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ENGINEERED NANOMATERIALS FOR BIOMEDICAL APPLICATIONS

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Cover: Confocal microscopy image of PBECs exposed to FITC labelled PAMAM-NH_2.
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To my family and friends

"No estimo res com la dolcesa
del cel blau del meu país;
ara en sóc lluny, però me'n recordo
dia i nit"

"No hi ha res que no em recordi
cada instant el meu país,
tot em fa pensar en els dies
que hi vaig viure tan felic"

El meu país, de Miquel Martí Pol, poeta català

ABSTRACT

Engineered nanomaterials (ENM) have emerged as attractive and promising candidates for a wide range of advanced applications including in particular in medicine. However, the increased development of ENM raises the need to carefully assess their potential impact on human health and environment. For that, detailed evaluation of the intrinsic and biological identity of ENM is required for the safe design and use of these materials. To this effect, the present thesis focuses on the synthesis and biocompatibility assessment of two different classes of nanomaterials, dendrimers and superparamagnetic iron oxide nanoparticles (SPIONs), promising future nanomedicines for drug delivery and imaging agents in magnetic resonance imaging (MRI). Assessment was performed on primary human monocyte derived macrophages (HMDM), primary human bronchial epithelial cells (PBEC), and cell lines. Hereby an insight on the impact of these materials on the immune system and on their promising and potential use as nanomedicines has been obtained. Furthermore, we attempted to use systems biology approaches as a novel tool to identify possible hazard of ENM by using next generation sequencing RNA-Seq and computational tools. Finally, we assessed the bio-nano-interactions by evaluating the effect of the protein corona on the targeting capabilities of ENM and their behaviour. Importantly, the ENM were extensively characterized, using different techniques prior to the toxicity studies. In Paper I, we evaluated the biocompatibility of a library of polyester dendrimers based on 2,2-bis(methylol)propionic acid (bis-MPA) including dendrimers with two different surface functionalization, hydroxyl and carboxylic end groups, and two commercial polyamidoamine dendrimers (PAMAM) with amine and hydroxyl end groups. We found excellent biocompatibility for the entire hydroxyl functional bis-MPA dendrimer library, whereas the cationic, but not the neutral PAMAM exerted dose and time dependent cytotoxicity in the cell models tested. In paper II, using system biology approaches and bioinformatics tools, we were able to identify and validate the toxicity mechanism of PBEC exposed to PAMAMs dendrimers at low doses. Our studies showed that PAMAM-NH_2, but not PAMAM-OH, caused down-regulation of cell cycle-related genes and cell cycle arrest in S-phase. Our findings provide evidence of the beneficial use of these new toxicology tools for the future risk assessment of nanomaterials. SPIONs have emerged as promising nanomaterials for biomedical applications, due to their excellent magnetic properties, chemical stability and biocompatibility. In paper III, ultrasmall superparamagnetic iron oxide nanoparticles (USIRONs) were prepared by a one-pot aqueous approach by using $Fe(OH)_3$ as iron precursor, vitamin C as reducing agent, and dehydroascorbic acid (DHAA) as capping agent. We showed that USIRONs present high crystallinity, long-term colloidal stability, enhanced saturation magnetization, and exhibit excellent biocompatibility as demonstrated in the toxicity evaluation using primary HMDM. When nanoparticles are in contact with physiological fluids, adsorption of proteins on the surface of the nanomaterial will occur, resulting in the establishment of a protein corona. Whether the protein corona will affect the targeting capabilities of the ENM was investigated. In paper IV, folic acid (FA)-conjugated iron oxide nanoparticles with poly(ethylene glycol) (PEG) or SiO_2 surface coatings were synthetized. We evaluated their biocompatibility and specific targeting effects on HMDM and on ovarian cancer cells, that over express the folic acid receptor. Notably, we demonstrated the nanoparticles (NPs) were non-toxic to cells and that FA specific uptake was observed only for the FA iron oxide SiO_2 coated NPs in the presence of serum proteins. Our studies contribute to the development of new nanomaterials and their applications, which may facilitate the clinical translation of the nanomedicines.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. Feliu N, Walter MV, Montañez MI, Kunzmann A, Hult A, Nyström A, Malkoch M, Fadeel B. Stability and biocompatibility of a library of polyester dendrimers in comparison to polymanidoamine dendrimers. Biomaterials 2012 33(7)1970-81.

II. Feliu N, Kohonen P, Palmberg L, Nyström AM, and Fadeel B. Next generation sequencing reveals low-dose effects of dendrimers in primary human bronchial epithelial cells: a systems biology study. [manuscript submitted 2014].

