopedia), dictionaries as well as internet sources, the terms "nanoscience" and "nanotechnology" are related to the study, understanding, controlled manipulation of structures and phenomena, and the application of extremely small things, which have at least one dimension less than 100 nm. Modern aspects of nanosciences and nanotechnologies are quite new and have been developing intensively in the last twenty to thirty years, but the nanoscale substances have been used for centuries, if not millennia. Particulate pigments, for example, have been used in ancient China, Egypt, etc., several thousands of years ago. Artists have decorated windows in medieval churches using silver and gold nanoparticles of various sizes and composition, without understanding the origin of the various colors. Nanoparticles that strengthen alloys of iron, aluminum and other metals, have been used for over a hundred years, although they have not been branded with a prefix "nano", but rather called "precipitates". Scientific disci-

plines, involved in significant research activities related to nanoscience and nanotechnology, are: physical metallurgy, materials science and materials engineering, chemistry, physics, biology, electrical engineering, and so on.

Where does the prefix "nano" come from? "Nano" comes from the Greek words *vāvoç*, which means a dwarf, indicating a dimension of one nanometer (1 nm), which represents one-billionth (10^{-9}) of a meter; Similarly, "nanosecond" (ns) denotes a billionth of a second, and so on. This sounds a bit abstract to many, but to put things into context with which we are familiar, we can mention that the diameter of a human hair, for example, is on average about 100.000 nm (10^5 nm = 100 microns = 0.1 mm), which is roughly the bottom threshold of human eye detection; Thickness of newsprint on average is also about 100.000 nm = 100 μ m = 0.1 mm; Person of 2 m height is 2.000.000.000 (2×10^9) nm high. For comparison, if we assume that the diameter of a children's glass marble was 1 nm, then the diameter of the Earth would be 1 m.

When we talk about the structures of inorganic, organic and bio-nanosystems, their dimensions are as follows: Diameter of carbon atom is in the order of 0.1 nm, or one-tenth of a billionth of a meter; Single-wall carbon nanotubes have a diameter of around 2 nm, or 2 billionth of a meter; The width of the deoxyribonucleic acid (DNA) chain is also about 2 nm, or 2 billionths of a meter; Proteins, which can vary in size, depending on how many amino acids they are composed of, are in the range mainly between 2 and 10 nm, or between 2 and 10 billionths of a meter (assuming their spherical shape); Diameter of individual molecules of hemoglobin is about 5 nm, or 5 billionths of a meter.

Indeed, these are small sizes, but why should they be important, or why does size matter? When analyzing physical systems on the nanoscale, their fundamental properties change drastically. Consider the example, melting point of gold: transition temperature of solid to liquid for gold nanoparticles ~ 4 nm in size, is about 400°C, while the melting temperature of bulk (macroscopic) gold is 1063°C. The same can be said for other properties: mechanical properties, electric conductivity, magnetism, chemical reactivity, etc., also may be drastically changed, which means that nanosystems deviate from the laws of classical physics that describe the motion of the planets, the direction of movement of a rockets which carry satellites to explore space, etc. The base of this fascinating behavior of nanostructures are bonds between the atoms. As structures become smaller, more atoms are present on the surface, hence the ratio of the surface area to volume for these structures increases dramatically. It results in a dramatic change of physicochemical properties of nanostructures from the bulk, as well as possible appearance of quantum effects: nanoscale structures become stronger, less brittle, demonstrate enhanced optical and catalytic properties, and generally, are very different compared to the usual, macroscopic system dimensions to which we are accustomed to in everyday practice.

This monograph comprises a number of contributions which illustrate the sparkling and fascinating world of nanoscience and nanotechnology.

Nanoporous organometallic materials, that can mimic the properties of muscles upon outside stimuli, are ideal actuators, thereby offering a unique combination of low operating voltages, relatively large strain amplitudes, high stiffness and strength. These phenomena are discussed in the manuscript of J. Th. M. DeHosson and E. Detsi.

Drugs in nanodimension range will become much more efficient with reduced adverse effects. A typical example are drugs, carried by various types of nanoparticles which have been previously functionalized, so as to only recognize diseased cells which is a highly selective medical procedure on a molecular level. Besides drugs, functionalized nanoparticles can carry radioactive material or a magnetic structure, which in a strong magnetic field develop high temperatures, and destroy cancer cells. Some aspects of electron microscopy utilized in the study of biological nanostructures are discussed in the paper of A. E. Porter and I. G. Theodorou.

Increased production of nanomaterials raises concern about their safety, not only for humans but also for animals and the environment as well. Their toxicity depends on nanoparticle size, shape, surface area, surface chemistry, concentration, dispersion, aggregation, route of administration and many other factors. The review by M. Čolić and S. Tomić summarizes the main aspects of nano-toxicity *in vitro* and *in vivo*, points out relevant tests of demonstrating toxicity and explains the significance of reactive oxygen species, as the main mechanism of nanoparticle cytotoxicity and genotoxicity through the complex interplay between nanoparticles and cellular or genomic components.

Carbon nanomaterials are a large group of advanced materials that are in focus of extensive research, due to their interesting properties and versatile applicability, especially carbon nanostructures doped by covalently bonded heteroatoms (N, B, P, etc.) which leads to improved properties. This topic is discussed in the manuscript by G. Ćirić-Marjanović.

Combinations of optical, magnetic and photocatalytic properties of nanomaterials, especially those with large energy gaps, are of great interest for nanoscience and nanotechnology. One of such systems are TiO₂ nanostructures with different crystal lattices and shapes (spheres, nanotubes, nanorods), either pure or hybrid, in the form of nanocomposites with matrices based on conducting polymers, which is presented in the work of Z. Šaponjić and coauthors.

Design and manufacturing of multifunctional nanomaterials is one of the most important trends in materials nanoscience, where combining nanomaterials of various characteristics, such as ferroelectrics, ferromagnetics and ferroelastics can lead to achieving adequate multifunctionality, a good example of which are multiferroic nanomaterials, presented in the work of V. Srdić and coauthors.

Materials containing crystal grains of nanodimensions can demonstrate dramatically improved properties. Theoretically as well as experimentally, it has been shown that metallic nanostructures can attain a high percentage of theoretical strength, which questions the classical definition of material strength, stated

until recently by textbooks that does not depend on size of a tested sample. Some aspects of mechanisms of formation, growth and shrinking of crystal grains are discussed in the paper of T. Radetić.

Computational methods, including first principal calculations, have been proven to be a powerful tool in allowing investigations of systems of various complexities, spatial and temporal scales. This allows for screening of a large number of systems, which is not experimentally feasible, and also the understanding of general trends which is of great importance for both theoreticians and experimentalists. The use of this concept in applications of metallic and oxide nanoparticles is described in manuscript of I. A. Pašti and coauthors.

Being aware of the importance of nanosciences and nanotechnologies and their global impact on humanity, in the autumn of 2017, Serbian Academy of Sciences and Arts launched a series of lectures dedicated to these topics from which this monograph arose. We hope that this monograph will be of interest to the reader and can serve as a motivation for creating opportunity for research to those who want to find out more about these fascinating fields of sciences and technologies.

