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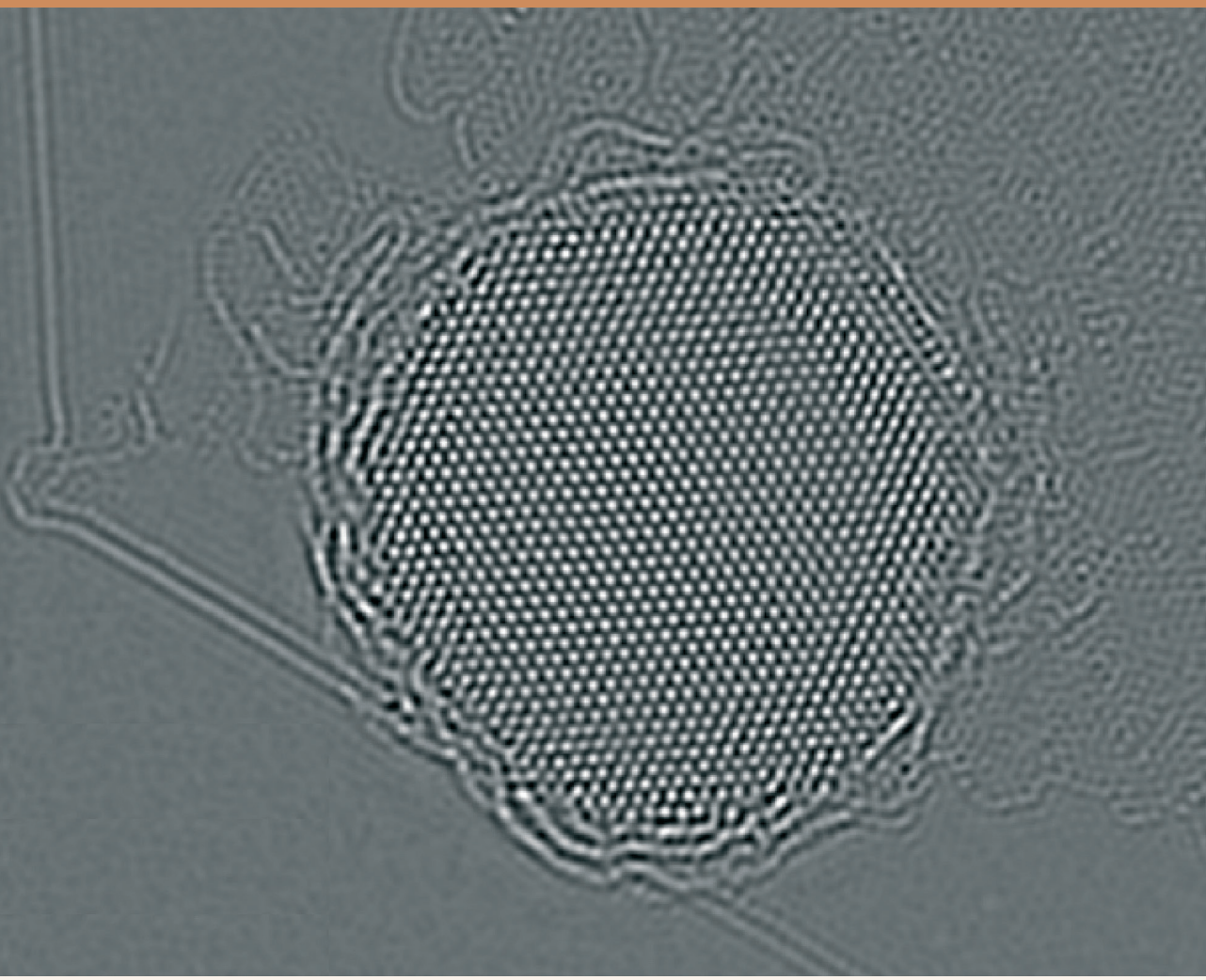

FASCINATING WORLD OF NANOSCIENCES AND NANOTECHNOLOGIES
ФАСЦИНАНТНИ СВЕТ НАНОНАУКА И НАНОТЕХНОЛОГИЈА

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ЦИКЛУС ПРЕДАВАЊА

Књига 6

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CONTENTS

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 *νᾶνος*, 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 μm = 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, „наносекунда" (ns) означава милијардити део секунде. Ово многима 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 μm = 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 амино киселина 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 чврше, 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 Ц. 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 виво, 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.

Угљенични 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 угљеничне 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 нанокомпозита 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 нанонауци 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 уџбенцима наводило да не зависи од величине испитиваног узорка. У раду Т. Радетић разматрани су неки аспекти механизама 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 нанонаука 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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TOXICITY OF NANOSTRUCTURES

MIODRAG ČOLIĆ,*[1, 2, 3] SERGEJ TOMIĆ[1, 2]

A b s t r a c t. – Due to their unique size (dimensions of 1 to 100 nm) and physicochemical properties, nanomaterials have found numerous applications in electronics, cosmetics, household appliances, energy storage, food industry, pharmacy and medicine. However, increased production of nanomaterials raises concern about their safety, not only for human beings but also for animals and the environment. Numerous studies confirmed that nanoparticles (NPs) can exert toxicity both, *in vitro* and *in vivo*, depending on their size, shape, surface area, surface chemistry, concentration, dispersion, aggregation, route of administration and many other factors, all of which are also relevant to desired biological properties of nanostructures. This review summarizes the main aspects of nanotoxicity *in vitro* and *in vivo,* points out relevant tests in order to demonstrate the toxicity and explains the significance of reactive oxygen species, as the main triggering factor of NP cytotoxicity and genotoxicity, acting through the complex interplay between NPs and cellular or genomic components, respectively. Special attention was devoted to the immunotoxic and immunomodulatory properties of NPs and their relevance for production of less immunogenic nanostructures capable of avoiding undesirable immune responses and the use of NPs as specific nanotherapeutics for drug delivery and vaccination strategy. Finally, ecotoxicological aspects of NPs are presented, showing why aquatic ecosystems are the most susceptible to environmental contamination and why studies on aquatic organisms are important for translational nanotoxicology.

Keywords: nanostructures, toxicity, immunomodulation, oxidative stress, ecotoxicology

INTRODUCTION

With the rapid development of nanotechnology, nanomaterials (dimensions of 1 to 100 nm) have become important in our everyday lives with numerous applications in electronics, energy storage, household appliances, food industry, cosmetics, pharmacy and medicine. They include inorganic and organic nanoparticles (NPs), nanofibers, nanotubes, quantum dots, nanocomposite materials, and many others. Increased production and intentional usage of NPs (cosmetics,

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drug delivery, implants) or unintentional exposure of NPs in the environment due to combustion processes (like diesel soot), manufacturing processes (such as spray drying or grinding), naturally occurring processes (such as volcanic eruptions or atmospheric reactions) are likely to increase the possibility of their adverse health effects. Therefore, nanomedicine and nanotoxicology have become two faces of the same coin. The contamination of aquatic ecosystems, soil and air by nanostructures and their subsequent uptake by biota is a major concern of environmental nanotoxicology (1, 2). Two major factors emphasize the importance of nanotoxicology and dictate the necessity of its rapid development: large-scale production of diversified nanomaterials, and remarkable progress in developing new types of nanomaterials with astonishing physical and chemical characteristics (3, 4). For instance, the development of graphene-like 2D layered nanomaterials, transition metal dichalcogenides or boron-nitride nanosheets has led to numerous new applications in nanophotonics. In the field of NPs bioapplications, protein-based biological machines (nanorobots) have been created in order to repair DNA damage (4). Medical products based on NPs consist of biological probes, drug carriers, biological sensors, implants and imaging agents while NPs used as therapeutics and diagnostic tools (theranostics), whether they are polymers, inorganic/metallic- or carbon-based or even novel complex nanocompounds, are of particular interest for nanotoxicology as their *in vivo* persistence can be prolonged (5). Examples of such nanomaterials, which illustrate why their application as theranostics agents is of particular interest, are noted in the following paragraphs.

Polymeric NPs, based on chitosan or poly-(lactic-co-glycolic acid) (PLGA) have been used as nanocarriers for drug delivery across the blood-brain barrier due to their biocompatibility and biodegradability, thereby ensuring safe therapy (6). Polystyrene nanospheres, when coated with streptavidin, offer greater sensitivity for biomarker discovery, compared to classical methods (7).

Inorganic (ceramic) NPs, including Silica (SiO_2), Titania (TiO_2) and Alumina (Al_2O_3) have been commonly used for drug administration in cancer therapy due to their porous nature, however, their applications are limited due to non-biodegradability (8, 9). Wide-spread use of silica NPs in fields of cosmetics or polishing, has transferred in the field of medicine. Due to the ease of surface modification, silica is widely used for coating materials in cancer therapy, drug delivery and DNA delivery (10, 11). Inorganic NPs, including superparamagnetic iron oxide NPs (Fe_2O_3/Fe_3O_4 or "SPIONs"), gadolinium-based paramagnetic NPs, gold (Au) shell NPs and Titania NPs, are routinely used for magnetic resonance imaging contrast enhancement and as cancer drug carrier systems, respectively (1, 12). Silver (Ag) NPs are being explored as antibacterial agents for treatment of infectious diseases, due to their ability to stabilize nanoparticles and favorable optical/chemical properties (13). Inorganic NPs such as quantum dots (QDs), which are essentially semiconductor nanocrystals, display excellent optical/chemical properties suitable for bioimaging. Although the first generation

QDs included compounds like CdSe, CdS, CdTe and PbS, due to toxicity of elements like Cd, a new generation QDs emerged with materials like InAs, InP, ZnSe. Initially, QDs have been explored for use as fluorescent markers, although recently they have found applications as contrast imaging compounds, tissue-specific vascular markers and theranostics agents (1).

The most widely recognized carbon nanostructures include nanotubes, fullerenes and graphene. Carbon nanotubes (CNTs) are tubular structures of about 0.3nm to 3nm or more in diameter and hundreds to thousands of nm in length (14). They can be grouped, depending on the number of layers, into single wall carbon nanotubes (SWCNTs) and multiwall carbon nanotubes (MWCNTs), displaying enormous potential for applications in electronics, optics, materials science, nanotechnology, biology, and medicine (14). These nanostructures have been widely explored in cancer therapeutics and imaging but can also be used as vascular stents and for neuron growth regeneration. Fullerenes contain multiple attachment points responsible for tissue binding and therefore can be very useful as carriers for drugs and biomolecules (15, 16). Graphene, an allotrope of carbon, is a thin two-dimensional nanomaterial which possesses excellent electronic, thermal and mechanical characteristics, and hence, has attracted intense interest in diverse areas such as nanoelectronics, solar energy harvesting, biology and medicine. In contrast graphene oxide can be exploited as a nanocargo to deliver different biomolecules including hydrophobic drugs, nucleic acids, and others suitable for therapeutic and bioimaging purposes (1).

The potential exposure routes of NPs in the body are through inhalation, ingestion, and dermal penetration. Nanomaterials used as theranostics are injected mostly directly into blood or lymph circulation. In this context, NP size, shape, surface area, and surface chemistry collectively define both, their desired biological effects and toxicity (17). Therefore, it is crucial that these nanomaterials must be biologically characterized for health hazards in order to ensure their risk-free and sustainable implementation. However, our knowledge of the harmful effects of these nanomaterials, starting from manufacturing processes up until their disposal, or from their entry into the organism up until their elimination, is still very limited. Currently no specific regulations have been developed for the usage of nanomaterials (18), which is the reason why this area is the focus of many research groups.