III. Xiao L, Li J, Brougham DF, Fox EK, Feliu N, Bushmelev A, Schmidt A, Mertens N, Kiessling F, Valldor M, Fadeel B, Mathur S. Water-soluble superparamagnetic magnetite nanoparticles with biocompatible coating for enhanced magnetic resonance imaging. ACS Nano. 2011 5(8) 6315-24.

IV. Krais A, Wortmann L, Hermanns L, Feliu N, Vahter V, Stucky S, Mathur S, Fadeel B. Targeted uptake of folic acid-functionalized iron oxide nanoparticles by ovarian cancer cells in the presence but not in the absence of serum. Nanomedicine. 2014 Jan 31[Epub ahead of print].

Additional relevant publications not included in the thesis:

I. Feliu N, Fadeel B. Nanotoxicology: no small matter. Nanoscale. 2010 2(12) 2514-20.

II. Fadeel B, Feliu N, Vogt C, Abdelmonem AM, Parak WJ. Bridge over troubled waters: understanding the synthetic and biological identities of engineered nanomaterials. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2013 5(2) 111-29.

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LIST OF ABBREVIATIONS

AuNPs	gold nanoparticles
bis-MPA	2,2 bis(hydroxymethyl)propionic acid
BSAI	biological surface adsorption index
CMC	critical micelle concentration
CM	Connectivity Map
CT	computed tomography
DI	deionized
DOX	doxorubicin
EA	elemental analysis
EMA	European Medicines Agency
ENM	engineered nanomaterials
EPR	enhanced permeability and retention
ER	endoplasmic reticulum
DHAA	dehydroascorbic acid
DENPs	dendrimer-entrapped gold nanoparticles
FA	folic acid
FcRn	the neonatal Fc receptor
FDA	Food and Drug Administration
FR	folate receptor
FTIR	Fourier transformed infrared spectroscopy
GO	gene ontology
GO	graphene oxide
HMDM	human monocyte derived macrophages
HTS	high throughput screening
ITS	intelligent testing strategy
IPA	Ingenuity Pathway Analysis
LDH	lactate dehydrogenase
MALDI-TOF	matrix assisted desorption ionization time of flight
MNPs	magnetic nanoparticles
MPIO	micrometre size paramagnetic iron oxide
MRI	magnetic resonance imaging
NAC	N-acetyl-L-cysteine
NIR	near-infrared
NMR	nuclear magnetic resonance
NPs	nanoparticles
PAMAM	polyamidoamine
PBEC	primary bronchial epithelial cells
PET	positron emission tomography
PDI	polydispersity index
PPI	poly(propylene imine)
QSAR	quantitative structure–activity relationship
REOs	rare earth oxide
ROS	reactive oxygen species
RNA-Seq	RNA sequencing
SPECT	single photon emission computed tomography
TEM	transmission electron microscope
TfR	transferrin receptor
TGA	thermogravimetric analysis
TNFα	tumor necrosis factor-alpha
rGO	reduced graphene oxide
SPIONs	superparamagnetic iron oxide nanoparticles
USIRONs	ultrasmall superparamagnetic iron oxide nanoparticles
UV-Vis	ultraviolet-visible spectroscopy
XRD	X-ray powder diffraction

1 INTRODUCTION

1.1 NANOMATERIALS

1.1.1 Biomedical applications of nanomaterials

Nanotechnology is a rapidly growing discipline that offers a wide range of applications. Their novel physicochemical properties make nanomaterials highly attractive and promising for medical development,[1] with potential benefits for treating and diagnosing cancer. Some of these materials already have reached the clinic.[2,3] However, their promising characteristics may also yield unexpected adverse effects on human health and the environment[4]. Therefore, careful toxicity evaluation is needed in order to provide knowledge for the safe design of these materials for such applications.

The unique chemical and physical properties of engineered nanomaterials are attributed to their small size that gives rise to significantly different properties from conventional materials on the microscale level.[5] These properties, such as narrow size distribution, composition, surface structure, shape, aggregation, and solubility, influence the mechanical, electrical, optical, and magnetic properties, as well as the chemical reactivity of these materials.[6,7] These distinct properties make ENM offer a wide range of opportunities and applications, covering both the engineering and biomedical sectors. Our curiosity is focused on the extraordinary potential that the utilization of ENM offers in improving clinical diagnosis and in various branches of biomedical research.

The field of nanomedicine encompasses the applicability of nanotechnology to medicine, with the goal to design multifunctional nanoparticles with desirable properties for medical applications, including diagnostics, imaging, drug delivery, and other health- associated applications.[8] Nanomedicines are generally based on multifunctional particles that can encapsulate active therapeutics within their nanoscale framework, and which are capable to release the therapeutic payload in a specific manner. Most often, such constructs increase the bioavailability of the drug and can be used to control the pharmacokinetics and biodistribution of the active compound. Liposomes, protein particles, and polymer-drug conjugates are examples of approved therapeutic nanomedicines used in clinics. Furthermore, nanoscale contrast agents are also used in clinical radiology, such as SPIONs and dendrimers that are used to enhance resolution in magnetic resonance imaging.[2, 9, 10] .