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ФАСЦИНАНТНИ СВЕТ НАНОНАУКА И НАНОТЕХНОЛОГИЈА

Истраживачи чији је рад довео до значајних открића гледају много даље, изван непосредног решавања техничких проблема, постављају себи важна питања, као што су: зашто се дешавају одређене појаве, како се оне развијају и на који начин функционишу? Кроз историју је развијен велики број експерименталних и теоријских метода, које се и дан-данас унапређују, како бисмо обогатили знање о свету који нас окружује и могли да видимо слике светова који измичу људском оку, укључујући ту и проналазак телескопа и микроскопа, који нам омогућавају да видимо „веома велике” и „веома мале” ствари.

Истраживачи који се баве „великим стварима” (универзумом, галаксијама, звездама и планетама) установили су да једна галаксија, око 100.000 светлосних година, у просеку садржи око једну билијарду (10^{15}) звезда. Истраживачи који се баве „малим стварима” (наноструктурама, молекулима, кластерима атома, појединачним атомима, атомским дефектима итд.) установили су да 1 cm^3 легуре алуминијума садржи око једну билијарду (10^{15}) наночестица које ојачавају ту легуру, како би могла да се користи као материјал за израду ваздухоплова, без којих је савремени транспорт незамислив. Како можемо пребројати звезде у једној галаксији или наночестице у једној легури алуминијума? Релативно лако, зато што уз помоћ електронских микроскопа можемо видети наночестице у легурама алуминијума, а звезде у галаксијама уз помоћ телескопа. Научна открића представљају основу научног и технолошког напретка, а један такав пример су открића у области нанонаука и нанотехнологија.

Зашто је ова монографија посвећена нанонаукама и нанотехнологијама?

Да бисмо одговорили на ово питање најпре морамо да установимо шта су то нанонауке и нанотехнологије? Према неизбежној Википедији, Енциклопедији Британици (или било којој другој енциклопедији), речницима, као и изворима са интернета, појмови „нанонаука” и „нанотехнологија” се односе на проучавање, разумевање, контролисано манипулисање структурама и појавама, као и на примену изузетно малих честица, чија је најмање једна димензија у опсегу до 100 nm. Иако су савремени аспекти нанонаука и нанотехнологија сасвим нови и интензивно се развијају у последњих двадесет до тридесет година, облици материје на нано скали користе се већ вековима, ако не и миленијумима. На пример, одређени пигменти коришћени су још у древној Кини и Египту, пре неколико хиљада година. Уметници су украшавали прозоре на средњовековним црквама користећи сребрне и златне наночестице различите величине и састава, при чему нису знали одакле потичу разне боје. Наночестице којима се ојачавају легуре гвожђа, алуминијума и других метала, користе се већ више од сто година,

иако у њиховом називу није садржан префикс „нано”, већ се обично називају „талози”. Научне дисциплине које су укључене у значајне истраживачке активности у области нанонауке и нанотехнологије су: физичка металургија, наука о материјалима и инжењерство материјала, хемија, физика, биологија, електротехника, и тако даље.

Одакле потиче префикс „нано”? Префикс „нано” потиче од грчке речи *νᾶνος*, што значи патуљак, указујући тако на димензију од једног нанометра (1 nm) која представља милијардити део метра (10^{-9} m). Слично томе, „нано-секунда” (ns) означава милијардити део секунде. Ово многим може звучати помало апстрактно, међутим, ствари можемо да поставимо у контекст који је нама познат, и да поменемо, на пример, да пречник власи људске косе у просеку износи 100.000 nm (10^5 nm = 100 микрона = 0.1 mm), што отприлике представља праг онога што може да се опази голим оком. Дебљина новинског папира у просеку такође износи око 100.000 nm = 100 μ m = 0.1 mm. Особа висине 2 m висока је 2.000.000.000 (2×10^9) nm. Поређења ради, ако претпоставимо да је пречник дечијег кликера 1 nm, онда би пречник планете Земље износио 1 m.

Када говоримо о структурама неорганских, органских и природних наносистема, њихове димензије су следеће: пречник атома угљеника је реда величине 0.1 nm, а то је једна десетина милијардитог дела метра; једнозидне угљеничне наноцеви имају пречник од око 2 nm, а то су два милијардита дела метра; ширина ланца дезоксирибонуклеинске киселине (ДНК) такође износи око 2 nm, а то су два милијардита дела метра; пречник протеина, чија величина често варира у зависности од тога од колико се аминокиселина састоје, реда је величине 2–10 nm, или између два и десет милијардитих делова метра (под претпоставком да су сферног облика); пречник појединачних молекула хемоглобина износи око 5 nm, или 5 милијардитих делова метра.

Уистину, ово су све мале димензије, али зашто би оне уопште требало да буду важне, или зашто је величина битна? Када се анализирају физички системи на нано скали, њихова основна својства се драстично мењају. Размотримо, на пример, тачку топљења злата: температура на којој наночестице злата реда величине ~ 4 nm прелазе из чврстог у течно стање износи око 400°C , док је температура топљења макроскопских узорака злата 1063°C . На исти начин мењају се и неке друге особине: механичке особине, електрична проводљивост, магнетизам, хемијска реактивност итд. могу драстично да се промене, што значи да наносистеми одступају од закона класичне физике који описују кретање планета, правац кретања ракета које носе сателите за истраживање свемира итд. Ово фасцинантно понашање наноструктура потиче од веза између атома. Што су структуре мање, то је више атома присутно на површини, услед чега се однос површине и запремине ових структура драстично повећава. Као последица јавља се драматична промена физичко-хемијских својстава наноструктура у односу на структуре макроскопских димензија, као и могућа појава квантних ефеката: структуре на нано скали

постају чвршће, мање крте, показују боља оптичка и каталитичка својства, и, уопштено, веома се разликују од структура уобичајених, макроскопских димензија, које сусрећемо у свакодневној пракси.

Ова монографија садржи низ радова који илуструју фасцинантан свет нанонаука и нанотехнологија.

Нанопорозни органометални материјали, који могу да опонашају особине мишића изложених спољашњим подстицајима, идеални су покретачи, који нуде јединствену комбинацију малих радних напона, релативно велике амплитуде напрезања, велику крутост и снагу. Ове појаве су описане у раду чији су аутори Џ. Т. М. ДеХосон и Е. Детси.

Лекови у области нанодимензија ће постати много ефикаснији и са смањеним штетним ефектима. Типичан пример су лекови које преносе различити типови наночестица, а које су претходно функционализоване тако да препознају само оболеле ћелије, што представља високо селективан поступак на молекуларном нивоу. Поред лекова, функционализоване наночестице могу да буду носачи радиоактивног материјала или магнетних структура, који у јаком магнетном пољу развијају високе температуре и тако уништавају ћелије рака. Одређени аспекти електронске микроскопије који се користе у проучавању биолошких наноструктура описани су у радовима чији су аутори А. Е. Портер и И. Г. Теодору.