SCREENING OF TOXICITY OF NANOSTRUCTURES

The tests used for the assessment of cytotoxicity of nanostructures are generally the same as those designed for studying the toxicity of drugs, as well as classical biomaterials. They can be generally divided into *in vitro* and *in vivo* tests. Toxicity tests *in vitro* are necessary in order to assess the cytotoxicity of nanostructures. They are used as general screening systems and can be also very use-

ful for understanding the mechanisms of cytotoxicity. *In vitro* tests are ethically less ambiguous, easier to control and reproduce, and less expensive than animal studies (19). For example, lactate dehydrogenase (LDH) release, as a quantitative measure of cell necrosis, is a very common assay employed in current toxicity studies (20-22). The most popular is the MTT test (termed by the substrate compound 3-(4,5 Dimethylthiazol-2-YI)-2,5 Diphenyltetrazolium Bromide) which measures the enzymatic activity of mitochondria in living cells. The assay gives information about cell viability but cannot discriminate between cell death and reduction of cell proliferation. Therefore, the MTT test should be combined with other tests, such as morphological cell analysis or specific tests for detection and quantification of apoptosis, necrosis or autophagy as well as proliferation assays. The latter assay is based on monitoring of the cell proliferation after several cycles of division (2-5 days) and subsequent cell analysis by flow cytometry or colorimetry. These tests are rapidly replacing previous proliferation tests which were based on incorporation of radioactive thymidine. However, care must be taken concerning the dyes used, since the degree of accuracy of toxicity assays greatly depends on the interaction between nanomaterials and dyes such as Alamar Blue and Neutral Red (19, 23-25). Cell cultures are very often influenced by fluctuations of external environment (temperature, waste concentrations, pH, etc.), so repeated experiments are necessary for obtaining accurate results. In addition, the cytotoxic response to NPs depends on target cell used. The cytotoxic effects of nanostructures *in vitro* can be also evaluated through studies of NP entry into cells together with the tests related to monitoring the integrity of cell membrane. The pathway of intracellular transport, subcellular localization or intracellular disintegration of nanostructures can be analyzed by specific morphological methods, including confocal and electron microscopy, together with measurement of the oxidative stress, lipid peroxidation mediators and other biochemical parameters associated with cellular and subcellular damages. When complex mechanisms are necessary to investigate, such as immunotoxicology or immunomodulation, different co-culture studies are required.

Compared with *in vitro*, *in vivo* tests are necessary for investigating blood contact response to NPs, biodistribution, toxicokinetic, systemic and local toxicity (acute, subacute, sub chronic and chronic toxicity), carcinogenicity, reproductive toxicity, inflammatory response and other reactions caused by NPs. Although animal experiments are costly and time-consuming, they cannot be replaced with *in vitro* tests, as a complete animal experiment offers a significant amount of important data with regard to the toxicity relevant to the entry of NPs in the organism, such as dermal and gastrointestinal toxicities or pulmonary accumulation of NPs. In addition, the toxic effects of nanomaterials on cardiovascular, immune, neuroendocrine, hepatobiliary, renal or reproductive systems can be investigated by specific *in vivo* studies, as mentioned above. In this context, measurements of specific biochemical parameters characteristic for the function of each organ together with histopathological analysis are the most explored methods (17).

CYTOTOXICITY OF NANOSTRUCTURES – *IN VITRO* STUDIES

As already mentioned, *in vitro* studies are the first screening system which assesses the cytocompatibility of nanostructures. The choice of the test depends on the general aim of the study, type of nanostructures and their physicochemical properties. In order to understand the mechanisms of cytotoxicity influenced by a particular nanostructure, the first step is usually related to the entry of NPs into a cell, a process which is largely governed by biological mechanisms of endocytosis (26). This process is based on the receptor-mediated entry, as the most prominent route, and requires recognition of some cell surface ligands by specific biological receptors, followed by the most common way of entry in the cell via clathrin- or caveolin-mediated endocytosis (27, 28). Besides the mentioned way of cell entry, NPs can also use passive diffusion, pinocytosis, as well as other clathrin- and caveolin-independent endocytic mechanisms (i.e. dynamin-independent processes). It is worth to mention that the interaction between NPs and cell membrane receptors could be also important for understanding the biological effects of nanomaterials, independently of their internalization. Endocytic routes of uptake are connected with the delivery of NPs into endosomes and lysosomes, where they are exposed to high concentrations of hydrolytic enzymes, followed by their degradation into ions. These ions can potentially pass through the nuclear or mitochondrial membrane and react with hydrogen peroxide (H_2O_2) and oxygen produced by the mitochondria thereby forming highly reactive hydroxyl (OH) radicals (29). Nanoparticles may also gather around the mitochondria, causing their dysfunction by breaking the balance between the production and consumption of reactive oxygen species (ROS), which generally includes superoxide anion radicals, hydroxyl radicals, singlet oxygen and H_2O_2 (30). In most cases, ROS are produced in the course of synthesis of adenosine triphosphate (ATP), accompanied by the transfer of protons and electrons in the mitochondria (31). ROS production is a normal cellular process which is involved in different aspects of cellular signaling, as well as in the defense mechanism of the immune system. However, when in excess, ROS cause severe damage to cellular macromolecules such as proteins, lipids and DNA (32), leading to additional oxidative stress (33, 34). Inactivation of specific proteins and DNA damage can result in inhibition of cellular proliferation, cell death (necrosis, apoptosis, autophagy) via various mechanisms and genotoxic effects. Another mechanism by which NPs contribute to the production of oxidative stress is their direct catalyzing of ROS, through NADPH, which in turn results in an additional oxidative stress. Cellular and nuclear damage can be also induced by signaling through different membranous receptors (35, 36). Nanomaterials with the most potent ability to induce oxidative stress by ROS, nuclear DNA damage and subsequent cell-cycle arrest, mutagenesis, and apoptosis are Ag NPs, cerium oxide (CeO_2) NPs, silica NPs, fullerenes, block copolymer micelles and CNTs (37). For example, CeO_2 NPs exert toxicity through oxidative stress, which in turn brings about Nrf2-mediated induction of heme oxygenase-1 (HO-1) (38). Ag NPs induce ROS formation, glutathione depletion, and inhibition of superoxide dis-

mutase (39). Studies with Au NPs have revealed that NPs with smaller diameters and hence larger surface area produce higher amounts of ROS (40). Studies with silica NPs indicated that single dose exposure to these nanostructures leads to ROS induction, and subsequent activation of pro-inflammatory responses (41). A ROS generation, a decreased mitochondrial membrane potential, increased levels of lipid peroxide and decreased enzymatic activities of antioxidants were shown to be induced by both, SWCNTs (42) and MWCNTs (43).

The proposed pathways of ROS induction by nanostructures are simplified, but the exact mechanisms by which ROS are generated are not fully understood. The existence of antioxidants can greatly decrease the accumulation of ROS, thus reducing their cytotoxic and genotoxic potential. Therefore, their quantification is of importance in studying the cytotoxicity of NPs. Organs like liver and spleen are the main targets of oxidative stress because of slow clearance of accumulated nanomaterials and prevalence of numerous phagocytic cells. Additionally, organs with high blood flow such as kidneys and lungs can also be affected by NPs e.g. in addition to ROS induction, NPs can induce perturbation of intracellular calcium [Ca^{2+}] which is associated with metabolic and energetic imbalance as well as cellular dysfunction (44). It has been shown that zinc oxide (ZnO) NPs increase intracellular [Ca^{2+}] levels, dependent on the extracellular [Ca^{2+}] entry through the disrupted membrane, as well as leakage of [Ca^{2+}] from the intracellular storage compartments due to endoplasmic reticulum (ER) stress. The interaction of [Ca^{2+}] and ROS involves various cross-talks, and as a result, amplification of cellular damages occurs. It has been shown that decrease in mitochondrial membrane potential (MMP) does not have to be always associated with the effect of ROS. A few examples include: the decrease of MMP in human bronchial epithelial cells (BEAS-2B) and human alveolar adenocarcinoma cells (A549) upon exposure to ZnO NPs (45), the decrease of MMP in neuronal cells (PC12) and A549 cells caused by TiO_2 NPs (46, 47), the decrease of MMP in human mesenchymal stem cells (hMSCs) (48) and human hepatoma cells (BEL-7402) (49) caused by Fe_3O_4 NPs. The binding of NPs such as ZnO, TiO_2, SiO_2, or FeO to proteins can result in irreversible binding processes and subsequent damage of those proteins due to release of metal ions (50). Furthermore, metal ions such as Zn^{2+} and Cu^{2+} released from ZnO and CuO can inactivate certain metalloproteins by dislodging metal ions within them (51).

Another pathway of nanotoxicity pertains to cell cycle arrest. Such a mechanism has been described for TiO_2, Fe_2O_3, CuO, NiO, ZnO, and Al_2O_3 NPs (52-54). Cells can be arrested in one or more cell cycle phases (most commonly in the G_2/M phase), but this process depends on the type of cells and the type of NPs. For instance, exposure to NiO NPs resulted in a significant increase in the G_0/G_1 in the BEAS-2B cell line with a simultaneous significant decrease of the G_0/G_1 phase in the A549 cell line (55). It is interesting to note that these NPs significantly suppressed the G_2/M phase in the BEAS-2B without significant change of S phase but have significantly increased the G_2/M phase in the A549 cells. On the other hand, exposure to ZnO NPs caused an increase in the population of cells in the G_2/M

phase in A549 cells but did not affect the cell cycle in BEAS-2B cells (45, 55). The arrest in cell cycle can also differ depending on the type of NPs. The exposure of BEAS-2B cells to NiO NPs led to an arrest in the G_0/G_1 phase, while exposure to ZnO and Fe_2O_3 NPs did not affect the cell cycle (45, 55). Upon exposure to ZnO and CuO NPs human immortal keratinocyte cells (HaCaT) were arrested in the G_2/M phase, while TiO_2 NPs induced the arrest in the S phase (52, 56, 57). With Al_2O_3 and Fe_3O_4 NPs, an increase in the sub-G_0 phase of human MSCs was reported (48, 58) while A549 cells were arrested in the G_2/M phase upon exposure to CuO (53), NiO (55), and ZnO NPs (59), in contrast to Fe_2O_3 NPs which did not change the cell cycle (45). The most dominant effect of cell cycle arrest is inhibition of cellular proliferation. However, the fate of such cells could greatly differ – damage can either be fixed or accumulated enough to undergo apoptosis. These processes are influenced by different genes, affected by NPs. For instance, exposure of A549 cells to CuO NPs was shown to downregulate about 90 genes involved in control of the cell cycle (53). Some of these genes code for proliferating cell nuclear antigen (PCNA), cell-division cycle protein (CDC2), and cyclin B1 (CCNB1) (53). ZnO NPs were shown to induce the p53 pathway in NCM460 cells, but not in DLD-1 or SW480 cells. These differences could be attributed to the differences in mutation of p53 in the cancerous cell lines. These NPs also caused DNA damage and the downregulation of cyclin B1 and cyclin-dependent kinase 1 (CDK1) in HaCaT, causing G2 arrest and PCNA down-regulation (52). NCM460, DLD-1, and SW480 cell lines up-regulated the expression of checkpoint kinase 1 (Chk-1), which was followed by cell cycle arrest. TiO_2 NPs were found to induce double-strand breaks and a down-regulation of cyclin B1 in A549 cells, leading to cell cycle arrest in the G_2/M phase (59).

Cells in cell cycle arrest can be recovered and continue proliferation upon removal of NPs. For example, A549 cells, whose proliferation was inhibited by CuO NPs, could start to proliferate again in culture upon addition of a fresh medium. Reduction of stress can also allow the cells to recover from the cell cycle arrest. In this context, ZnO NPs exposure induces G_2/M arrest in intestinal cell lines and the addition of antioxidant N-acetylcysteine can reverse this arrest by approximately 50–70% (32).