Nanotechnology is expected to make an important contribution to cancer prevention, detection, diagnosis, imaging, and treatment (*cf.* Figure 1). Today all conventional cytotoxic therapies are not completely effective.[11] Large proportions of new emerging drug candidates are only poorly water-soluble such as docetaxel (Taxotere), which is commonly used for the treatment of breast and lung cancer. Therefore, the development of drug nanocarriers for poorly soluble pharmaceuticals is an important task.[12] Theragnostics is the treatment approach that combines therapeutic delivery systems

with diagnostics (imaging), with the aim to monitor the treatment and to improve drug efficacy and safety,[13] developing both prognosis and therapy.[14] Nanomaterials are particular relevant for cancer treatment, which may offer advantage over traditional therapies by providing higher efficacy and lower toxicity of treatment.

Nanomedicines that exhibit extended circulation time may accumulate passively into tumor tissue. This process is known as enhanced permeability and retention (EPR) effect.[15,16] It refers to the propensity to nano-and micro- size therapeutic agents to accumulate in tumor tissue, providing significant advantages compared with low molecular weight agents[17]. However, for a successful delivery of nanomedicines, several factors should be taking consideration: i) the influence of the EPR effect as previously mentioned, ii) the permeability of the tumor vasculature, iii) the blood flow to the tumor, iv) the intratumoral pressure, and v) the nature of the barrier between the tumor cells and the extracellular matrix.[15]

In recent years, there has been an increase in the different imaging technologies and their applications.[18, 19] Molecular imaging offers the opportunity to obtain information on cellular function and molecular processes in living organisms by both *in vitro* and *in vivo* systems, with high sensitivity and specificity. Examples of molecular imaging systems include computed tomography (CT) imaging, optical imaging, magnetic resonance (MR) imaging, single photon emission computed tomography (SPECT), and position emission tomography (PET). Notably, among the different techniques, CT and MRI provide higher resolution than the other modalities. Moreover, molecular imaging techniques can be used in diagnoses of diseases such as cancer, by enabling to visualize tumours in the body.[20] Current advances in nanotechnology offer to use engineered nanomaterials as molecular imaging agents. Due to their possibility to be designed with multifunctionality, which permits different imaging modalities,[21] and to perform target diagnosis and therapeutics, several nanomaterials have been studied for cancer treatment[22] including polymers, dendrimers, liposomes, micelles, magnetic nanoparticles, *etc.*[23] A detailed description of the materials used in this thesis and their biomedical applications is given in the following.

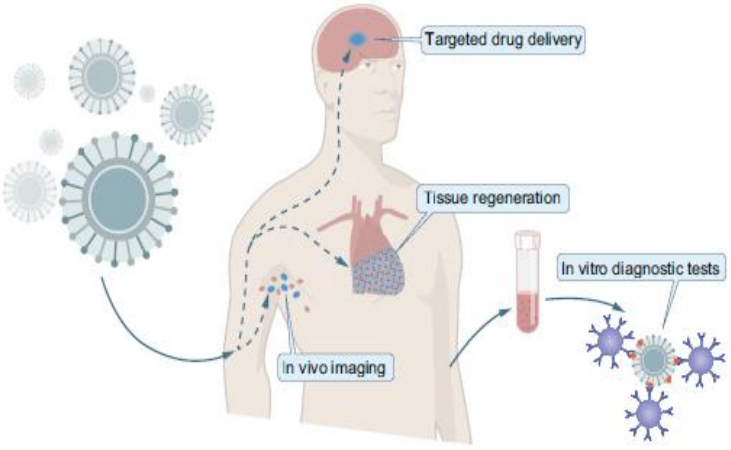

Figure 1. The application of nanotechnology in medicine. The image is reproduced from Fadeel *et al.*[24] with permission from Journal of Internal Medicine.

1.1.2 Dendrimers

Dendrimers are highly branched and monodisperse macromolecules with exact structure, placement of functionally and dimensions, whose name comes from the Greek word *"Dendros"* meaning tree and "meros" meaning part. In 1978, when Vögtle[25] *et al.* described the first synthesis of dendritic materials based on polypropylene-amine structure. A few years later, in 1985, two parallel studies published by Tomalia *et al.*[26] and Newkome *et al.*[27] , pioneers of dendrimer technology, described, polyamidoamine (PAMAM) dendrimers and poly(etheramide) structures, respectively. In 1990, a new type of dendrimer based on the convergent method was presented by Hawker and Frechét, the poly(benzylether) dendrimer.[28] Today, the dendritic family can be divided into sub-groups based on their disperse frameworks, *i.e.* monodisperse dendrimers and dendrons, and polydisperse dendrigraft, hyperbranched polymers, and dendritic linear hybrids[29] (*cf.* Figure 2).