Повећана производња наноматеријала изазива забринутост везану за њихову безбедност, не само по здравље људи, већ и за животиње и животну средину. Њихова токсичност зависи од величине наночестица, њиховог облика, величине и хемије површине, концентрације, дисперзије, склоности ка стварању агломерата, начина примене, као и многих других фактора. Рад чији су аутори М. Чолић и С. Томић даје преглед главних аспеката нанотоксичности ин витро и ин vivo, указује на релевантне тестове за утврђивање токсичности, појашњава значај реактивности молекула кисеоника, као главног механизма цитотоксичности и генотоксичности наночестица кроз сложено међудејство наночестица и ћелијских или генских компоненти.

Угњенични наноматеријали представљају велику групу напредних материјала, који због својих занимљивих својстава и широке примењивости заузимају централно место у опсежним истраживањима, нарочито када су у питању угњеничне наноструктуре допиране разнородним атомима, повезаних ковалентним везама (N, B, P итд.), што доводи до побољшања њихових својстава. Ову тему обрађује рад чији је аутор Г. Ћирић-Марјановић.

Комбинације оптичких, магнетских и фотокаталитичких својстава наноматеријала, нарочито оних са великим енергијским процепом, од велике су важности за нанонауке и нанотехнологије. Један од таквих система су TiO_2 наноструктуре са различитим кристалним решеткама и облицима (наносфере, наноцеви, наноштапићи), у чистом или хибридном облику, у облику нанокompозита са основама које су на бази проводних полимера, што је представљено у раду З. Шапоњића и сарадника.

Пројектовање и производња мултифункционалних наноматеријала представљају један од најважнијих трендова у наноуци о материјалима, где комбиновање наноматеријала који поседују различита својства, попут фероелектричности, феромагнетизма и фероеластичности, може довести до постизања одговарајуће мултифункционалности, чији су добар пример мултифероични наноматеријали, који су представљени у раду В. Срдића и сарадника.

Материјали који садрже кристална зрна нанодимензија показују знатно побољшане особине. Теоријски и експериментално је показано да металне наноструктуре могу да достигну висок проценат теоријске чврстоће, што доводи у питање класичну дефиницију чврстоће материјала, којом се, до скоро, у уџбеницима наводило да не зависи од величине испитиваног узорка. У раду Т. Радетић разматрани су неки аспекти механизма формирања, раста и смањивања кристалних зрна.

Показало се да рачунарске методе, укључујући ту и прорачуне на бази првог принципа, представљају моћну алатку која омогућава истраживање система различитих комплексности, како на димензионој тако и на временској скали. Оне омогућавају и преглед великог броја система, што експериментално није изводљиво, као и разумевање општих трендова који су од великог значаја, како за теоретичаре тако и за експериментаторе. Коришћење овог концепта у примени металних и оксидних наночестица описане су у раду чији су аутори И. А. Пашти и сарадници.

Свесна значаја наноука и нанотехнологија, као и њиховог глобалног утицаја на човечанство, Српска академија наука и уметности је у јесен 2017. године покренула серију предавања посвећену овим темама, на основу којих је настала и ова монографија. Надамо се да ће ова монографија бити занимљива читаоцу и да ће моћи да послужи као мотивација за стварање прилика за истраживања онима који желе да сазнају нешто више о овим фасцинантним областима наука и технологија.

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EMERGING RISKS AND OPPORTUNITIES FOR ZINC OXIDE-ENGINEERED NANOMATERIALS

ALEXANDRA E. PORTER*¹, AND IOANNIS G. THEODOROU¹

A b s t r a c t. – Nanostructures of zinc oxide (ZnO) are at the forefront of application-driven nanotechnology because of their unique piezoelectric, semi-conducting and catalytic properties. Despite the fact that the functional properties of ZnO nanomaterials (NMs) are being exploited and developed, little is known about their bioreactivity. Inhalation of airborne ZnO nanomaterials (ZnONMs) represents a key route of human exposure, both from the perspective of intentional (diagnostic and therapeutic applications) and unintentional scenarios. However, the toxicology of this class of material to the lung has not been reviewed thoroughly in the past. The purpose of this review is to discuss the bioreactivity of ZnONMs, and especially the current status of our understanding of their toxicology in the lung, in light of ZnO dissolution in biological environments. The ability to better anticipate hazard and risk will prevent unwanted outcomes, while allowing to efficiently harness ZnONM properties for novel applications.

Keywords: zinc oxide, bioreactivity, inhalation, dissolution, nanobio interface

INTRODUCTION

In the last three decades, there has been a surge in the application of nanotechnologies to a wide range of human endeavour. Increasing commercialization of solid-state particulate material of nanoscale dimension (nanoparticles) in a growing inventory of biomedical, electronic device, manufacturing, agricultural and common consumer products has brought many advantages to every-day life. However, their unique properties, such as nanosize and high surface reactivity – seized upon or carefully engineered to render their use so advantageous for many applications – may also increase their toxicity to the environment and confer toxicity to humans (through inhalation, ingestion or dermal routes) in the course of their synthesis, use and disposal. Thus, there is a concomitant need to understand and quantify the occupational health, public safety and environmental implica-

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tions of these currently ubiquitous material forms. Nanoparticulates are not only sourced from a variety of familiar naturally-occurring forms but increasingly engineered from targeted materials systems using novel processing routes whose nanoscale products (e.g. carbon-based materials, including nanotubes, fullerenes and graphene, as well as rare-earth oxide particulates, nanosilver, nanogold, metal oxides, and a variety of nanoscale ceramic and polymeric drug-delivery particles) have not been systematically – or in many cases, never – evaluated for toxic responses. At issue is nanoparticulate toxicity to human health and to a wide range of natural and agricultural environments. Due to the enormous number of permutations of nanoparticle (NP) shape, dimensions, composition, and surface chemistry, only a fundamental understanding of the critical biological interactions with such particulates can permit a realistic, practical assessment of the risks associated with the wide range of possible product types.

Metal oxide nanostructures, specifically, are receiving increasing attention in nanotechnology research because they offer distinct advantages over their bulk counterparts in a large variety of applications. Nanostructures of zinc oxide (ZnO), are attracting particular interest because of their unique piezoelectric, semiconducting and catalytic properties [1]. 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, nanotransducers, photocatalysts and nanogenerators and have made ZnO one of the most important products in nanotechnology, besides carbon and silicon NMs [2, 3]. ZnO has a rich family of nanostructures, including particles, rods, wires, plates, dendrites, tubes and hierarchical structures. Among them, one-dimensional (1D) ZnO nanostructures, such as nanowires (NWs), are increasingly being studied due to the novel properties observed with the formation of high aspect ratio structures [3]. Despite the fact that the functional properties of ZnO nanomaterials (NMs) are being exploited and developed, little is known about their *bioreactivity*.