One of the mechanisms by which oxidative stress induces cytotoxicity is up-regulation of pro-inflammatory mediators through NF-κB (Nuclear Factor-κB), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3-K) pathways (60, 61). Oxidative stress supports inflammation by degradation of κB (IκB) inhibitor and thus allowing subsequent translocation of NF-κB into the nucleus in order to regulate the transcription of its target genes (62). In support of this hypothesis, the OH, HOCl, and $^1O_2$ reactive species have been shown to induce nuclear translocation and activation of NF-κB (63). ROS-mediated activation of NF-κB is directly linked to the production of pro-inflammatory mediators such as TNF-α, IL-8 and IL-6 (64, 65). Several metal oxide NPs including those of Zn, Cd, Si and Fe have also been shown to exert the toxicity via the activation of NF-κB and subsequent production of inflammatory cytokines (66-69). CNTs were

also shown to promote inflammation by augmenting the secretion of TNF-α and Monocyte Chemoattractant Protein-1 (MCP-1/CCL-2) (70). Some of their effects in mesothelial cells are mediated by signaling molecules, such as ARP, AP-1, NF-κB, p38 and Akt (71).

C-Jun NH2-terminal kinases (JNK) and p38 mitogen activated protein kinases (MAPK) from the MAPK signaling pathway are known to regulate responses to cellular stresses (72). In this context, TiO_2 NPs were shown to mediate toxicity in a human bronchial epithelial cell line by augmenting the IL-8 production via the p38 MAPK pathway. Furthermore, Ag NPs induced the toxicity *in vivo* by increasing ROS formation and subsequent expression of p38 MAPK and hypoxia-inducible factor (HIF-1) (73). Similar results were published for of SiO_2 NPs, whose toxicity was associated with the activation of JNK, p53 and NF-κB pathways and the increased production of pro-inflammatory cytokines such as IL-6, IL-8 and MCP-1 (74). On the other hand, ZnO NPs caused an overexpression of Cox-2, iNOS, pro-inflammatory cytokines (IL-6, IFN-γ, TNF-α, IL-17) and unexpectedly, IL-10, a regulatory cytokine, in macrophages through the PI3-K signaling pathway (75). Furthermore, SiO_2 NPs were shown to induce inflammation and activate autophagy via the PI3-K/Akt/mTOR pathway (76). The inflammation and ROS are tightly connected processes. As already mentioned, ROS are potent inducers of inflammation. On the other hand, inflammation has been shown to directly cause toxicity and promote cell death through the induction of ROS production (77). A simplified view of the NP cytotoxicity, including the main mechanisms involved, is presented in Fig. 1.

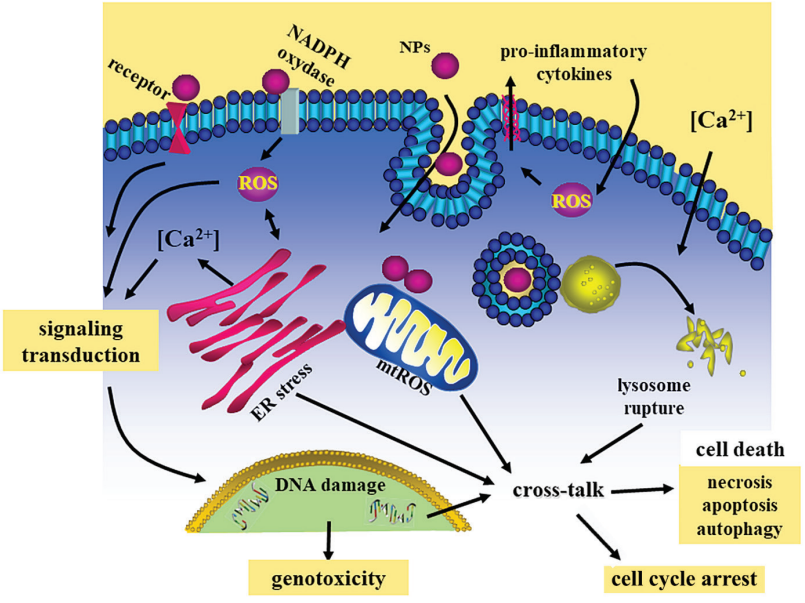

Figure 1. The simplified view of the NP cytotoxicity

NPs enter cell membrane through different mechanisms, but the most common route involves the endocytic pathway. Once localized intracellularly, NPs interfere with different organelles and induce ROS production by dysregulating mitochondria, lysosomes and endoplasmic reticulum (ER). These processes were followed by an increase in intracellular [$Ca2^{+}$] from the ER stores and by the flux of [$Ca2^{+}$] from extracellular spaces. ROS can be also generated by a direct effect of NP to the membranous NADPH. Cellular damages can be also generated through interactions of NPs with particular membranous receptors. ROS together with [$Ca2^{+}$] generates different signaling pathways leading to transcription of different genes, including those for pro-inflammatory cytokines. These cytokines may stimulate inflammation and more ROS production. NPs can damage DNA and stimulate genotoxic mechanisms, directly or indirectly via ROS. In a complex cross-talk between NPs and different biomolecules, cells undergo to cell cycle arrest or death by different mechanisms such as apoptosis, necrosis or autophagy.

INFLUENCE OF PHYSICOCHEMICAL PROPERTIES OF NANOSTRUCTURES ON THEIR CYTOTOXICITY

It is of considerable importance to perform physicochemical characterization of NPs, in terms of their surface properties, surface charge, size, shape, structure, composition, crystalline, and other parameters, since these characteristics influence the interaction of nanostructures with cells and, thus, their overall potential toxicity. Understanding these properties can lead to the development of safer NPs from the same source.

The most important property of NPs contributing to their cytotoxicity is particle size. The size plays a critical role in cellular uptake of nanomaterials, efficiency of NP processing in the endocytic pathway and physiological response of cells to NPs (33, 78-83). Given the same mass, smaller NPs have a larger specific surface area (SSA) and thus more available surface area to interact with cellular structures such as nucleic acids, proteins, fatty acids, and carbohydrates. It has been shown that smaller sized NPs enter the cell more easily than the larger ones. In contrast, larger sized NPs may absorb different proteins on their surface, making the reactivity of NPs with cells in a specific manner. An example how the size of NPs influences their cytotoxicity was published by Kim et al. (84). The authors investigated cellular toxicity of Ag NPs using three different characteristic sizes on several cell lines. They demonstrated that the toxicity was clearly size- and dose-dependent in terms of cell viability, intracellular ROS generation, LDH release, induction of apoptosis and ultrastructural changes in cell morphology. Smaller sized Ag NPs were much more cytotoxic than the larger-sized NPs. Pan et al. (85) demonstrated that Au NPs sized from 0.8-1.8nm induced toxicity in HeLa, SK-MEL-26, L929 and J774A1 cells unlike larger (>15nm) Au NPs. We showed that Au NPs were not cytotoxic for L929 cells, but decreased their growth, whereas

Au NPs smaller in size had a stronger effect (86). Other papers also suggested that chemically reduced Au NPs larger than 4nm did not reduce viability significantly in the array of cells tested, but could reduce cellular proliferation (87-91), and confirmed once again that smaller nanoparticles had stronger anti-proliferative effects (88). Regarding the mechanisms involved, it has been shown that non-toxic Au NPs can reduce cellular proliferation by inducing a transient oxidative stress (92), affecting cytoskeleton architecture (93) or interacting with negatively charged DNA (94).

Dey et al. (95) also showed that hydroxyapatite (HAp) NPs were more cytotoxic to HCT116 cells than the micron-sized HAp particles. Similar results were published by Yuan et al. (96) who showed that HAp NPs induced apoptosis in tumor HepG2 cells, which was strongly size-dependent. For some NPs, toxicity was found to be a function of both size and SSA. For instance, the size of TiO_2 was shown to correlate with ROS production when comparing the amount of ROS produced per surface area within a certain size range (97). It is interesting that TiO_2 NPs below 10 or above 30 nm in size produced similar levels of ROS per surface area. However, there was a dramatic increase in the ROS production per unit surface area when the size of these NPs increased from 10 to 30 nm. In line with these findings, Yin et al. (98) examined the effects of particle size and surface coating on the cytotoxicity of nickel ferrite *in vitro* using the Neuro-2A cell line as a model. They concluded that nickel ferrite NPs without oleic acid coating, induced cytotoxicity independently of particle size within the given mass concentrations and surface areas. However, nickel ferrite NPs coated with oleic acid, induced cytotoxicity that increased significantly when one or two layers of oleic acid were deposited. It is interesting that large NPs with coatings of oleic acid, showed a higher cytotoxicity than smaller particles.

In addition to size, the structure and shape of nanomaterials are two additional crucial factors that influence their toxicity. NPs may have different shapes and structure, such as tubes, fibers, spheres, planes, and polyhedral shape. These distinctions may lead to differences in their toxicity effects. An example is the effect of different carbon-based NPs on mouse keratinocytes, published by Grabinski et al. (99). The carbon materials tested included carbon nanofibers, MWCNTs, and SWCNTs. The authors concluded that carbon nanofibers did not significantly affect cell viability. However, MWCNTs and SWCNTs reduced cell viability in a time-dependent manner, due to the ROS production, so that cells exposed to MWCNTs produced three times higher levels of ROS than those exposed to SWCNTs. Zhang et al. (100) compared the cytotoxicity of graphene and CNTs. They found that both graphene and SWCNTs induced cytotoxic effects, which were concentration-dependent and shape-dependent. The finding that graphene induced a stronger metabolic activity than that of SWCNTs at low concentrations, suggests the importance of shape on cellular toxicity. In this context rod-shaped Fe_2O_3 NPs were found to produce much higher cytotoxic responses than sphere-

shaped Fe_2O_3 NPs in a murine macrophage cell line (RAW 264.7), including higher levels of LDH release, inflammatory response, ROS production, and necrosis (101). Amorphous TiO_2 was found to generate more ROS than anatase or the rutile forms of similar structure (97). It is likely that amorphous TiO_2 has more surface defects, and therefore more active sites that are capable of causing ROS. The anatase form of TiO_2 was also significantly more toxic to PC12 cells than the rutile form (47). Rod-shaped CeO_2 NPs produced significant LDH release and TNF-α in RAW 264.7 cells, while neither octahedron nor cubic forms induced significant responses. Different toxicity behavior has also been shown for TiO2 NPs with different crystal structures (102). Gold nanorods are reported as more toxic than spherical Au NPs (103), but the mechanism of toxicity seems to include toxic contaminants from the nanorod synthesis, rather than a direct toxicity (104). In contrast to spherical Au NPs, the data on cytotoxicity of gold nanocages and gold nanostars *in vitro* is quite scarce. Why the physical shape of a nanoparticle influences cytotoxicity remains to be elucidated.

The concept of "nanomaterial surface" includes different aspects, such as surface area, pore, surface chemical bond, potential (charge) and surface changes by covalent attachment of different molecules or chemical groups (functionalization). Of them, particle surface charge was mostly investigated. The surface charge may affect the cellular uptake of particles, their interaction with organelles and biomolecules as well as the degree of cytotoxicity. The surface charge is also a major determinant of colloidal behavior, which influences the cellular response by changing the shape and size of NPs through formation of aggregates or agglomerates. An example is the experiment when three similarly sized iron oxide particles with different charges were shown to have differential toxicities on a human hepatoma cell line (BEL-7402) (49). The toxicity of oleic acid-coated Fe_3O_4, carbon-coated Fe, and Fe_3O_4 NPs increased with an increase in surface charge. This suggests that the higher positive charge the NPs have, the greater electrostatic interactions they have with the cell, which is followed by greater endocytic uptake. Another example is the experiment showing that positively charged ZnO NPs produce stronger cytotoxic effects in A549 cells than negatively charged NPs of a similar shape and size (105). The phenomenon can be explained, in part, in the context of cellular membrane composition. It is known that negatively charged glycosaminoglycans are abundant on the cell surface and could interact electrostatically with positively charged NPs (106). If the electrostatic interactions are higher, the more likely NPs could be internalized (107). Shahbazi et al. (108) evaluated the impact of mesoporous silicon NPs surface chemistry on immune cells and human erythrocytes both *in vitro* and *in vivo*. They concluded that negatively charged hydrophilic and hydrophobic mesoporous silicon NPs caused less ATP depletion and genotoxicity than the positively charged amine modified hydrophilic mesoporous silicon NPs, thus proving the significance of surface charge on the cytotoxicity of examined cells.