Divergent and convergent growths are two conventional approaches used in the synthesis of dendritic structures. In essence the divergent method grows the structure from the interior outwards, and in the convergent method the structure is grown from the exterior inwards.[30] Several more optimized methods for dendrimer synthesis have since then been evolved, including the orthogonal growth, double exponential growth, and double stage convergent growth strategies.[29]

Today several dendrimers have reached the market, including PAMAM (Dendritech), which is composed of a diamine core linked with methyl acrylate, poly(propylene imine) (PPI) (Symo-Chem BV), and 2,2 bis(hydroxymethyl)propionic acid (bis-MPA) dendrimers (Polymer Factory).

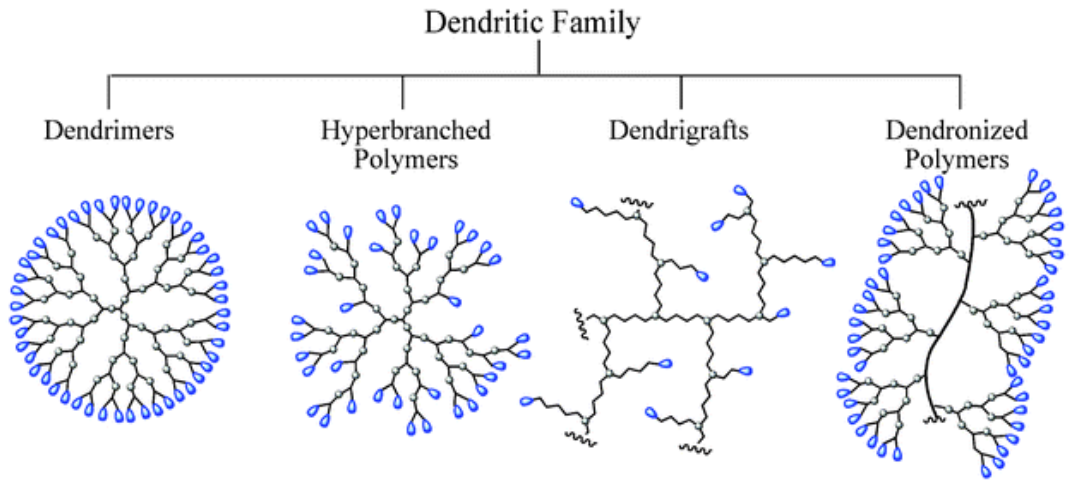

Figure 2. Schematic picture of sub-classes in the dendritic family. The image is reproduced from Carlmark *et al.*[30] with permission of The Royal Society of Chemistry.

For therapeutic applications, dendrimers offer advantages over other classes of nanomaterials. Unlike traditional polymers, dendrimers are monodisperse, which is a crucial property to ensure the reproducibility of the PK and BioD studies[31]. They present broad design possibilities with high control of size, shape, solubility, molecular weight, and specific control of the exact position of functional groups.[32] In addition, dendrimers with sufficient hydrophilicity, unlike polymer micelles and liposomes, do not have a critical micelle concentration (CMC). Therefore, dendrimers cannot disintegrate upon dilution. All these features clearly distinguishes these structures as unique carriers in therapeutic applications.[33]

Dendrimers have often been referred to as equivalents of "proteins" due to their size, globular structure, and exact molecular weight.[34] Despite the similarities between dendrimers and globular proteins, dendrimers have rigid structures as determined by covalent bonds, and represent a 3D structure with interior core and an exterior with controlled surface functionality.

The polyester dendrimers based on the bis-MPA scaffold were originally described by Ihre *et al.*[35] and are considered promising materials for biomedical applications[30], due to their hydrolytically degradable structure and good biocompatibility.[36, 37] Indeed, the group of Fréchet *et al.* have investigated the use of polyester dendrimers for therapeutic applications in several publications, such us efficient drug delivery carriers of doxorubicin (DOX) *in vivo*[38] and dendrimers for targeted positron emission tomography (PET).[39-41] Our recent studies further demonstrated their biocompatibility in both, primary human derived macrophages (HMDM) and cancer cell lines.[37] Furthermore, *Wu et al.* explored the formation and the cytotoxicity of chemotherapeutic delivery vehicles constructed from four different amphiphilic linear-dendritic hybrid block copolymers in primary human macrophages on breast cancer cells. Overall, the study showed that micelles in the size range of 100 nm had a loading efficiency of (DOX) up to 22%. The resulting carriers were evaluated as passive drug delivery vehicles and the results demonstrated that they exhibit superior drug efficacy and a higher apoptosis inducing capacity as compared to free DOX.[42] These results further confirm the promising use of these materials as drug delivery agents. To date, the FDA has not yet approved any dendritic carrier for drug delivery applications.[43]