On the one hand, there has been an increased interest in the use of inorganic NMs, including ZnO, which have demonstrated broad-spectrum antibacterial properties, for the control of microbes. Bacterial infections are a major cause of chronic infections and mortality, with the rise of multidrug-resistant bacterial strains representing an emerging challenge. Consequently, metal oxides like ZnO, with their ability to withstand harsh processing conditions, have attracted attention for the development of novel antibacterial agents. On the other hand, with the continuous growth in the production of ZnONMs (particulate and wire forms) 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. The purpose of this review is to discuss the bioreactivity of ZnONMs, and especially the current status of our understanding of their toxicology in the lung, in light

of ZnO dissolution in biological environments. Inhalation of airborne ZnONMs represents a key route of human exposure, both from the perspective of intentional (diagnostic and therapeutic applications) and unintentional scenarios. However, the toxicology of this class of material to the lung has not been reviewed thoroughly in the past. The ability to better anticipate hazard and risk will prevent unwanted outcomes whilst enjoying the societal benefits of a dawning nanomedicines era.

ANTIBACTERIAL ACTIVITY OF ZINC OXIDE NANOMATERIALS

Over the last two decades, there has been a growing interest in the use of inorganic antimicrobial agents, including ZnONMs, for the control of microbes. For spherical ZnONPs, more detailed reviews on their antibacterial activity already exist elsewhere [4] but in summary, antibacterial effects have been reported for both Gram-positive and Gram-negative bacteria [5-7]. Meanwhile, the antibacterial activity of ZnONPs is considered to be greater than that of titania (TiO₂) or silica (SiO₂) NPs [8]. However, several inconsistencies still remain in published results. In most cases, studies have suggested a higher toxicity on Gram-positive bacteria than on Gram-negative bacteria [7-9]. In contrast, the opposite effect has also been observed for ZnO nanoparticles capped with poly ethylene glycol (PEG) [6]. In general, antibacterial activity has been shown to increase only slightly with decreasing particle size but more prominently with particle concentration [6, 10]. For instance, 13 nm ZnONPs completely inhibited *E. Coli* and *S. Aureus* growth at concentrations higher than 13.4 mM and 1 mM, respectively [9]. On the other hand, a separate study reported an even stronger antibacterial activity for 93 nm ZnONPs against *E. Coli*, at concentrations as low as 1.25 mM [11].

With regards to 1D ZnONMs, ZnO nanorod antibacterial coating approaches have been investigated for the development of antimicrobial fabrics (e.g. facemasks or tissues) [12] or implant modifications that 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 NW orientations [13]. Jansson *et al.* evaluated bacterial adhesion and viability of common implant-associated pathogens, including *P. aeruginosa* and *S. epidermidis*, on ZnONW surfaces compared to sputtered ZnO thin films and glass substrates [14]. Adherent *P. aeruginosa* were reduced on ZnONW surfaces compared to glass and sputtered ZnO, but the number of adherent *S. epidermidis* on ZnONWs was equivalent to glass. Both ZnONW and sputtered ZnO films demonstrated a significant bactericidal effect on adherent *P. aeruginosa*, killing around 2-fold more bacteria compared to glass. A more pronounced bactericidal effect was observed against *S. epidermidis*, with sputtered ZnO and ZnONW substrates killing 20-fold and 30-fold more bacteria than glass, respectively. More recently, ZnONWs grown on glass substrates exhibited significant antimicrobial effects to *B. Subtilis* and *E. Coli* and prevented biofilm formation [15]. *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.

In earlier studies, the generation of reactive oxygen species (ROS) was considered as the main mechanism responsible for the antibacterial activity of ZnONMs [5, 10, 11] but lacked solid supporting evidence. More recently, several findings support the notion that antibacterial activity may be attributed to ZnONM dissolution and release of Zn²⁺ ions [16-19]. Since several factors may affect ZnONM dissolution (*e.g.* size, shape, pH, organic components) [20, 21], characterization of ZnONMs at the local *point of exposure* in cellular microenvironment and measurement of dissolution kinetics are essential to resolve the discrepancies that remain in published results, and be able to effectively harness the antibacterial properties of ZnONMs. The effects of ZnONM dissolution on their bioreactivity are discussed in more detail in Section 4.

THE BIOREACTIVITY OF ZINC OXIDE NANOMATERIALS IN THE LUNG

In Vivo effects of Zinc Oxide Nanomaterials in The Deep Lung

In a previous review we discussed the deposition behaviour of silver (Ag) NPs and NWs in the lung and their interaction with cells, proteins and lipids in the alveolar unit [22]. For a more detailed description of the behaviour of NMs in the lung, the reader should refer to this review [22]. In the case of ZnONMs, epidemiological data on human exposure and health effects are currently lacking for these materials. Ultrafine ZnO particles are known to reach the alveoli in the deep lung and cause pulmonary inflammation and symptomatic responses [23, 24]. These responses, for instance, manifest as metal fume fever in welders [25]. However, only a few studies have investigated the *in vivo* pulmonary toxicity of engineered ZnONMs.

According to one study, rats exposed to 50-70 nm ZnONPs by intratracheal instillation expressed potent but reversible pulmonary inflammatory responses [26]. However, *in vivo* and *in vitro* toxicology measurements in this study 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 ZnONPs. Wang *et al.* examined the toxic effects of 20 nm ZnONPs following inhalation [27]. A significant increase in zinc content was observed in liver tissues, while histopathological examination showed that ZnONPs caused a severe damage in liver and lung tissues. This led to the conclusion that ZnONPs may be able to translocate from the lung into the blood circulation after pulmonary exposure. Additionally, the deposition in the lungs and the speed of penetration were more rapid for ZnO than iron oxide (Fe₂O₃) NPs [27].

In a work by Cho *et al.* 10 nm ZnONPs intratracheally instilled into female Wistar rats were inflammogenic to the lungs both acutely (24 h) and chronically (4 d) [28]. In a similar study by the same group, ZnONPs induced pulmonary

eosinophilia, goblet cell hyperplasia, severe fibrosis and airway epithelial injury [29]. Similar pathologies were also observed with the instillation of dissolved Zn^{2+} into rat lungs, which indicated that one main cause of toxic effects could be ZnO dissolution in the acidic environments of lysosomes following cellular uptake. Intracellular dissolution could have caused lysosomal destabilization and cell death, leading to the subsequent pathogenicity observed. In support of this paradigm, doping ZnONPs with iron, which decreases the rate of ZnO dissolution, led to lower acute pulmonary inflammation and cytotoxicity [30]. However, discrepancies still remain in the published literature regarding the respective role of particulate and ionic zinc. In a study attempting to separate particulate from ionic effects, *in vitro* and *in vivo* exposures were performed with ZnONPs as well as the aqueous extracts (ZnOAEs) obtained from their dissolution [31]. ZnOAEs and ZnONPs had similar toxic and pro-inflammatory effects on the human alveolar type II-like epithelial cell line A549, but the strength of the effects was higher for ZnONPs [31]. 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 were rapidly cleared by adsorption to systemic circulation [32]. 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 [26].