The same was true for positively charged NPs, which interact with negatively charged DNA, leading to DNA damage (17). It is interesting that negative charged PLGA NPs led to a higher inflammatory response, which is associated with their higher uptake by immune cells (109). Moreover, Calatayud et al. (110) studied the effect of the surface charge of functionalized Fe_3O_4 nanoparticles on protein adsorption and cell uptake. It was demonstrated that the functional groups on the magnetic NP surface determined the formation of protein-magnetic NP clusters. This experiment suggests the ability to modify the surface of magnetic NPs in order to control the non-specific protein adsorption. El Badawy et al. (111) studied the various factors that influence toxicity of Ag NPs and concluded that the surface charge was one of the most important parameters. Furthermore, it was shown that Ag NPs exhibited an obvious surface charge dependent toxicity on the different bacillus species.

For NPs with the same composition, surface properties may also affect their cytotoxicity. It was demonstrated that CNTs, fullerenes, Au NPs, and silica NPs could be modified with diverse surface chemistry. This may alter cytotoxicity both *in vitro* and *in vivo*. In this context, functionalized MWNTs induced different levels of protein binding, cytotoxicity, and immune responses (112). The modification of MWCNTs significantly inhibited NF-κB activation and reduced immunotoxicity of MWCNTs in BALB/c mice (113). We showed that the surface functionalization of MWCNTs with amino groups via chemical modification of carboxyl groups introduced on the nanotube surface exhibited much better dispersibility and biocompatibility than non-functionalized, row MWCNTs. Functionalized MWCNTs, at the concentrations between 1 and 50 μg/ml, were not cytotoxic for the fibroblast L929 cell line. However, the concentrations of MWCNTs greater than 10 μg/ml reduced cell growth and this effect correlated positively with the degree of their uptake by L929 cells (114). MWCNTs functionalized with polyethylene glycol (PEG) induced less generation of ROS and cytotoxicity in macrophages than MWCNTs-COOH, which correlated with the lower cellular uptake of MWCNTs-PEG (97). Silica NPs (70 nm) induced strong inflammation in mice after intraperitoneal administration, but this reaction was dramatically suppressed by surface modification by carboxyl groups (115).

IMMUNOTOXICITY AND IMMUNOMODULATORY PROPERTIES OF NANOSTRUCTURES

The term immunotoxicity refers to the cytotoxic effect of different substances on the immune system components. Immunotoxicity can be considered together with general cytotoxicity, but some specific characteristics of the immune response to NPs, may significantly modify their cytotoxic potential. Under cer-

tain circumstances, NPs could not damage or kill immune cells, but modulate their functions towards down-regulation (immunosuppression), or up-regulation (immunostimulation). Therefore, it is important to consider immunotoxicity and immunomodulation as two complementary phenomena (116).

When applied *in vivo*, NPs may be recognized as foreign antigens by the immune system and provoke the immune response. Usually, NPs are not immunogenic themselves, but act as haptens when attached to large molecules, such as serum albumin. Certain nanostructures, like Au NPs, have been used as adjuvants to potentiate the production of antibodies to small antigens with low immunogenicity (hormones, peptides, antibiotics, vitamins, etc.) (117). Such properties of gold nanostructures may have a role in vaccination strategy. NPs may suppress general immune responses without manifesting cytotoxicity, by interfering with complex signaling pathways regulating both inflammation and immune responses (118). Certain NPs could have specific immunotolerogenic properties, a phenomenon which has been described recently by our research group (119). So, it is important to consider all these effects very carefully because they could bring unexpected side effects in the clinical treatment. These facts are also relevant for the preparation of less immunogenic or tolerogenic NPs and for the adequate modifications of existing NPs to reduce their proinflammatory, immunosuppressive and immunotoxic properties (118). In addition, understanding the effects of NPs on immune cell functions is essential in designing safe and effective NP-based *in vivo* drug delivery systems. There are many research papers related to the immunotoxic and immunomodulatory properties of different NPs and our research group provided additional evidence on the complex immunomodulatory mechanisms of CNTs, Au NPs, graphene quantum dots (GQD) and nanocellulose fibrils (91, 119-121)

CNTs have been shown to induce systemic immunosuppression in mice after inhalation (122-124), including production of prostaglandin E2 (PGE2) and IL-10 (122, 124) and T cell dysfunction (123, 125). Subcutaneous administration of MWCNTs in BALB/c mice was followed by the activation of complement, augmented production of proinflammatory and Th2 cytokines (124) and increased number of $CD4^+$ and $CD8^+$ T cells in the spleen (126). In other studies, MWCNTs showed allergy adjuvant effects in OVA^-sensitized mice, induced fibrosis in lungs and aggravated asthma (70, 127). Laverny et al. (128) showed that MWCNTs increased the release of a series of cytokines in peripheral blood mononuclear cells (PBMCs) from healthy donors after stimulation with toll-like receptor (TLR) agonists or T-cell mitogen. However, the nanostructures suppressed the immune responses in PBMCs from mite-allergic subjects.

Dapsone is an anti-microbial and anti-inflammatory drug with low solubility. We showed that MWCNTs conjugated with dapsone (dap-MWCNTs) were highly soluble, and they were rapidly ingested by rat peritoneal macrophages (PMØ) as were the control, oxidized o-MWCNTs. Neither dap-MWCNTs, nor o-MWCNTs, at lower concentrations (up to 50μg/ml), affected the viability of

PMØ, while higher concentrations triggered apoptosis. Apoptosis of PMØ induced by o-MWCNTs was higher than apoptosis induced by dap-MWCNTs and it correlated with the induction of oxidative stress in PMØ (121). It is interesting that equivalent concentrations of soluble dapsone induced oxidative stress, possibly due to its low solubility in non-conjugated form, but not apoptosis of PMØ. A number of excellent review articles have addressed the toxicity of Au NPs, including immunotoxicity (89, 129-131). It has been shown that bulk gold and many synthesized Au NPs are not toxic *in vitro* (1) and *in vivo* (132). However, cytotoxicity of some Au NPs samples observed *in vitro* vary greatly with several key parameters, such as their purity, size, shape, charge, stabilization agent, incubation conditions, type of cells used, and interference with the assay readout (131). Furthermore, the presence of cytotoxic contaminants during synthesis of Au NPs appears fundamental for their toxicity profile (104, 133). In our previous studies (86, 134) we showed that the synthesis of Au NPs from the gold scrap precursor by ultrasonic spray pyrolysis (USP) provided 5 fractions of Au NPs with increasing amounts of alloying contaminants. Two fractions (1 and 2) of Au NPs composed of pure gold were not toxic for rat thymocytes and splenocytes, even after 3 days of culture with up to 100 μg/ml of Au NPs, as assessed by MTT and cell viability assays. Other three fractions were cytotoxic.

Au NPs have a strong adjuvant effect on the immune response, however, the mechanisms involved are not yet fully understood. Some experiments have suggested that Au NPs can stimulate B cells directly, as demonstrated in a study with a CH12.LX B cell line, which increased NF-kB expression after Au NPs treatment (135). Additionally, Au NPs were shown to up-regulate blimp1 and down-regulate pax5 expression in B cells, leading to secretion of IgG by these cells (136). It has been suggested that innate immunity cells, such as macrophages and dendritic cells (DCs), are also involved in the adjuvant effect of Au NPs.

Innate immunity cells, such as granulocytes, monocytes, macrophages and DCs, are the first cells to interact with NPs upon their entry into the body, each having a specific tissue distribution and a set of functional responses at its disposal. The reported immunological effects of Au NPs vary greatly, and their effects were described as adjuvant (stimulation of the innate immune response), proinflammatory (activation of phagocytes to produce IL-1β, IL-6, TNF-α, etc.) (137), immunosuppressive due to immunotoxicity or down-regulation of the immune response (138), immunomodulatory (139), neutral or anti-inflammatory (91, 140). The critical parameters for different immunological effects of Au NPs on macrophages and DCs seem to be their size and coating. Upon placement into biological solutions, Au NPs immediately receive corona made of a dozen different proteins, predominantly albumin, apolipoprotein, immunoglobulins and fibrinogens (141, 142). Walkey et al. (143) showed that the composition of corona on PEG-coated Au NPs depends on PEG density and Au NPs size. This subsequently determines the predominant mechanism of Au NPs uptake by macrophages, possibly via involvement of different receptors. It has been suggested that the uptake

of Au NPs via scavenger receptors by macrophages does not induce release of proinflammatory cytokines (144). In contrast, Au NPs coated with mannose-containing polysaccharides are able to trigger the activation of mannose receptors on macrophages, leading to proinflammatory response, which can be harnessed for development of Au NPs-based vaccines (145). Similarly, coating of Au NPs with TLR agonists, such as TLR-9 agonist cytosine-phosphate-guanosine (CpG) oligonucleotide (ODN), was shown to induce immunostimulatory effects in macrophages, observed as an increased production of TNF-α and IL-6 by these cells (146). Interestingly, un-conjugated Au NPs impaired CpG-induced production of TNF-α and IL-6 (147) and LPS-induced production of NO and IL-6 (139) by RAW274.7 murine macrophages in a size-dependent manner. We showed that Au NPs made of pure gold inhibited proliferation of splenocytes stimulated with Concanavalin A (Con-A), and that smaller Au NPs had a stronger effect (134). These results correlated with a decreased production of IL-2, and an increased production of IL-10 in Con-A-stimulated cultures treated with smaller Au NPs. An increased IL-10 production upon Au NPs treatment was also observed by Liptrott et al. (148) in an equivalent model of phytohemagglutinin-activated human lymphocytes cultures. IL-10 is a known anti-inflammatory cytokine with anti-proliferative effects, produced by both regulatory T cells and antigen-presenting cells (APC) with tolerogenic potential (predominantly M2 macrophages and tolerogenic DCs) (149). Devanabanda et al. (150) investigated the immunomodulatory potential of Au NPs and Ag NPs *in vitro* using murine splenic and human peripheral blood lymphocytes (PBL) in terms of effects on viability and mitogen-induced proliferation. They reported that lymphocyte proliferation was significantly inhibited by Au NPs (25–200 μg/ml) and Ag NPs (12.5–50 μg/ml) in a dose-dependent manner.

Monocytes represent a major APC population in blood, which give rise to both macrophages and DCs upon extravasation (151), both of which are responsible for the regulation of inflammatory responses to different nanostructures in tissues (118). There are three functionally different subpopulations in human blood, classical $CD14^{+}CD16^{-}$, inflammatory $CD14^{+}CD16^{+}$ and regulatory $CD14^{low}CD16^{+}$ monocytes. Regulatory monocytes do not extravasate into tissues, and they were shown to regulate blood vessel repair upon damage (152, 153). We showed that citrate-stabilized Au NPs of different sizes produced by USP (~10nm and ~40nm, both at 50μg/ml) were internalized by human monocytes after 24h in culture, without eliciting any toxic effects. However, phenotypic analysis of monocytes revealed that the percentage of inflammatory $CD14^{+}CD16^{+}$ subpopulation was reduced significantly compared to non-treated monocytes, whereas the $CD14^{low}CD16^{+}$ population was not affected. Furthermore, both Au NPs reduced significantly the expression of key molecules responsible for antigen-presentation and costimulation (HLA-DR and CD86, respectively), as well as proinflammatory cytokines (TNF-α) and p40 subunit (IL-12/IL23) by monocytes. Again, smaller Au NPs exhibited a stronger effect. These results suggested for the first time that citrate

capped Au NPs exhibited anti-inflammatory effects on human blood monocytes (154). Considering that TNF-α, IL-12 and IL-23, as well as increased expression of HLA-DR and CD86 are considered proatherogenic (155), the down-modulatory effects of Au NPs could be beneficial for further development of theranostics for atherosclerosis.