PAMAM dendrimers are one of the most studied dendrimer structures. The surface of PAMAMs can be modified with different functional groups that allow them to be explored in a multitude of biomedical applications including; i) drug delivery systems (DDS), ii) transfection systems (Suprafect), and iii) contrast agents for PET, MRI, and CT. PAMAMs are non-degradable materials and typically present amine groups on the surface. Cationic PAMAM dendrimers have been associated with higher cytotoxic response than non-anionic, small cationic or neutral dendrimers.[44] In order to use PAMAMS as nanomedicine, further surface functionalization is required to achieve superior biocompatibility. PAMAMs have been used i) as templates or stabilizers for preparation of inorganic nanomaterials,[45] ii) to encapsulate or conjugate a wide range

of drugs, such as paclitaxel,[46, 47] methotrexate[47] among others, and iii) for targeted drug delivery. For instance, intravenously injected N-acetyl-L-cysteine (NAC) conjugated polyamidoamine dendrimers were able to effectively accumulate in activated microglia and astrocytes in the brain of young rabbits with cerebral palsy. The drug delivery vehicle suppressed neuroinflammation and improved their motor function as compared with the free-drug or PAMAM alone.[48] Dendrimer-based contrast agents for MRI have remarkable potential due to their flexible nature and high relaxivity properties.[49] Dendrimers provide advantages compared to gadolinium chelated agents due to their; i) superior r_1 relaxivity values[50] and ii) tunable kinetics (archived by specific chemistry)[51]. Some examples illustrating these features have been reported. For instance Peng *et al.* showed that G5-PAMAM dendrimer-entrapped gold nanoparticles (DENPs) can be used for tumor imaging by CT contrast agents. [52] Li *et al.* synthesized multifunctional Gd-Au DENPs that were effectively used for dual-mode MR/CT imaging of tumors.[53] At present, no targeted contrast agent is available for clinical MRI. Wi *et al.* synthesized a new CLT1 targeting contrast agent based on a stable macrocyclic Gd(III) chelate, Gd-DOTA. The CLT1 targeted contrast agent CLT1-dL-(Gd-DOTA)4 linked *via* a generation 1 lysine dendrimer presented high specificity for a prostate tumor model and effective MR imaging *in vivo*.[54] However, further studies are required to develop multifunctional nanoparticles for molecular imaging of different types of cancer.

1.1.3 Magnetic nanoparticles

Magnetic nanoparticles (MNPs) are a class of engineered nanomaterials that can be manipulated by external magnetic fields and are considered as one of the most important classes of nanomaterials today due to their unique physical properties[55]. MNPs are commonly composed from magnetic elements such as iron, nickel, cobalt, or metal oxides. Indeed, superparamagnetic iron oxide nanoparticles (SPIONs) have emerged as one of the key nanomaterials for biomedical applications, with a potential to create future medicines such as cancer theranostics. This is due to their excellent magnetic properties, high chemical stability, excellent colloidal stability, and good biocompatibility.[56] Altogether, SPIONs are potential candidates for improving contrast enhancement in magnetic resonance imaging (MRI), hyperthermia[57], molecular detection and drug delivery.[58]

Iron oxide NPs are being used in clinical trials as contrast agents in magnetic resonance imaging (MRI),[59] a powerful tool for non-invasive and non-destructive diagnostic applications in various clinical and biomedical settings. Briefly, imaging contrast of MR is based on the different relaxation times of water protons in tissue. To achieve superior images, contrast agents are used to induce changes in the relaxation time.[60] SPIONS are known as T_2 (transversal relaxation time) agents (negative contrasts agents) and paramagnetic agents such as gadolinium and manganese chelates are considered T_1 (longitudinal relaxation time) agents (positive contras agents). Indeed, most of the paramagnetic contrast agents are based on gadolinium ions Gd^{3+}, which accelerate proton relaxation, and which are often employed to reveal physiological and pathological structural details of tissues. Their efficacy will depend on the values of the

r_1- and r_2-relaxivities of these chelates and their pharmacokinetics.[61] Besides Gd^{3+}-assisted ^1H MRI techniques, contrast agents based on ^{19}F-NMR agents[62] are emerging as attractive candidates due to the high sensitivity of the ^{19}F, low background signal, extraordinary gyromagnetic ratio, good biocompatibility, and high natural abundance.[63-65] However, in this section emphasis is given on SPIONs, as they represent part of the materials evaluated in this thesis.
Several methods for the synthesis of magnetic nanoparticles have been proposed. SPIONs are based on an iron oxide core and are coated by either inorganic materials such as gold or silica or organic materials such as polymers, phospholipids, or polysaccharides.[66] The two major iron oxide phases used are mainly composed by magnetite (Fe_3O_4) and maghemite (γ-Fe_3O_4) and are often synthetized in sizes smaller than 5-20 nm (the ultra small yes, but those are not clinically approved). In these sizes, smaller than the single domain limit, iron oxide NPs exhibit superparamagnetic behaviour at room temperature.[55] Therefore, in absence of an electromagnetic field, the overall magnetic moment will be zero.[67]