A more recent work has also highlighted the ability of ZnONPs to cause cardiopulmonary impairments [33]. In this work, Sprague-Dawley rats were exposed to ZnONPs *via* either intratracheal (IT) instillation or inhalation of occupationally relevant concentrations [33]. In contrast to previous findings, instilled ZnONPs predominantly accumulated in the lungs over 24 h, 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 h. 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 [33].

In Vitro Bioreactivity of Zinc Oxide Nanomaterials in pulmonary exposure models

Meanwhile, the toxicity of ZnONPs has been reported for various *in vitro* mammalian cell systems, as described in a relevant review article [34]. For in-

stance, exposure of mouse neuroblastoma Neuro-2A cells to 50-70 nm ZnONPs led to cell death, with half-maximal inhibitory concentration (IC_{50}) values between 50 and 100 $\mu\text{g/ml}$ [35]. ZnONPs caused significant decrease in mitochondrial function while transmission electron microscopy (TEM) imaging revealed nanoparticles attached to the cell surface as well as internalized into the cells [35]. The same study also showed that ZnONPs were more toxic than other metal oxide nanoparticles (TiO_2 , Fe_3O_4 , Al_2O_3 and CrO_3) [35]. Similarly, ZnO nanorods (20-70 nm \times 100-200 nm) were more toxic than Fe_2O_3 and Y_2O_3 NPs on human aortic endothelial cells [36]. Inflammatory responses of the cells initiated at a threshold concentration of 10 $\mu\text{g/ml}$ and were dose-dependent above this value, with an IC_{50} of 50 $\mu\text{g/ml}$. Another comparative study revealed that ZnONPs were more toxic than carbon black, single walled carbon nanotubes (SWCNTs) and SiO_2 on primary mouse embryo fibroblast cells [37]. In accordance with other findings, cell viability decreased in a dose-dependent manner above a threshold exposure dose of 10 $\mu\text{g/ml}$ and was linked to the generation of intracellular oxidative stress.

Since *in vivo* work indicates that airway exposure to ZnONPs poses an important hazard, several *in vitro* studies have specifically 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) have been used most frequently. The cytotoxicity of ZnONPs was mediated through reactive oxygen species (ROS) generation and oxidative stress [21, 38], apoptosis [39, 40] or genotoxicity [41, 42]. 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 $[\text{Ca}^{2+}]$ [38]. 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 [43]. ZnO exposure (2-8 $\mu\text{g/ml}$) increased IL-8 mRNA and protein expression, and this induction was mediated by p65 phosphorylation and I κ B α phosphorylation and degradation. Furthermore, Ng *et al.* explored the activation of cellular DNA damage pathway through the tumour suppressor p53 gene by ZnONPs [44]. 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 mitochondrial activity and chemokine production of A549 cells, with smaller particles being more toxic than larger ones [45]. Lin *et al.* demonstrated that the exposure of A549 cells to ZnONPs (70 and 420 nm) induced dose-dependent cytotoxicity over a narrow concentration range (8-18 $\mu\text{g/ml}$) [46]. 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 similar lines, 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 [47]. Other authors have suggested that the secondary aggregate size may be more important than the primary ZnONP size in determining their toxicity profile [48]. 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 [49].

Although the main paradigm explaining the toxicity of ZnONPs appears to be their dissolution and release of Zn^{2+} ions, many studies were unsuccessful in clarifying whether ZnO dissolution occurred extracellularly, intracellularly or both. One study showed that virtually all human mesothelioma MSTO-211H and rodent 3T3 fibroblast cells died after incubation with 19 nm ZnONPs at concentrations higher than 15 $\mu\text{g}/\text{mL}$ [50]. The authors inferred that this sharp concentration dependence of the cytotoxic response might be due to the presence of a critical Zn^{2+} concentration in the tissue culture medium and the subsequent toxic effects of aqueous Zn^{2+} [51]. In an attempt to quantify the role of dissolved Zn^{2+} ions to the cellular toxicity, Xia *et al.* measured the solubility of 13 nm ZnONPs in water and two cell-free tissue culture media (MDEM and BEGM) [21]. ZnONPs readily dissolved in the culture media, with more than 80% of the maximum dissolved Zn^{2+} concentration reached within 3 h. 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 could possibly facilitate 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 tissue culture medium and found that the Zn^{2+} equilibrium concentration was around 10 $\mu\text{g}/\text{mL}$ when 40 $\mu\text{g}/\text{mL}$ of particles were used [52]. Their results implied that dissolved Zn^{2+} in the tissue culture medium or inside cells played the main role in ZnONP toxicity. Deng *et al.* demonstrated that ZnONPs of several sizes (10, 30, 60 and 200 nm) were toxic to mouse neural stem cells at concentrations higher than 12 $\mu\text{g}/\text{mL}$ in a dose-dependent manner, and that the same concentrations of $ZnCl_2$ – which is highly soluble in aqueous solutions and therefore often used as a control for free Zn^{2+} ions – had similar toxic effects [48]. Iron doping of ZnONPs, which changes the material matrix to slow Zn^{2+} release, decreased ZnO cytotoxicity [53]. 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 [54]. ZnONPs with thicker shells were less cytotoxic, presumably because of a lower rate of Zn^{2+} release by ZnONPs. Similarly, encapsulation of ZnO nanorods in a nanothin amorphous SiO_2 coating resulted to slower dissolution kinetics and significantly lower DNA damage in lymphoblastoid cells [55].

To further investigate the role of extracellular vs. intracellular zinc, high resolution X-ray spectromicroscopy and high elemental sensitivity X-ray microprobe analyses have been used to determine the fate of ZnO and iron-doped ZnONPs following exposure to BEAS-2B cells [56]. The cells accumulated zinc from the cell culture solution and only organic-complexed, ionic Zn^{2+} was present intracellularly one hour following exposure. Although this finding indicates that ZnONP toxicity is caused by free or complexed Zn^{2+} ions within cells, the study did not directly show whether the uptake of Zn^{2+} ions or ZnONPs was the dominant pathway for internalizing zinc. Other reports have indicated that the cytotoxicity of ZnONPs was independent of the amount of extracellular soluble Zn^{2+} in the tissue culture medium and that direct NP contact with the cells was required [30, 57, 58]. A study performed by our group, showed that the toxicity of ZnONWs (100-300 nm \times 2-10 μ m) on human monocyte macrophages (HMMs) was similar to equivalent concentrations of $ZnCl_2$ [59]. Quantification of the amounts of Zn^{2+} released in simulated bodily fluids (SBFs) of extracellular pH (ex-SBF, pH 7.4) and lysosomal pH (lyso-SBF, pH 5.2) by inductively coupled plasma mass spectrometry (ICP-MS), revealed that ZnONWs are expected to be relatively stable extracellularly but dissolve rapidly in lysosomal environments. Confocal laser scanning microscopy (CLSM) and transmission electron microscopy showed that HMMs had taken up ZnO nanowires by phagocytosis and that the acidic pH of the lysosomes triggered ZnONW dissolution. Intracellular ZnO dissolution was measured by CLSM of live HMMs and revealed that an increase in intracellular Zn^{2+} concentrations was followed by cell death, which had features of both apoptosis and necrosis. The role of ZnONP dissolution in their toxicity against mammalian cells is further discussed in Section 4.