In contrast to Au NPs, Ag NPs seem to be more immunotoxic and pro-inflammatory, partly owing to the stimulation of ROS production in immune cells (156, 157). As per other mechanisms involved in the modulation of the immune system, it has been shown that Ag NPs (22 nm) exposure caused the downregulation of Malt1 and Sema7a expression. The inhibition of these gene products is associated with immune cell dysfunctions, such as aberrant T cell differentiation (158).

Some *in vitro* studies showed that iron oxide NPs did not induce inflammatory response on human macrophages (159) and aortic endothelia cells (160). However, high doses of iron oxide NPs may induce ROS production (161). The immunomodulation of the iron oxide NPs was also seen *in vivo*. For example, intratracheally administrated high and intermediate dose of iron oxide NPs with a diameter of 35 ± 14 nm or 147 ± 48 nm inhibited the allergic Th2-dominated response induced by ovalbumin (OVA). The low dose of these larger size NPs particles had no significant effect, while the low dose of smaller size particles had an adjuvant effect on the Th2 response to OVA. (162). Another study showed that intratracheal instillation of AgNPs with a diameter of 52.25 ± 23.64 nm induced inflammation in the respiratory tract by affecting alveolar macrophages and epithelial cells, which generated ROS and produced inflammatory cytokines. (163).

Intravenously injected iron oxide NPs (58.7 nm) shifted the Th1/Th2 balance towards the Th2-dominant direction and suppressed the delayed-type hypersensitivity in OVA-sensitized mice (164). In addition, repeated administrations of these NPs suppressed inflammation more strongly than single instillation (165).

Instillation of CeO2 NPs with a diameter of 8 nm in mice was followed by inflammation in pulmonary system (166). In a similar study the lung toxicity was accompanied by oxidative stress and up-regulation of IL-1β, TNF-α and IL-6 (167). However, some *in vitro* studies indicated that CeO2 NPs with a small diameter (3–5 nm) caused a significant anti-inflammatory effect in a murine macrophage line by scavenging ROS (168). A recent study reported that these NPs, of the same size, stimulated the production of IL-10, and induced a Th2-dominated T cell proliferation (117).

Amorphous silica NPs were investigated in PBMCs and purified monocytes. These NPs could induce inflammatory response by augmenting the production of IL-1β, IL-8, and ROS through MAPKs (169). However, modification of carboxyl groups on silica particles dramatically suppressed the inflammatory responses (115). Other reports showed that 30 and 70 nm silica NPs induced higher production of TNF-α in RAW264.7 cells and stronger immune/ inflammatory responses than 300 and 1000 nm particles *in vivo* (117). Intravenous injections of silica NPs

with a single dose of 50 mg/kg caused hepatic inflammation and oxidative stress in mice (170). Similarly, Kupffer cells stimulated by 15 nm silica NPs released large amounts of ROS, TNF-α and NO (117).

Two studies with graphene NPs showed their proinflammatory effects in macrophage cell lines (171, 172). In this context Yue et al. (172) showed that larger, micro sized particles (2μm) induced stronger inflammation responses compared to nanosized (350 nm) particles. When graphene oxide NPs were coated with PVP oxide, the physiological activity of macrophages was enhanced by these NPs (173). Graphene may stimulate the immune response. In this context, intravenously delivered graphene nanosheets induced Th2 inflammatory responses in the lungs via the IL-33/ST2 axis (126), which was a phenomenon that could be associated with the exacerbation of allergic diseases. Quantum dots were shown to be immunotoxic due to the oxidative stress (174, 175). They can also suppress the immune response, as demonstrated for CdTe quantum dots on a macrophage cell line, by reducing NO, TNF-α, KC/CXCL-1, and IL-8 production (176).

Some studies showed that C60 fullerenes have immunostimulatory properties (177-181). In addition, after instillation, C60 upregulated gene expression of various proinflammatory cytokines (IL-1, TNF-α, IL-6) and Th1 cytokines (IL-12, IFN-γ) in mice. The carboxyfullerenes could stimulate the extravasation of neutrophils and enhance their bactericidal activity (181). An interesting study showed that immunization of mice with a C_{60} fullerene conjugated to bovine thyroglobulin stimulated the production of fullerene-specific antibodies (178). *In vivo* studies indicated that polymer-based NPs inhibited inflammation but had no effect on host immunity (182, 183). On the other hand, some polymer-based NPs, such as carboxymethyl chitosan were shown to be effective adjuvants in vaccination (184) due to the activation of cellular immune responses (185). However, some polymeric NPs, such as polystyrene, had opposite effects. These NPs induced T-cell tolerance and ameliorated experimental autoimmune encephalomyelitis by inactivating pathogenic T cells (186).

The described immunomodulatory effects of different NPs give us just a simplified view of their complexity, where different cells and different mechanisms are involved. Therefore, each nanostructure deserves to be thoroughly investigated, because the response of the immune system to a particular nanostructure may have unexpected health effects.

DENDRITIC CELLS AS A KEY TARGET OF THE IMMUNOMODULATORY *Effect of nanostructures*

DCs are key cells of the innate immunity with antigen-presenting functions that regulate the immune response on the crossroad between immunity and tolerance (187). DCs are of hematopoietic origin and are located as precursors in bone

marrow and blood, as immature APC in peripheral tissues and as mature state in peripheral lymphoid organs. In their immature state, DCs take up and process antigens, but cannot prime T cells efficiently, leading to T cell anergy (188). In contrast, the activation of DCs via innate immunity receptors, such as TLR4 by LPS or dead cells (189), triggers the signaling mechanisms involved in their transition towards mature DCs, such as the loss of spontaneous Ca^{2+} oscillations and nuclear transportation of Ca^{2+}-sensitive transcription factor NF-kB and NFAT (190). This leads to phenotypic maturation of DCs, increased expression of co-stimulators and their migration from the periphery into draining lymph nodes. There, mature DCs induce proliferation of T cells and produce cytokines that are responsible for T cell differentiation. Some of the cytokines are IL-12p70 which induce differentiation of IFN-γ producing T helper (Th) 1 cells and cytotoxic $CD8^+$ T cells (CTLs). IL-23 is responsible for the expansion of Th17 cells, and IL-10 is the main cytokine responsible for the induction of regulatory T cell populations (Tregs) and expansion of Th2 cells (187, 188, 191). Th1, Th17 and CTLs are required for pathogen clearance, efficient anti-tumor response but also for the induction of many autoimmune diseases. On the other hand, Tregs and Th2 cells suppress inflammation, leading to repair of damaged tissues, but they also lead to tumor progression and the establishment of chronic infections (192).

There is a large number of published papers on the effect of different nanostructures on DCs and their effects may be generally viewed as immunotoxic and immunomodulatory (117). The cytotoxic response of DCs to NPs is similar to the response of other cells. However, modulation of DC functions greatly depends on the type of NPs, their doses and physicochemical characteristics. Our several papers describe molecular mechanisms involved in immunomodulatory activity of CNTs, Au NPs, cellulose nanofibrils (CNFs) and graphene quantum dots (GQDs) and outline their main similarities and differences (91, 119, 120, 193). Some of these properties, related to the internalization of NPs and their Th polarization capability, are given in Fig.2 and Fig.3. respectively.

As already mentioned earlier, CNTs have been considered as a promising tool in delivery of drugs and biomolecules to specific cellular and intracellular targets. We have studied the response of human monocyte derived dendritic cells (MoDCs) to MWCNTs functionalized with 7-thia-8-oxoguanosine (7-TOG), which is a selective Toll-like receptor (TLR) 7 agonist or dapsone, an anti-infectious and an anti-inflammatory drug. Functionalization was performed by covalent attachment of these compounds to oxidized (o)-MWCNTs. Using confocal laser microscopy and transmission electron microscopy, we showed that MoDCs efficiently ingested MWCNTs (Fig.2) and that neither control nor functionalized MWCNTs were cytotoxic. 7-TOG-MWCNTs induced maturation of MoDCs and potentiated the allostimulatory and Th1 and Th17 polarizing capability of these cells, similarly as did the soluble 7-TOG at 4-10 times higher concentrations. These findings could be important for tumor immunotherapy, bearing in mind that Th1- and Th17-mediated immunity suppress tumor growth and potentiate the

cytotoxic mechanisms by immune cells. We also showed that dap-MWCNTs inhibited maturation of MoDCs and suppressed proliferation of alloreactive CD4+ T cells as well as T cell-mediated immune responses, more strongly than equivalent concentrations of soluble dapsone or soluble dapsone combined with o-MWCNTs (data not shown). Cumulatively, the obtained results show that the interactions between functionalized MWCNTs and DCs are very complex and they differ from the effects of non- functionalized MWCNTs and soluble conjugates. The results also pointed out the possibility to use conjugated MWCNTs in the development of tumor vaccines or for the treatment of dapsone-sensitive intracellular microorganisms or inflammatory diseases responding to dapsone therapy.

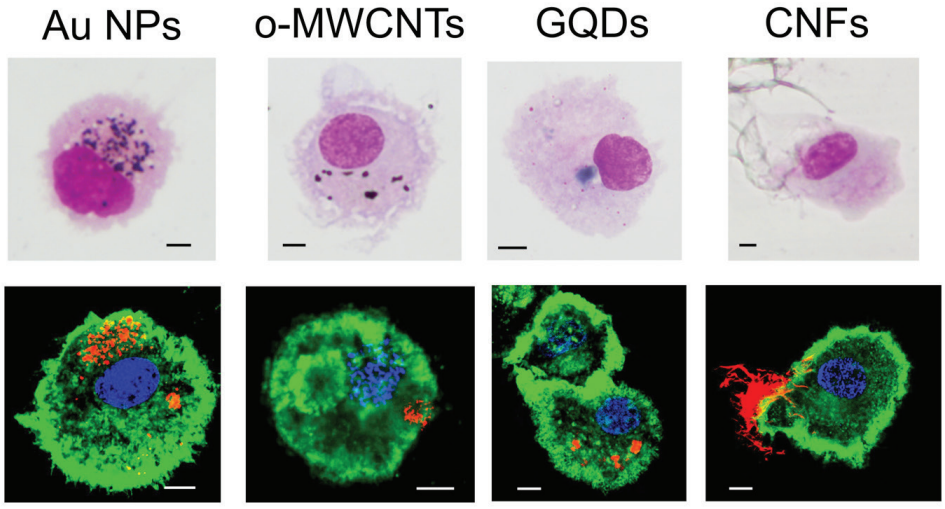

Figure 2. Interaction between human MoDCs and nanostructures

Images show human MoDCs after the cultures with different NPs (Au NPs, GQD), nanotubes (o-MWCNTs) or nanofibers (CNFs), prepared for light microscopy (upper row-MGG staining) or confocal microscopy (lower row- staining of MHC class II-green, and nuclei-blue. Nanoparticles were detected by light scattering (Au NPs, GQDs, o-MWCNTs) or after Calcofluor staining (CNFs), and are marked in red. Scale bars represent 10 μm. MoDCs either completely internalized nanoparticles (Au NPs, o-MWCNTs and GQDs), or just partially (CNFs), due to large size. The internalized nanostructures were present in the cytoplasm perinuclearly, or under the cell membrane depending on their size, surface charge and intracellular trafficking. Fibrillar nanoparticles (CNFs) interact predominantly via membrane surface, and small protrusions of CNFs can be seen partially internalized in the cytoplasm of MoDCs. The mechanism of nanostructures internalization may point to the signaling molecules involved in the interaction with DCs. See further discussion in the text.