Different methods are applied to synthetize iron oxide nanoparticles. Among them, the most commonly used approaches include i) co-precipitation;[68] that allows for the preparation of large quantities of nanoparticles in one single batch, ii) sol-gel synthesis,[69] iii) thermal decomposition of precursors, iv) hydrothermal reactions,[70] *etc.* Additionally, a wide range of strategies has been proposed to functionalize MNPs for their adequate biomedical applications. [71] Furthermore, targeting ligands, such as peptides, monoclonal antibodies, coupled to the polymer surface of SPIONs, allow the nanoparticles to recognize specific tissue signatures and to act as drug-delivery system.[72] As a result of their final size, MPNs can be classified as a: i) micrometre size paramagnetic iron oxide (MPIO), ii) superparamagnetic iron oxide, SPIO particles with diameter $\geq$ 50 nm, and iii) ultrasmall superparamagnetic iron oxide, USPIO with smaller diameters.[73]

To date, several classes of SPIONs have been developed. The medical use of magnetic iron oxide nanoparticles has been approved by the European Medicines Agency (EMA), and the US Food and Drug Administration (FDA). [74] Hofmann-Amtenbrinka *et al.* review examples of commercial iron oxide NPs used as contrast agents. This includes Lumiren® ($\geq$ 300 nm (+ agent)) and Gastromark® (ferumoxsil) for bowel imaging, Endorem© and Feridex IV© used as a spleen and liver imaging agents, USPIO such as Sinerem© (40 nm (- agent)) and Supravist© (25nm), and SPIONs like Resovist© (100-200 nm) (- agent)[66] . However, some of them have been currently removed from the market.[2]

The current research of SPIONs mainly involves *in vivo* drug delivery, MRI, and hyperthermia.[73] The final applicability of the MNPs in biological systems mainly depends on their plasma half-life, their final biodistribution, and their possible side effects. Moreover, it is still not well understood which are the parameters responsible for preventing or allowing the carriers to reach the specific target. One possible explanation could be the different biological identity formation (protein corona) of

nanoparticles when they are in contact with biological fluids. Further studies are required to address this important question. However, several ligands have been coupled to nanoparticles for enhancing drug delivery efficiency. Tietzer *et al.* synthetized a novel magnetic chemotherapeutic carrier-system with a size < 200 nm, that targets mitoxantrone efficiently in tumours *in vivo* and efficiently treated those tumours implanted in the limbs of rabbit, leading to complete tumour remissions.[75] Targeted therapy with MRI nanoparticles provides a suitable tool for local energy deposition and heating of cancer cells.[2]

Despite the fact that the FDA and EMA have already approved MNs as MRI contrast agents, the intrinsic proprieties of this material that determine the optimal factors required for biomedical applications are still not sufficiently well understood.[55] For the safety evaluation of these nanomaterials, cytotoxicity end points need to be considered. This includes cellular uptake, final fate of nanoparticles, and mechanism of action and biodegradation of the nanomaterial. More details on the design of safe nanomaterials will be discussed in the following section.

1.2 NANOTOXICOLOGICAL ASSESSMENT OF NANOMATERIALS

1.2.1 Overview and immunotoxicity

Nanomaterials offer a great potential for a wide range of medical applications. However, there is the concern that human exposure of nanoparticles could lead to significant adverse biological outcomes in health and environment. [2] Nanotoxicology is emerging as a scientific discipline attempting to investigate the interactions of engineered nanomaterials with biological system at the nanoscale level by providing knowledge on the safety assessment of nanomaterials.[3] Oberdörster *et al.* identified essential factors in nanotoxicology that need to be addressed for the safety assessment and future development of nanotechnologies. This includes dose metrics, exposure routes, and biodistribution of nanomaterials. Remarkably, he emphasized the importance of nanoparticles with well-defined physicochemical properties, and the validation of the conventional *in vitro* assays employed.[76]

Understanding the interactions of nanomaterials with relevant biological systems is a critical factor in the safe and sustainable development of nanotechnology for biomedical use.[77] Indeed nanomaterials may be distributed in the body *via* the lymphatic system where immunological reaction can occur. The primary focus of the immune systems is to protect against foreign materials, including particles. Therefore, is important to investigate the nano-immuno interactions, especially for nanoparticles that are intended to administration into systemic circulation as it is well known that particles in the blood are rapidly cleared by cells of the reticuloendothelial system in the liver and spleen.[78] Notably, the use of *in vitro* tests involving primary cells is of particular relevance, as these model systems are closer to the *in vivo* situation.[79] Exposure of nanomaterials may induce immunotoxicity resulting in harmful effects to the immune system. Notably, macrophages are key players in the innate immune response, as these cells are capable of phagocytosing micro- and nano-particles. The

recognition of the nanoparticles by the immune cells may play an important role. Based on the intrinsic properties of nanomaterial, the nano-immuno interactions can be controlled.[80] Some examples are illustrated (*cf.* Figure 3).