Finally, the effect of shape on ZnONM bioreactivity with alveolar cells has not been completely elucidated. 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) [45]. The authors suggested that the contact area between a single NP and a single cell may be 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 [39]. ZnO nanorods induced apoptosis involving the p53, survivin, bax/bcl-2 and caspase pathways. Some studies have attempted to examine the effect of 1D ZnONMs using ZnO nanorod-coated substrates. Li *et al.* investigated the biocompatibility of ZnO wire surfaces (1 μ m \times 200 μ m) on HeLa (cervical cancer) and L-929 (subcutaneous connective tissue) cell lines [60]. Cell viability at 48 h was reduced only for concentrations higher than 100 μ g/mL, and was 75% for HeLa and 50% for L-929 cells. HeLa cells phagocytosed some fragments of ZnO wires. In contrast to previous findings where mesenchymal stem cells were able to survive on silicon NWs for several days [61], 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 h on ZnO nanorod (50 nm × 500 nm) surfaces [62]. 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 [63]. However, a significant degree of necrosis was apparent after 6.5 h of incubation. After 16 h, the number of live cells adherent on flat ZnO was 52% but only 12% on ZnO nanorods, indicating the effect of both nanotopography and material on cell viability. Apart from these studies, investigation of the pulmonary toxicity of freestanding high aspect ratio ZnONWs are generally lacking from the literature. In our own work, the cellular toxicity of ZnONWs with two different lengths (“short”: S-ZnONWs, 30 nm × 1 μm; and “long”: L-ZnONWs, 30 nm × 3 μm) was studied in relation to cell viability of human alveolar epithelial type 1-like cells (TT1 [64]) [65]. Alveolar epithelial type 1 cells cover 95% of the total alveolar surface making them susceptible to inhaled NPs, and may govern uptake and potential translocation of inhaled NPs across the pulmonary epithelial barrier. To compare with the effects of Zn²⁺ ions, ZnCl₂ was used as a source of soluble ionic zinc. We demonstrated similar time- and concentration-dependent decrease in cell viability following exposure of TT1 cells to all Zn compounds. The median lethal doses (LD₅₀) after 24 h were estimated to be 10.13 μg/mL for ZnCl₂, 10.90 μg/mL for S-ZnONWs and 11.18 μg/mL for L-ZnONWs, in terms of ionic Zn²⁺ concentration. Therefore, both lengths of ZnONWs exhibited similar effects on the viability of TT1 cells, in accordance to similar dissolution kinetics measured *in vitro* in pH7 and pH5 buffers. Using confocal laser scanning microscopy imaging of live TT1 cells, we showed that cellular damage correlated with an increase in intracellular ionic Zn²⁺ following uptake and dissolution of ZnONWs inside cells. As discussed in more detail in Section 4, our study also highlighted that detailed characterization of ZnONMs at the local *point of exposure* in cellular microenvironment is paramount for allowing accurate conclusions about their bioreactivity *in situ*.

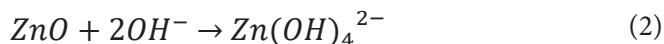
THE ROLE OF ZINC OXIDE NANOMATERIAL DISSOLUTION ON THEIR BIOREACTIVITY

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 [66-68]. Zinc is a component of over 300 enzymes, as either a catalytic or regulatory cofactor or in a structural capacity [66]. A severe zinc deficiency in humans may 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 [69]. Oral zinc supplementation has proven beneficial in a number of infectious diseases, particularly in acute lower respiratory infections, leprosy

and tuberculosis [68], while topical application of ZnO has been shown to facilitate the healing process of chronic and surgical wounds [70]. It is also becoming increasingly clear that Zn^{2+} , similar to 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 neuro-generation in conditions such as Alzheimer's disease, amyotrophic lateral sclerosis and ischemia [71-73].

Despite the importance of zinc in biological systems, the role of ZnONP dissolution on their toxicity against mammalian cells has not been completely elucidated. As already discussed, zinc has been shown to induce cytotoxicity and apoptosis in mammalian cells, however few 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 dys-homeostasis can induce cell death. A rise in the intracellular Zn^{2+} concentration is often associated with high levels of ROS production [21]. These oxygen radicals could originate at the particle surface but also from biological substrates such as damaged mitochondria [21]. 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 [74-76]. Increased zinc could also inhibit key enzymes in the glycolytic pathway, leading to ATP depletion in cells [77, 78]. 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 [79, 80].

There is already much discussion in the literature regarding ZnO dissolution in biological environments and whether ZnONM cytotoxicity arises from extracellular or intracellular dissolution or both, or from a combination of particulate and ionic effects. Several attempts have been made to quantify ZnONM dissolution and link this to the magnitude of cytotoxic effects; however, several discrepancies still exist between published results. The phase diagram of ZnO indicates that it is an amphoteric, meaning ZnO can dissolve according to either reaction (1) or (2) depending on the solution pH [81]:



The rate of dissolution can also be affected by several factors, such as size, doping, surface coating or the presence of biological components. Table 1 summarizes the results of dissolution studies performed on ZnONMs in relation to toxicological studies. Lack of sufficient controls over the particles used and the cellular systems investigated, however, as well as differences in the analytical meth-

odologies used, make it hard to compare between experiments performed by different groups.

Table 1. Summary of ZnO dissolution studies reported in the literature.

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).	[82]
50–70 nm	Osterhout's medium	Recombinant metal sensor bacteria (<i>E. coli</i> MC1061)	- No difference in dissolution between nano and bulk ZnO particles (up to 80% dissolution).	[83]
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).	[84]
50–70 nm	LB Broth	Membrane filtration ICP-AES	- Dissolution rate decreased with increasing NP concentration (~ 3.2% dissolution).	[85]
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	[86]
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.	[20]

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 μm). - Citric acid significantly enhanced dissolution for all NP sizes.	[87]
20 nm	- Deionized water (DI) - Tris buffer with varying pH (pH 7, 8, and 9) - Artificial seawater (ASW \pm 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.	[88]
100-300 nm \times 2-10 μm 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 $^{\circ}\text{C}$ for 30 min. - ZnONWs dissolved rapidly in lyso-SBF, with complete dissolution after the same incubation conditions.	[59]
13 nm	- Ultrapure water - DMEM with 10% FBS - BEGM (contains growth factors, cytokines and supplements)	Centrifugation ICP-MS	- Dissolution followed the order: DMEM (90 μM) > BEGM (80 μM) > H_2O (60 μM). - Organic components enhanced dissolution, with up to 80% of total dissolved Zn within 3 h.	[21]
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_2O (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.	[18]