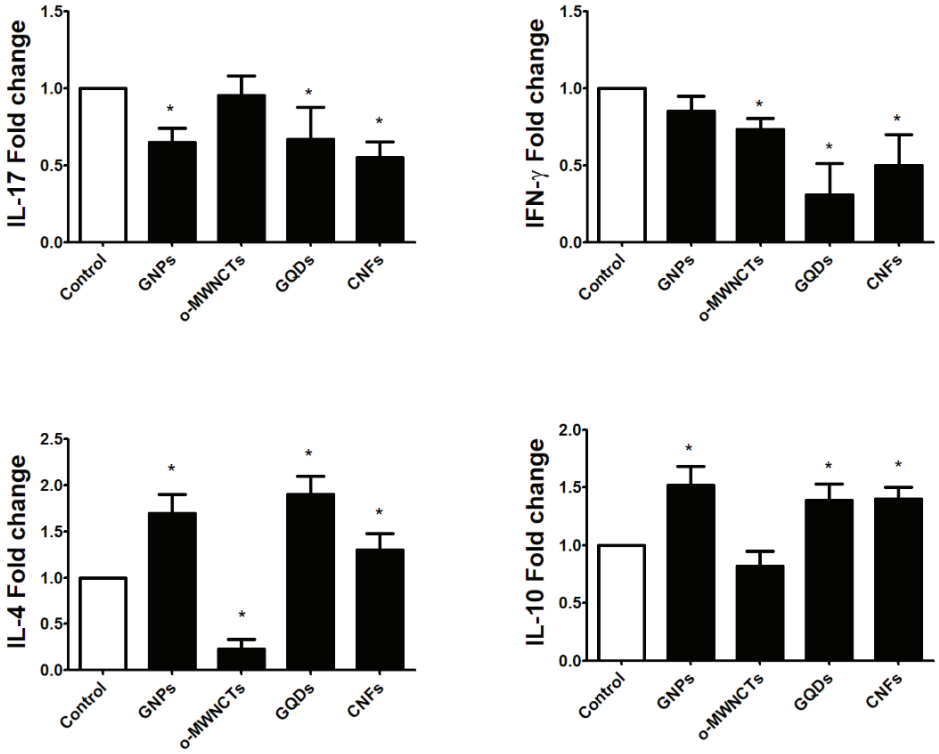

Figure 3. MoDCs pre-treated with nanostructures trigger different cytokine responses in co-culture with T cells

The study model usually involves the treatment of MoDCs in the stage of maturation or differentiation, with non-toxic concentrations of nanostructures, followed by thorough washing of DCs from free NPs and stimuli before the co-culture with T cells. Upon interaction with DCs, T cells differentiate into IFN-γ-producing (Th1), IL-17-producing (Th17), IL-4 producing (Th2), or IL-10-produing regulatory T cell populations in the process of Th polarization. DCs treated with nanostructures may reduce inflammation by inhibiting the production of pro-inflammatory cytokines (IFN-γ and IL-17), by up-regulating the production IL-4 and IL-10 by T cells, or both. In our experiments proinflammatory Th subsets (Th1 and Th17) were down-regulated by DCs pre-treated with all nanoparticles, whereas IL-10 and IL-4 were best induced by GNPs-, GQD- and CNF-treated DCs.

The studies with other carbon NPs showed that fullerenes may enhance the production of IL-6, activate NK cells and DCs, which stimulate subsequently T-cell mediated immune responses (177, 180). Graphene oxide NPs could induce DCs to differentiate and mature at varying degrees (173) but suppress the anti-

gen-delivering ability of OVA-loaded DCs to T cells (180). This mechanism was associated with down-regulation of LMP7 subunit of immunoproteasome in cells, which is responsible for antigen processing in DCs (180). When coated with polyvinilpyrrolidone (PVP), graphene exhibited lower immunogenicity than non-coated graphene, regarding maturation and differentiation of DCs (173). Similarly, PVP-coated iron oxide NPs, showed a decreased antigen processing and CD4+ T cell stimulation capacity of human MoDCs (194).

Graphene quantum dots (GQDs) are atom-thick nanodimensional carbon sheets with excellent physico-chemical and biological properties, which make them attractive for application in theranostics (1). However, their immunoregulatory properties are insufficiently investigated, especially in human primary immune cells. We found that non-toxic doses of GQDs were able to inhibit the production of proinflammatory and Th1 cytokines, and augment the production of anti-inflammatory and Th2 cytokines by human PBMNCs. While unable to affect T cells directly, GQDs impaired the differentiation and functions of MoDCs, lowering their capacity to stimulate T cell proliferation, development of Th1 and Th17 cells, and T-cell mediated cytotoxicity (Fig.3). Additionally, GQD-treated DCs potentiated Th2 polarization, and induced suppressive $CD4^{+}CD25^{high}Foxp3^{+}$ regulatory T cells. After internalization (Fig.2) in a dynamin-independent, cholesterol-dependent manner, GQDs lowered the production of ROS and nuclear translocation of NF-κB in DCs. The activity of mammalian target of rapamycin (mTOR) was reduced by GQDs, which correlated with the increase in transcription of autophagy genes and autophagic flux in DCs. In addition, genetic suppression of autophagy impaired the pro-tolerogenic effects of GQDs on DCs. Our results suggest that GQD-triggered autophagy promotes tolerogenic functions in MoDC, which could be beneficial in inflammatory T-cell mediated pathologies but could be harmful in GQD-based anti-cancer therapy (120).

Several studies investigated the effects of metallic NPs on DCs and most of them were related to gold. As shown in the studies with macrophages, the effects of Au NPs on DC functions depend on the physicochemical properties of surfaces of these particles. For example, positively charged Au NPs were shown to induce IL-1β and IL-6 production by DCs (195). Au NPs coated with adjuvant molecules or immunogenic antigens were shown to induce activation and pro-inflammatory phenotype of DCs. In line with this, Cheung et al. (196) showed that the Epstein-Barr virus peptide can be delivered successfully to DCs via 15nm Au NPs, leading to activation of antigen-specific CTLs. Similarly, 13nm Au NPs conjugated with prostate cancer antigens and Fc fragments of IgG, were internalized effectively by DCs in an FcγR-dependent manner, leading to their increased capacity to stimulate proliferation of lymphocytes (197). However, different effects of Au NPs on DCs could be expected when using un-coated Au NPs. Villiers et al. (140) showed that non-coated 10nm Au NPs did not affect IL-6 production by DCs, but reduced IL-12p70 production upon LPS stimulation.

Our study showed that the size of Au NPs could be critical for the selection of proper nanoparticles for tumor diagnostics and therapy, as well as for the treatment of inflammatory conditions including autoimmune diseases (91). Namely, using two sizes (10nm and 50nm) of chemically reduced Au NPs at non-toxic concentrations, we found that smaller Au NPs suppressed phenotypic maturation of DCs, as judged by a lack of up-regulation of CD86, HLA-DR and CD83 expression. This correlated with an impaired capacity of 10nm Au NPs -treated DCs to induce T cell proliferation *in vitro*. Additionally, DCs treated with 10nm Au NPs, unlike 50nm GNPs, produced more IL-10, and less IL-12 and IL-23, leading to an increased Th2 polarization in co-culture with T cells (Fig.1). The question arose as to why Au NPs with different sizes displayed such different effects on DC maturation, and therefore we observed that smaller Au NPs were taken up by DCs (Fig. 2) using both dynamin-dependent and dynamin-independent mechanisms, whereas larger Au NPs entered predominantly in a dynamin-dependent manner. Additionally, a higher number of smaller Au NPs per DCs was found, as evaluated by micro particle induced X-ray spectroscopy (91, 198). Smaller Au NPs also escaped endosomes more often as determined by TEM and focused-ion beam Scanning Electron Microscopy (91). All of this could lead to stronger alteration of signaling processes by smaller Au NPs, as well as to activation of additional signaling pathways, such as those involved in dynamin-independent internalization processes (199, 200). We also found that DCs internalized GNPs from necrotic cells that, in the case of smaller GNPs, led to a weaker maturation of DCs, their lower capacity to stimulate proliferation of T cells, and an increased capacity to induce Th2 cells` differentiation. Consequently, the internalization of smaller Au NPs derived from necrotic tumor HEp-2 cells by DCs impaired their capacity to induce CTLs capable of killing HEp-2 cells. In contrast, larger Au NPs did not impair the maturation and anti-tumor functions of DCs. These results suggested that smaller Au NPs could be potentially hazardous if applied for tumor diagnostics and therapy, as they could trigger DC-mediated up-regulation of pro-tumorigenic Th2 cells (201). On the other hand, smaller Au NPs could be potentially beneficial for the treatment of inflammatory conditions and autoimmune diseases. Our studies suggest that larger GNPs are safer for cancer therapy and diagnostics, since they do not induce adverse immune effects. However, further improvement and optimization of their application in cancer therapy will be required, such as functionalization with agents that possess immunostimulatory effects. In line with these observations, Lin et al. (202) reported that Au NP delivery of modified CpG (a TLR9 agonist with immunostimulant activity) could stimulate macrophages and inhibit tumor growth for immunotherapy. Ahn et al. (203) recently demonstrated that Au NPs enabled efficient tumor-associated self-antigen delivery to DCs and then activated the cells to facilitate cross-presentation, inducing antigen-specific cytotoxic T cell responses for effective cancer therapy.

Some polymer NPs were reported to activate the immune system through modulating the functions of immune cells, such as DCs and T cells (204-206).

Amphiphilic NPs possessed pathogen-mimicking properties and could activate DCs similarly as LPS and other agonists of pathogen associated pattern recognition receptors, such as TLRs (206). Similar effects were published for poly(methyl vinyl ether-co-maleic anhydride) NPs (149 ± 2 nm) which activated DCs through the TLR stimulation (204). In addition, sulfonate (245 nm) and phosphonate-functionalized (227 nm) polystyrene NPs induced the maturation of DCs, significantly enhanced their T cells stimulatory capacity, and shifted Th1 response (205).

Other NPs have also been shown to modulate the immune response mediated by DCs. For instance, dendrimers conjugated with maltose have been shown to stimulate the immune response by activating DCs, suggesting its potential use as candidates for vaccines (207). Protein NPs exerted immunostimulant properties as reported in recent studies (143, 208, 209). Some of them, which mimic the effect of viruses, can facilitate the activation and cross-presentation of DCs. This type of NPs delivered with peptide epitopes, which are recognized by DCs, showed an increased and prolonged CD8+ T cell activation (209). Polystyrene NPs (50 nm) have been shown to inhibit lung inflammation when administrated intratracheally in a dose of 200 μg/mouse after allergen challenge and this suppression was due to the modulation of DCs functions. Furthermore, these NPs inhibited the expansion of CD11c+MHCII^{hi} DCs in the lungs and draining lymph node and allergen laden CD11b^{hi}MHCII^{hi} DCs in the lungs (182).