As summarized by Kunzman *et al.* nanoparticles can be modulated for desired applications; i) functionalization of USPIONs with PEG may reduce uptake by macrophages leading to increase circulation time and ii) functionalization of SPIONs with ligands that are recognized by immune cells may enhance the uptake in cells.[79] Bhattacharjee *et al.* showed the effect of surface charge and cellular uptake. Demonstrating that negatively charged silicon nanoparticles increased the phagocytosis of silicon nanoparticles by rat alveolar macrophages while positively charged and neutral nanoparticles reduced the phagocytic ability of macrophage.[81]

Importantly, Jones *et al*. recently, showed that the rate of nanoparticle clearance depends on the global status of the immune system. The study highlighted the importance of choosing appropriate *in vivo* models for pre-clinical evaluation of nanomedicines.[82]

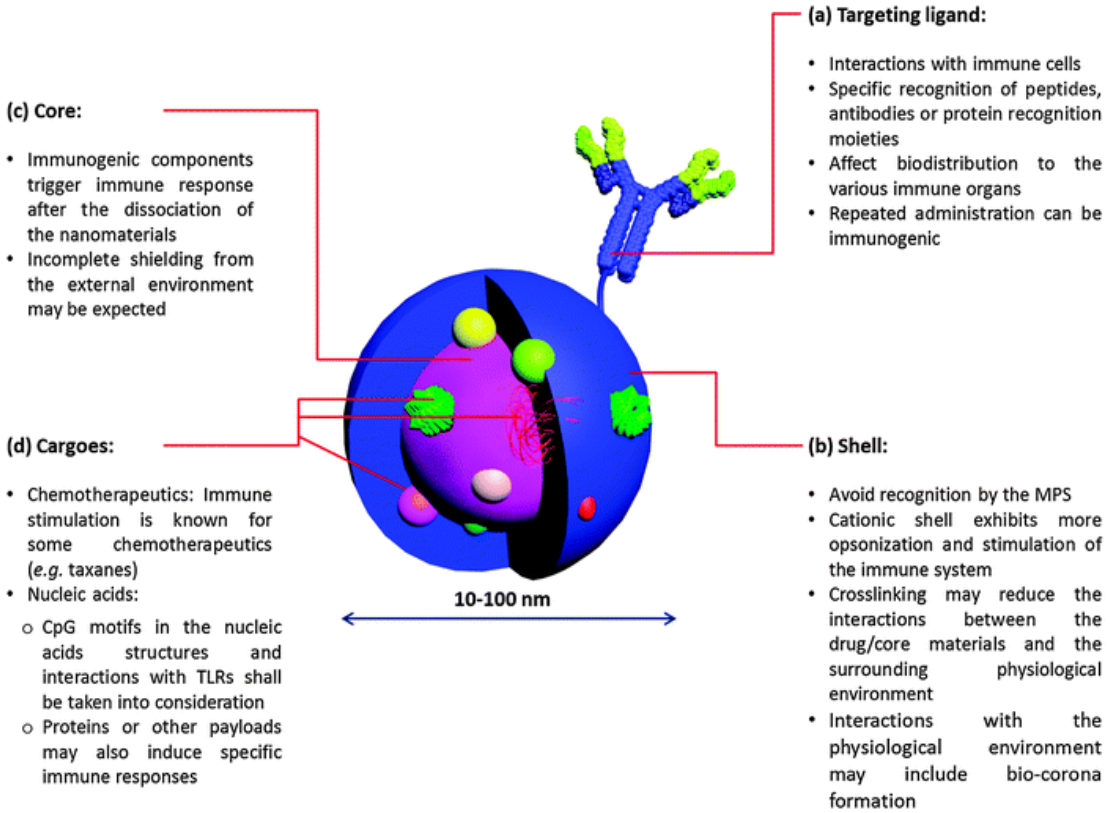

Figure 3. The effect of the intrinsic properties of nanoparticles on immunogenicity. The image is reproduced from Elsabahy *et al.* [83] with permission of The Royal Society of Chemistry.