25, 40 and 70 nm	<ul style="list-style-type: none"> - Ultrapure water - Environmental Protection Agency moderately hard water - DMEM and RPMI-1640 (\pm BSA) 	Membrane filtration ICP-AES	<ul style="list-style-type: none"> - Equilibrium solubility followed the order: DMEM (34 mg/L) > H₂O (7.18-7.40 mg/L) > RPMI-1640 (5 mg/L) > moderately hard water (0.7-1 mg/L) - BSA enhanced NP dissolution. 	[89]
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	<ul style="list-style-type: none"> - Low concentrations of phosphate rapidly and substantially reduced Zn²⁺ 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. 	[90]
30 nm	10 mM NaNO ₃ solutions with varying concentrations of sodium sulphide (Na ₂ S)	Centrifugal Ultrafiltration ICP-AES	<ul style="list-style-type: none"> - Increasing Na₂S concentrations reduced the rate and extent of Zn²⁺ 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. 	[91]
13 nm	<ul style="list-style-type: none"> - Ultrapure water - DMEM with 10% FBS - BEGM (contains growth factors, cytokines and supplements) 	Centrifugation ICP-MS	<ul style="list-style-type: none"> - Dissolution followed the order: DMEM (90 μM) > BEGM (80 μM) > H₂O (60 μM). - Organic components enhanced dissolution, with up to 80% of total dissolved Zn within 3 h. 	[34]

Furthermore, the dissolution of 1D ZnO nanostructures, which represent an emerging class of commercially relevant NMs, has hardly been investigated. For ZnO nanorods with the hexagonal wurtzite crystal structure, the top (001) plane is comprised of either O²⁻ or Zn²⁺ and is polar. The (110), (100) and (010) side planes are comprised of an equal number of O²⁻ and Zn²⁺ 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 [92]. Thus, the polar faces, having the highest surface energy, are metastable and may be dissolved preferentially. However, such selective dissolution has not always been experimentally observed. In one study, ZnO nanorods with diameters of 100 nm or smaller could not be etched in alkaline solutions [93]. This could be because the energy of the polar faces is only smaller 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 [81]. 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 [81]. Consequently, ZnO rods with a small diameter (<100 nm) dissolved homogeneously whereas anisotropic dissolution was observed for rods with higher diameters (>100 nm), which led to the formation of ZnO tubular structures [81].

Despite the fact that the use of ZnONWs is becoming more widespread, potential adverse effects to human health upon exposure are still unclear [34]. In a previous study within our group, ZnONWs were toxic to human monocyte macrophages (HMMs) at similar concentrations as ZnCl₂ [59]. 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. In our own work, we have further investigated the bioreactivity of ZnONWs with the pulmonary epithelium, in order to elucidate the mechanisms that control their toxicity [65]. Since several complex factors may affect ZnONW dissolution, we sought to characterize ZnONWs at the local point of exposure in cellular microenvironment, in an attempt to draw accurate conclusions about their bioreactivity. Therefore, we examined the impact of pulmonary surfactant (PS) on the stability of ZnONWs (Figure 1) and the subsequent effects on their interaction with alveolar epithelial cells. PS covers the entire alveolar region to decrease surface tension in the alveoli and prevent alveolar collapse, and consists of a mixture of lipids and surfactant-specific proteins. PS therefore represents a first line of defense of the lungs against inhaled NMs. Our results revealed adsorption of PS lipids on the surface of ZnONWs (Figure 1). This lipid corona delayed the kinetics of Zn²⁺ ion release from ZnONWs at acidic pH, by blocking direct contact between the ZnONW surface and the aqueous environment. TEM imaging indicated that surfactant molecules adsorbed and formed lipid bilayers on the surface of the particles, possibly by virtue of their amphiphilic nature, thus sterically stabilizing the particles. Dynamic light scattering (DLS) and ζ-potential measurements confirmed that PS prevented the agglomeration of ZnONWs, possibly through contributions of both steric and charge stabilization. Confocal laser scanning microscopy imaging of live TT1 cells showed that cell damage correlated with an increase in intracellular ionic Zn²⁺ 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, also delaying the onset of cellular damage. Despite delaying this onset of cellular damage, pre-incubation of ZnONWs with PS led to uptake of the ZnONWs by a higher percentage of TT1 cells within 24 h; this increase in cellular uptake dominated over their delayed intracellular dissolution and resulted to a higher reduction in cell viability after 24 h. This work highlights that combinations of spatially resolved static and dynamic techniques are required to develop a holistic understanding of the complex interplay of parameters that govern ZnONM bioreactivity at the point of exposure and to accurately predict their risks on human health.

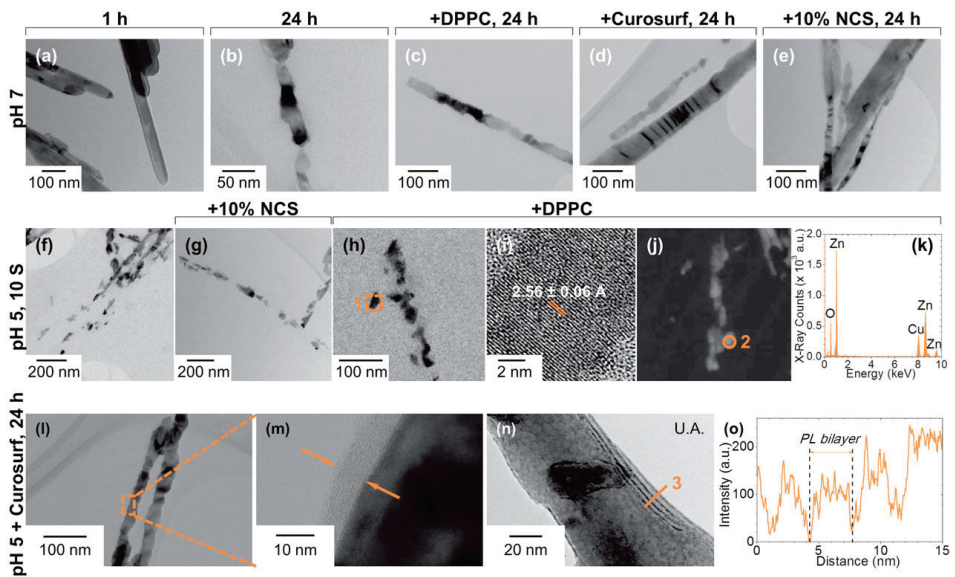


Figure 1. Pulmonary surfactant (PS) reduces the etching of zinc oxide nanowires (ZnONWs) at lysosomal pH and forms a phospholipid corona around ZnONWs [65].