Cellulose nanofibrils (CNFs) are very attractive natural nanomaterials for wide biomedical applications. Previously, we showed that CNFs possessed good biocompatibility and anti-inflammatory effects (210). Here we further explored the effects of CNFs on the immune response, using a co-culture model of human monocyte derived dendritic cells (DCs) and CD4^{+}T cells. We found that CNFs, applied at non-toxic concentrations during DC differentiation, impaired up-regulation of CD1a expression. After a stimulated maturation, CNF-treated DCs expressed lower levels of co-stimulatory and maturation molecules, possessed weaker allostimulatory, T helper (Th)1 and Th17 polarizing capacity, and increased the frequency of interleukin (IL)-10-producing and Th2 cells. Furthermore, CNF-treated DCs were able to expand FoxP3^{+}, IL-10^{+} and transform growth factor-β^{+}CD4^{+} T regulatory cells that were hyporesponsive to polyclonal stimulation and possessed augmented suppressive capacity. These findings correlated with an increased immunoglobulin-like transcript-4 and indolamine dioxygenase-1 expression by CNF-treated DCs. Internalization studies revealed predominantly partial internalization of CNFs by DCs, an increased CD209 expression at the place of contact, and the accumulation of actin bundles around the CNFs. Cumulatively, our results indicate that CNFs induce an active immune tolerance by acting on DCs, and that this property is very desirable if CNFs are to be used as an implantable biomaterial (119). Functionalization of CNFs via oxidation and subsequent modification with phosphonates provides a good platform for bone tissue regeneration therapy (211). Using the same model of MoDCs and T cells coculture we found that CNFs functionalized with 3-Amino Propylphosphonic

Acid (APA) significantly inhibited down-regulation of CD14 and up-regulation of CD1a expression during their differentiation, whereas control, oxidised CNFs (cCNFs) only inhibited the up-regulation of CD1a. APAcCNFs impaired LPS and IFN-γ-stimulated maturation, allostimulatory and Th1/Th17-polarisation capacity of DCs, more than cCNFs, which correlated with higher capacity of APAcCNF-treated DCs for inducing IL-10 production and $CD8^{+}IL\text{-}10^{+}$ Tregs in an ILT-3 dependent manner (212). These results have opened new perspectives for the application of functionalized CNF-based nanomaterials, not only for well tolerated scaffolds in tissue engineering, but also as scaffolds for controlled DC-mediated induction of tolerogenic immune response.

TOXICITY OF NANOSTRUCTURE – *IN VIVO* STUDIES

In comparison with nanotoxicity *in vitro* studies, there is a much smaller number of nanotoxicity *in vivo* studies, whereas very few studies examine chronic toxicity. Many of them investigate the local or systemic adverse effects of nanomaterials. In this context, it is very important to choose a suitable study design, depending on whether potential nanomaterial will enter the human body unintentionally or will be used for theranostic purpose. In any case, the mode of entry into the body, absorption, biodistribution, accumulation and elimination are very important parameters.

The possible routes of entry of NPs into the body include inhalation, absorption through the skin or digestive tract, direct injection into circulation, and absorption or implantation for drug delivery systems. In particular, inhalation and ingestion are likely to be the major routes of NP uptake in terrestrial organisms (213). Inhalation is the most common way of entry of NPs into the organism during the manufacturing process and therefore this problem is of specific importance and concern. Similarly, diesel exhaust NPs, which are very toxic, are one of the major compounds responsible for air pollution (5). Several studies examined lung toxicity following inhalation. An example is the demonstration of lung damage by CNTs in mice and rats followed by the inflammatory granulomatous response (20, 214). Pulmonary toxicity was also shown for TiO_2 NPs by Li et al. (92), who examined different biochemical parameters in bronchoalveolar lavage. Some of the observed parameters of toxicity were also associated with the change of pH value of the medium with dispersed NPs. An interesting study showed that intranasally instilled TiO_2 NPs could be translocated into the CNS, where they may cause some brain lesions due to the induction of oxidative stress (215). The intratracheally implanted Fe_2O_3 nanoparticles in rats enter the systemic circulation after crossing the alveolar-capillary barrier and are selectively taken up by spleen, kidney and testicles (216). The particles induced size- dependent toxicity due to oxidative stress, implying that more severe lung injury was caused by nanosized particles

than by submicron-sized particles. Lung injury was followed by fibrosis and one of proposed mechanisms was related to the pro-inflammatory response triggered by alveolar macrophages after the phagocytosis of Fe_2O_3 NPs. Furthermore, Fe_2O_3 nanoparticles delay the coagulation process by prolonging the prothrombin time and activated partial thromboplastin time (216).

NPs can reach the gastrointestinal tract through the ingestion of food, water, cosmetics, drugs, and by the use of drug delivery devices (29, 217-219). The toxicity experiments related to NPs that are administrated via the gastrointestinal tract are designed to focus primarily on systemic toxicity. In this context, metallic NPs have been the most thoroughly investigated. It has been shown that Cu NPs induced both gastrointestinal damage like loss of appetite, diarrhea and vomiting, but also systemic effects due to the damage of CNS. LD50 in mice for these NPs was much lower (413 mg/kg) compared with >5000 mg/kg for the bulk Cu (220). Wang et al. assessed the acute toxicity of Zn powder by gastrointestinal administration at a dose of 5 mg/kg body weight in mice that exhibited severe symptoms of lethargy, anorexia, vomiting, and diarrhea (221). Similar findings were shown for CdSe quantum dots (QD) on enterocyte-like Caco-2 cells, as a small intestine epithelial model (222). The authors concluded that Caco-2 cell viability correlated with the concentration of free Cd2þ ions present in cell culture medium. Exposure to low (gastric) pH affected cytotoxicity of CdSe QDs, indicating that the route of exposure may be an important factor in QD cytotoxicity. It is interesting that selenium, administrated orally, had less toxic effect on liver in mice in the form of a NP than sodium selenite (223). Orally ingested NPs may cause cardiac cytotoxicity. Such a study was conducted in mice, where particles of two different sizes, Zn NPs and microsized Zn powder, at a dose of 5 mg/kg body mass were administered via the gastrointestinal tract (18). The obtained results showed that both types of particles induced fatty degeneration, which can be caused by anemia. Nevertheless, it was Zn NPs that led to cardiac impairment, not microsized Zn particles, as judged by an increase in certain biochemical blood parameters, which eventually suggested that the size of these particles played a major role in toxicity.

Dermal exposure has been hypothesized as one of the most significant route of exposures to NPs (220). Nevertheless, the literature reports about the absorption and effects of NPs in the skin are very scarce. Dermal absorption and skin penetration of NPs need a better evaluation because only a few, contradictory data are available in the literature, mainly on TiO2 (29, 33, 217, 224). A study on cultured keratinocytes exposed to the extracts of several types of Ag containing dressings showed that Ag NPs were the most toxic (225), which might have implication in infected wound.

The systemic toxicity of nanostructures can be easily evaluated by their direct administration into the bloodstream or lymph circulation. The injected NPs can damage red blood cells and circulatory immune cells. Administration of nanostructures into the bloodstream is also an excellent model to examine the accumulation of NPs in different organs, their biodistribution, kinetics and elimination.

However, such studies are relatively rare and therefore, if conducted in a proper way, are very valuable. The experiments with Au NPs are one example, which confirmed that the size of Au NPs was critical for their biodistribution and cytotoxicity *in vivo*. Smaller particles were distributed to several different tissues (blood, liver, lung, spleen, kidney, heart), while larger particles (200-250 nm) showed very minute presence in blood, brain and spleen (226-228). Particles larger than 10 nm were shown to persist in the liver and spleen of mice for up to 6 months without any consequences (229, 230), but it was not clear whether such a long retention induced any adverse effects. Other *in vivo* models suggested that Au NPs could have a significant impact on the life span and fertility of experimental animals (231). Based on these and many other *in vivo* data (130), it could be expected that Au NPs would not induce an acute toxicity *in vivo*, nevertheless, some further research, investigating the long persistence of NPs in the organism, should be conducted for acquiring a thorough understanding of their safety. In contrast, exposure of experimental animals to Ag NPs has been shown to cause anemia, cardiac enlargement, growth retardation and degenerative changes in their liver (232). Another study with silica NPs showed that intravenous injections of silica NPs with a single dose of 50 mg/kg caused hepatic inflammation and oxidative stress in mice (170). Superparamagnetic iron oxide nanoparticles (SPIONs) were taken up by liver, spleen and lymph node within 24 hours after *in vivo* administration and experienced progressive metabolism. In addition, dose-dependent toxicity was observed with repeated injection (233). Feng et al. reported that injected Fe_2O_3 NPs induced metabolic abnormalities in spleen and kidney of rats (234). Other investigators reported that iron oxide NPs can cross the blood-brain barrier and may cause CNS toxicity (235). The kidneys, liver and spleen are the main target organs for QDs toxicity because of the accumulation of QDs within these vital organs (1). A study by Sadaf et al. (236) showed the significant renal injury of mice after the intravenous administration of QDs. A noticeable rise in the blood levels of urea, nitrogen and creatinine indicates the risk of QDs in nephrotoxicity (236). The toxicity of hematological system was also observed after the intravenous injection of QDs coated with either carboxyl or amine groups in mice. The injected QDs were capable to activate the coagulation cascade and induce pulmonary vascular thrombosis (237). Moreover, exposure of QDs (Cd–Se) to the eyes led to corneal damage (238).

Wang et al. (239) demonstrated that intravenous administration of graphene oxide NPs in mice was followed by granuloma formation in the lungs, kidneys, liver and spleen. Another similar study reported by Zhang et al. (240) on mice showed the uptake of radiolabeled graphene oxide in the lungs. Furthermore, at the dose of 10 mg/kg, toxic signs were observed, such as pulmonary edema, inflammation and granuloma formation in the lungs. Silica NPs accumulate mainly in the lungs, liver, and spleen (241-243). After intravenous administration of these NPs in mice, they are taken up by macrophages, and could potentially cause liver injury (244). Similarly, Hassankhani et al. (245) provided evidence that oral

administration of silica NPs caused significant changes in the levels of albumin, cholesterol, triglyceride, total protein, urea, HDL, LDL, alkaline phosphatase and aspartate aminotransferase.

The mechanisms of toxicity of particular organs (the brain, cardiovascular system, liver, spleen, kidneys, reproductive system) can be subsequently modeled using cell lines established from such organs and tissues, including endothelial cells, astrocytes, glial cells, liver cells and others (18). An example is the paper of Hussain et al. (246) who observed that the circulatory Ag NPs (15 and 100 nm) accumulated in the liver and induced cytotoxicity. Based on a complementary *in vitro* study on BRL 3A liver cells it can be concluded that Ag NPs-induced oxidative stress *in vitro* and most probably *in vivo* contributes to the toxicity (247).

GENOTOXICITY OF NANOSTRUCTURES

Genotoxicity of nanostructures is one of the most important problems in nanotoxicology, because genotoxic mechanisms, if not reparable, are associated with carcinogenesis, reproductive toxicity and different chronic diseases (1, 17, 18). Therefore, each nanostructure, regardless of whether it is considered as an environmental, occupational or intentional hazard, must be thoroughly investigated in terms of its genotoxic potential. Unfortunately, many nanostructures have been shown to cause different genotoxic pathways *in vitro*, which are tightly connected with ROS production and defect anti-oxidative mechanisms in cells. At the same time, these findings suggest that overproduction of ROS could be a common causative factor involved both in cytotoxicity and genotoxicity.

Genotoxicity can be checked by performing different assays, such as those detecting chromosomal aberrations, DNA strand breakages, point mutations and increased expression of DNA repair proteins. In this context, metallic NPs have been the most thoroughly investigated. Using a micronucleus assay, Colognato and coworkers (248) demonstrated that Co NPs (size range from 100 nm to 500 nm) were genotoxic for human peripheral blood leucocytes. The frequency of micronucleus formation in these cultures depended on the concentration of applied Co NPs. Au NPs have been shown to induce DNA damage in embryonic lung fibroblasts (92). It is important to stress that the applied concentration (25 mg/ml) of Au NPs (size 20 nm) is significantly above the concentrations causing cytotoxicity (134, 249). Similar genotoxic manifestations were observed with Ag NPs at the doze of 50 mg/ml (18). Genotoxic mechanisms of Ag NPs were indirectly investigated in mouse embryonic stem cells and embryonic fibroblasts (250). Namely, it was shown that Ag NPs increased the expression of p53 and its phosphorylation, as well as the phosphorylation of H_2AX protein, and both parameters are very confirmative that under such experimental conditions, Ag NPs are genotoxic. TiO_2 NPs have been described to exhibit genotoxic effects in lung epithelial cells us-

ing a comet assay (251, 252). Similar phenomenon was described before for TiO_2 NPs illuminated with UV light (253). This result is of particular importance when considering TiO_2 NPs as a component of sunscreens. The smaller TiO_2 NPs (less than 10 nm) had greater genotoxic effect than those that were larger (> 200 nm).

Chromosomal damages (254) or DNA damages (78), associated with numerous cellular and nuclear alterations were documented for SiO_2 NPs at the concentration of 25 mg/ml. These findings are in accordance with the results of other authors (255, 256) showing oxidative stress-induced cellular alterations by SiO_2 NPs. Genotoxicity was also detected using CuO NPs (257), colloidal antimony pentoxide, α- and β- alumina, amorphous ferric oxide, where the induction of DNA strand breakage was induced upon NPs internalization (18). It is important to stress that smaller sized NPs and surface coated NPs produce higher reactivity and induce higher genotoxicity (258-260). Hong et al. (261) reported that positively charged coatings of iron oxide NPs caused DNA strand breaks, in contrast to the negatively charged counterparts. The genotoxicity of these NPs also depended on the type of coating. For example, polyethylene (PEG) coated iron oxide NPs were mutagenic, whereas solid electrolyte interphase NPs did not exert genotoxicity (259). Most of these mechanisms correlated with better uptake of NPs and stronger oxidative stress–induced molecular changes in examined cells. Other types of nanostructures have been much less investigated in relation to genotoxicity. For instance, SWCNTs, fullerenes and other CNPs are less genotoxic than metallic NPs (18). However, QDs have been demonstrated to be genotoxic. For instance, small sized QDs were found to be actively distributed inside the nucleus and damaged nucleoli and nuclear histones (262). Similarly, Hoshino et al. (263) reported the specific localization of mercaptoundecanoic acid coated QDs in the nucleus and subsequent modification of DNA methylation. Potential genotoxicity of Cd–Te QDs was reported in human breast cancer cells (MCF-7) (264). Treatment of MCF-7 with QDs induces global hypoacetylation with alteration in DNA helix, implying a global epigenomic response of these NPs (264).

The introduction of NPs into the environment upon their disposal may also cause genotoxicity in aquatic organisms and therefore, the studies conducted on aquatic organisms such as *Daphnia* or zebrafish are important for translational medicine. Wu et al. (265) showed that Ag NPs induced different malformations in *Oryzias latipes*, such as spinal, heart and eye abnormalities. Park and Choi (266) showed a higher degree of DNA damage in *D. magna* induced by Ag NPs compared to Ag ions. Coated Ag NPs are also able to penetrate the nucleus of zebrafish embryo (*Danio rerio*) and to cause DNA breakage (267). Recent data showed that TiO_2 NPs induced omphalocele in chicken embryo by disrupting the Wnt signaling pathway (268).

Even though the number of experiments on the toxicity of NPs in the reproductive system of animals has increased, this field of study is still in its preliminary stage. In female animals, targeting of the uterus and ovaries is shown for a

variety of NPs, including TiO_2, Cd, and Au, but there is a wide variation among the obtained results. In males, there is evidence that NPs accumulate in the testes. In this context, TiO_2-based NPs may be more dangerous than other metal NPs, with an impact on cells in the seminiferous tubules, sperm motility and morphology. The transplacental transfer of many types of NPs, including Au, TiO_2, SiO_2, C, and QDs, has been shown in animal models, suggesting their toxic effects on the fetal brain and nerve development. In addition, TiO_{2-} and Cd-based NPs have been shown to have fetotoxic effects when inhaled by pregnant animals (269).

ECOTOXICITY

The rapid development of nanotechnology and the large-scale production of nanomaterials have led to a significant increase in the quantity of NPs that are released into the aquatic environment. Pollution from nanoparticles originates from a variety of sources, production facilities, manufacturing processes, wastewater treatment plants, and accidents during their transport. Aquatic ecosystems are most vulnerable to environmental pollution because they receive many contaminants, sequester them and transport the contaminants further. The fate of NPs in aquatic ecosystems can be different, depending on the physicochemical parameters of the environment and biodiversity of the system. For example, NPs may adhere to algae that may then be consumed by filter-feeders and transfer to higher trophic levels (270). NPs can be immobilized as a result of their sorption or binding to other particles or molecules, such as organic matters (271). NPs may aggregate and sediment and thus their concentrations in the local microenvironment may be significantly increased. In addition, sediments represent porous environmental matrices that typically have large specific surface areas. The aggregation processes as well as the release of NPs from these aggregates and sediments into the environment depends on the environmental factors such as pH, temperature, and presence of organic matter. However, these processes are dictated by the type of NPs. For example, Gilbert et al. (272) showed a pH-driven aggregation and disaggregation of NPs with larger aggregate radius at higher pH. In contrast, other reports showed an increased mobility of NPs under increased acidification that is followed by the change in the surface charge (273).

Temperature is also known to affect aggregation. Walters et al. (274) reported the formation of smaller NP aggregates at higher temperatures that can exert higher toxicity. Temperature may increase the dissolution of NPs, as reported for Ag NPs (275). Before analyzing the ecotoxicity of NPs it is important to consider the routes of their entry in aquatic organisms and target organs. The major routes of entry are via ingestion or direct passage across the gills and other external surface epithelia, whereas the cellular immune system, gut epithelium, and hepatopancreas are the main target (276, 277). Some NPs, which are able to penetrate

the semipermeable membranes of aquatic organisms, may form aggregates around the exoskeleton of the organisms (270). The hepatopancreas is of particular importance because it is responsible for metabolism and detoxification and because NPs, which are taken up via ingestion through the digestive tract, may accumulate in the hepatopancreas (278). At the cellular level, endocytosis (<100 nm) and phagocytosis (100–100,000 nm) represent two processes by which NPs might be absorbed into eukaryotic cells (279).

There has been extensive research investigating the toxicity of NPs to aquatic organisms with several recent reviews reporting on the ecotoxicology of NPs (280, 281). The authors, by summarizing the data on the biological effects of NPs, have shown that NPs can be toxic to bacteria, algae, invertebrates, fish, and mammals. However, the studies remain poorly and unevenly distributed and most of them have been done on *Daphnia magna*, the crustaceans, which represent the food and energy link between algae and fish (282). In this context, Park and Choi (266) reported increased mortality of *D. magna* by the influence of Ag NPs, whereas Asghari et al. (283) showed abnormal swimming of *D. magna* following exposure to these NPs. Scown et al. (284) reported size-dependent uptake of Ag NPs (10–35 nm) and subsequent induction of oxidative stress in the gills of *Danio rerio,* while Maria et al. (285) reported reduced levels of enzymes involved in the anti-oxidative defense in the gills and hepatopancreas of female *Carcinus maenas*. Wu et al. (265) recently showed that Ag NPs induced a variety of morphological malformations such as edema, spinal and fin fold abnormalities, heart malformations, and eye defects in *Oryzias latipes*. Ultrastructural changes in the midgut of *D. magna* upon exposure to CuO NPs were published by Heinlaan et al. (286). Significant differences in toxicity, as judged by LC50 after 24 hours of exposure, between Al_2O_3 NPs (82 mg/L) and bulk Al_2O_3 (153 mg/L) and between TiO_2 NPs (80 mg/L) and bulk TiO_2 (136 mg/L) were also demonstrated (287). Wiench et al. (288) performed a 21-day chronic reproduction experiment on *D. magna* using coated TiO2 NPs and reported that the lethality dose for adults was 30 mg/L, while the dose for offspring production was 3 mg/L. The 21-day EC10 and EC50 values for reproductive effects were 5 mg/L and 26.6 mg/L, respectively.

CdTe QDs were shown to significantly decrease the viability of hemocytes, as well as the number of hemocytes capable of ingesting fluorescent beads in *Elliption complanata* mussels (289). The immunosuppressive effects of these NPs were also observed in Juvenile rainbow trout. When CdS/CdTe QDs were exposed to Juvenile rainbow trout, the leukocyte counts, viability, and phagocytic activity were significantly reduced (282). Size of QDs aggregates may affect the immune response of QDs. For instance, large CdS/CdTe QDs aggregates (25–100 nm) reduced phagocytosis more than smaller NPs (<25 nm) on bivalves (*Mytilus edulis* and *Elliptio complanata*) and fish (*Oncorhynchus mykiss*) (290).

There have been significant attempts to use nanomaterials to remove toxic NPs from ecosystems or to construct new NPs that are non-toxic. Some of the examples have been recently published by our research group using functionalized

CNTs (114). In addition, a new class of low-cost superhydrophobic materials have been synthesized (291).

CONCLUSION

Given the increased production and intentional use of NPs (cosmetics, drug delivery, implants), as well as the exposure to unintentionally released NPs in the environment (combustion, manufacturing and naturally occurring processes), their adverse health effects are expected to evolve. The potential exposure routes of nanostructures in the body are inhalation, ingestion, and dermal penetration, whereas nanomaterials used as theranostic agents are injected into the blood circulation. In this context, particle size, shape, surface area, surface chemistry, concentration, dispersion, aggregation, route of administration and many other factors determine both the desired biological effects and the toxicity of nanostructures. Therefore, it is of the utmost importance to carry out the biological characterization of nanomaterials to determine their health hazards and ensure risk-free and sustainable implementation of nanotechnology. *In vitro* toxicity studies have been used as general screening systems and as specific assays to elucidate the mechanisms of NP cytotoxicity. In contrast, *in vivo* tests are necessary to investigate the blood contact response to NPs, biodistribution, toxokinetics, systemic toxicity, carcinogenicity, reproductive toxicity or the inflammatory response. Special tests have been designed for studying the ecotoxicity of nanostructures. Many published reports clearly indicate that NPs exert different levels of toxicity *in vitro* and *in vivo*, which depends on a number of experimental factors originating from NPs themselves, target cells or tissues and general experimental conditions. Although the exact molecular mechanisms underlying nanotoxicity are not fully understood yet, these complex processes are closely associated with increased ROS production, changes in intracellular [Ca^{2+}] homeostasis, cell cycle arrest and induction of a pro-inflammatory response. As a result, cell injury and death, suppression of cell proliferation or genotoxic manifestations occur. The immunotoxicity and immunomodulatory properties of NPs are of particular importance, especially for the preparation of less immunogenic nanostructures capable of avoiding the undesirable immune responses, so as to minimize the systemic side effects of the application of NPs in specific nanotherapeutics for drug delivery and in the development of novel vaccine strategies.

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ТОКСИЧНОСТ НАНОСТРУКТУРА

Р е з и м е

Због своје јединствене величине (димензије од 1 до 100 нм) и физичко-хемијских својстава, наноматеријали су нашли бројне примене у електроници, козметици, кућним апаратима, складиштењу енергије, прехрамбеној индустрији, фармацији и медицини. Међутим, повећана производња наноматеријала изазива забринутост у погледу њихове сигурности, не само за људска бића, већ и за животиње и животну средину. Бројна истраживања су потврдила да наночестице (НЧ) могу да испољавају токсичност, *in vitro* и *in vivo*, која зависи од њихове величине, облика, површине, хемијског састава површине, концентрације, степена дисперзије и агрегације, начина примене и многих других фактора. Сваки од њих је релевантан за очекивано биолошко својство одређене наноструктуре. Овај прегледни чланак приказује сажето главне аспекте нанотоксичности *in vitro* и *in vivo* и истиче битне тестове којима се доказује токсичност НЧ. Посебно се наглашава значај реактивних врста кисеоника, као главног покретачког фактора цитотоксичности и генотоксичности НЧ, које делују у сложеној међусобној интеракцији НЧ са ћелијским, односно геномским компонентама. Посебан аспект се односи на имунотоксична и имуномодулацијска својства НЧ, што је од велике важности за производњу мање имуногених наноструктура. На тај начин

се може избећи нежељени имунски одговор и омогућити примена НЧ као специфичних нанотерапеутика за доставу лекова и развој нових стратегија вакцинације. На крају су приказани екотоксиколошки аспекти НЧ, указано је зашто су водени екосистеми најосетљивији када се разматра контаминација животне средине наночестицама и зашто су истраживања на воденим организмима важна за област транслацијске нанотоксикологије.