(a–e) Bright field transmission electron microscopy (BFTEM) images of ZnONWs incubated in normal saline (NS) at pH7 for 1 h (a) or 24 h (b–e), in the absence (a, b) or presence of dipalmitoylated phosphatidylcholine (DPPC; the main lipid component of PS) (c), Curosurf (a natural surfactant from porcine lungs) (d), or 10% newborn calf serum (NCS) (e). (f–h) BFTEM images of ZnONWs incubated in NS at pH5 for 10 s, 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 s (k) STEM-EDX spectrum collected from the area marked 2 in (j). (l–n) BFTEM images of ZnONWs incubated in NS at pH5 in the presence of Curosurf for 24 h. (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 ZnONWs. (o) Intensity line profile collected from the line marked 3 in (n).

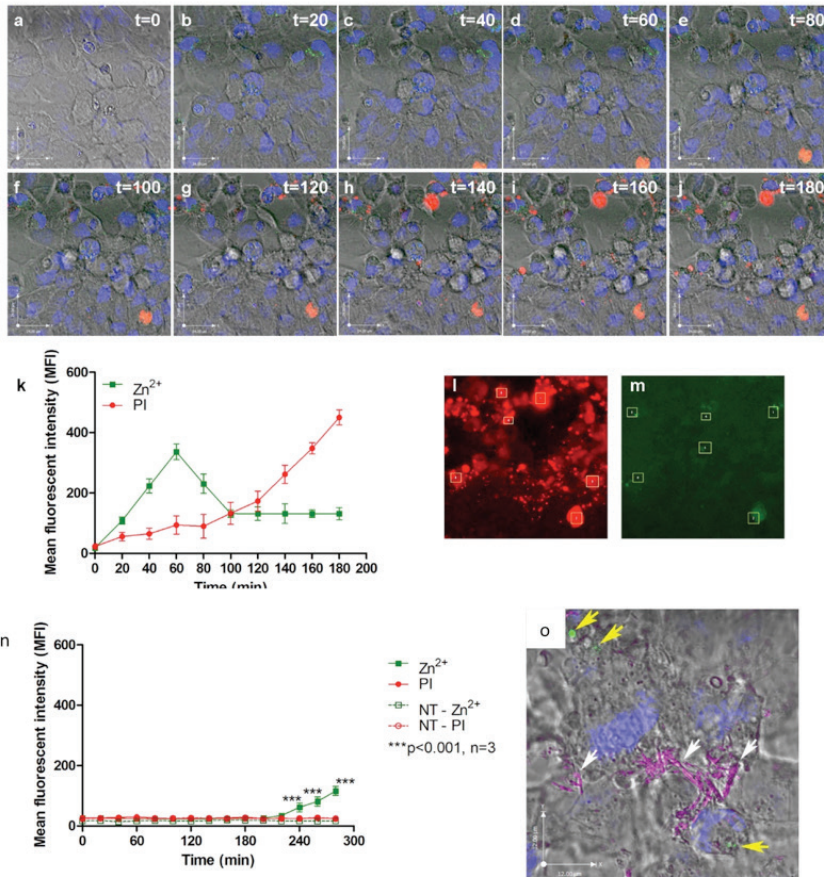


Figure 2. Intracellular dissolution of ZnONWs within live human alveolar epithelial type 1-like (TT1) cells correlates to cell viability, while PS delays intracellular dissolution of ZnONWs and the onset of cell damage [65]. (a–j) Live TT1 cells were incubated with ZnONWs at 37 °C with 5% CO₂ and imaged by confocal laser scanning microscopy every 20 min (a–j) up to 3 h. Cells were stained with Hoechst 33342 (blue, nuclei) and FluoZinTM-3, AM (a Zn²⁺-selective indicator, green) prior to exposure to ZnONWs in propidium iodide-containing (PI, red, necrotic cells) serum free RPMI cell culture medium. (k) The dynamic intracellular dissolution of ZnONWs (squares, green line) and their effect on cell viability (circles, 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. Regions of interest (ROI 1–6, squared in l, m) indicate the areas where the fluorescent signals were measured and plotted in k. (n) When live TT1 cells were incubated with ZnONWs pre-incubated with Curosurf, there was no significant Zn²⁺ release (green line) and no necrotic cell death (red line) was observed within 3 h of exposure. A significant release of Zn²⁺ ($p < 0.001$; $n = 3$ with a total of 21 cells observed) was only observed after 4 h of exposure (green line in n, yellow arrows in o indicate areas of Zn²⁺ release), with no significant necrotic cell death (red line in n). ZnONWs, imaged in reflectance mode (magenta in o, white arrows) remained intact in the system close to areas of Zn²⁺ release.

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 [24, 25], while ZnONPs have been shown to cause cardiopulmonary impairments [33]. *In vitro*, the toxicity of ZnONPs has been reported for various mammalian systems [34], including lung epithelial cells and alveolar macrophages [21, 38]. Their cytotoxicity was mediated through reactive oxygen species (ROS) generation and oxidative stress [21, 38], apoptosis [39, 40] or genotoxicity [41, 42]. 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 [59, 65]. However, to fully elucidate the mechanisms of biological action of ZnONMs, additional efforts in particle characterization through all stages of *in vitro* or *in vivo* experiments are required. Clarifying how the physicochemical properties of ZnONMs evolve as they interact with biological systems (*e.g.* aggregation state, morphology, formation of biomolecule coronas), will help to better understand the characteristics of the system that the cells actually encounter. Further characterization of the particle-cell interface and quantification of ZnONM dissolution kinetics at this interface may also be useful in drawing accurate links with relevant mechanisms of cellular damage. Novel approaches based on the correlative application of high spatial and energy resolution analytical microscopy techniques, and the development of new metrology methods to quantify the amount of intracellular Zn²⁺ ions, may offer an improved understanding of the mechanisms by which ZnONMs interact with cells. The ability to better anticipate hazard and risk will prevent unwanted outcomes, while allowing to efficiently harness ZnONM properties for novel applications.

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САГЛЕДАВАЊЕ РИЗИКА И МОГУЋНОСТИ ЗА ЦИНК-ОКСИДНЕ НАНОМАТЕРИЈАЛЕ

Резиме

Наноструктуре цинк-оксида (ZnO) у самом су врху нанотехнологија освојених услед захтева за њиховом применом, због својих јединствених особина, пиезоелектричних, полупроводничких и каталитичких. Независно од чињенице да су ZnO наноматеријали (NMs) развијени и коришћени, мало се зна о њиховој биореактивности. Удисање честица ZnO наноматеријала (ZnONMs) присутних у ваздуху, представља основни начин на који су људи изложени њиховом утицају, и то у оба случаја: из перспективе намерног излагања контакту (као код дијагностичких и терапеутских примена), или ако

такве намере нема. Међутим, за токсикологију плућа за ову класу материјала до сада није учињен преглед. Циљ овог прилога је да дискутује о биореактивности ZnONM, посебно о тренутном стању ствари и нашем разумевању њихове плућне токсикологије, у светлу растварања ZnO у биолошким срединама. Могућност бољег сагледавања хазарда и ризика помоћиће спречавању нежељених исхода, док ће истовремено омогућити ефикасније искоришћавање особина ZnONM за нове примене.