Understanding the process of clearance is a key to controlling the biodistribution of nanoparticles for medical imaging and drug delivery applications, and may also be relevant to the understanding of adverse effects of engineered nanomaterials. Importantly, nanomaterials could lead to indirect "secondary" effects. For that reasons evaluations of nanoparticle safety should consider both, direct and indirect effects, in order to avoid any potential risk to targets on the distal side of cellular barriers.[84] Sood and co-workers demonstrated that nanoparticles can cause indirect DNA damage *in vitro* across trophoblast and corneal barriers, and cause cytokine and chemokine release across corneal barrier. This indirect effect depends on the thickness of the cellular barrier through a pathway that involves gap junctions.[85] Serag *et al.* demonstrated that the traffic of single wall carbon nanotubes (SWCNTs) into different cell subcellular structures can be facilitated or inhibited by attaching a specific functional tag and by controlling medium components.[86]

Most of the nanotoxicology studies are based on understanding the acute response of nanomaterial exposure. However, the impact of chronic exposure of nanoparticles on human health is an important factor that should be taken in consideration. Recently, Mahler and co-workers showed the effects of oral exposure of polystyrene nanoparticles on the absorption of iron by using an *in vitro* model of the intestinal epithelium as well as an *in vivo* chicken intestinal loop model, demonstrating that acute exposure can disturb iron transport.[61] Recently it has been shown that acute zebrafish embryonic exposure of nanoparticles could be used as a model to predict potential long term effects. Troung *et al.* exposed 1.5 nm gold nanoparticles (AuNPs) with different surface functionalization; negatively charged 2-mercaptoethanesulfonic acid and positively charged trimethylammoniumethanethiol (TMAT) and neutral 2-(2-(2-mercaptoethoxy)ethoxy) ethanol (MEEE) to embryos showing that acute exposure of MES- and TMAT–AuNPs during embryonic development results in larval behavioral abnormalities that persist into adulthood and that was driven by the different surface functionalized gold nanoparticles.[87]

In order to understand mechanism of the molecular pathogenesis in the mouse spleen caused by TiO_2 NPs, Sang et al. exposed mice to 2.5, 5, 10 mg kg body weight of TiO_2 NPs for 90 days and investigated the inflammatory and immunological responses and apoptosis in the mouse spleen. They found that following exposure for 90 consecutive days disrupted the immunological function and toxicity in the spleen of mice, concluding that long-term exposure to low dose of TiO_2 NPs could cause chronic splenic injury in mice.[88] In addition, in a recent *in vivo* study, Sun and coworkers investigated the mechanism by which TiO_2 NPs activates, Nrf2, in the mouse lung by exposing mice to 10 mg/kg body weight of TiO_2 nanoparticles for 15–90 days. They founded that TiO_2 NPs accumulated and increased the reactive oxygen species (ROS) production in lung provoking severe pulmonary edema, inflammatory response and pneumonocyte apoptosis.[89] Cui *et al*, evaluated the gene expression profile in the livers of mice exposed to 10 mg/kg body weight TiO_2 NPs for 90 days. Their results showed that titanium accumulated in the liver, and liver damage caused by long-term exposure to TiO_2 NPs may be associated with significant changes in the expression of genes

involved in immune and inflammatory responses, apoptosis, oxidative stress, and the metabolism process, and in particular, a reduction in complement factor expression. [90]

Understanding which of physicochemical properties of engineered nanomaterials leads to cytotoxicity remains a challenge, as these parameters are interconnected. [91] Therefore, there is a need to develop new approaches to further evaluate the interactions and toxicology effects of nanomaterials with relevant biological systems**.** The following section will discuss the impact of physicochemical properties of the nanomaterials on their cytotoxicity.

1.2.2 Role of the physicochemical properties

Understanding which of the physicochemical properties of engineered nanomaterials determines their toxic response, uptake, and biodistribution in living organisms is not straightforward and still needs to be improved.[92] Numerous studies have attempted to address this issue. Nevertheless, a brief overview on how the intrinsic physicochemical properties of nanomaterials affect cytotoxicity and how they can be tuned to control toxicity, uptake, and fate, are discussed in the following. The physicochemical properties of nanomaterials include size, shape, surface chemistry, rigidity, colloidal stability.[93]

Size represents an important parameter to be considered from a toxicology point of view, as in case the size of nanoparticle decreases their surface area to volume ratio increases and will determine the number of surface groups present on the surface of the material and its reactivity.[94] In general, for smaller nanomaterials a higher toxic response is observed.[6] The role of size in particle uptake has been previously discussed. It has been suggested that for higher intracellular doses of nanoparticles a higher toxic response is observed.[92] Furthermore, there are several *in vitro* studies showing size dependent uptake. For instance, Zhu *et al.* described size-dependent cellular uptake efficiency of silica NPs in HeLa cells.[95] In addition, there are reports in the literature describing the effect of nanoparticle shape, and how this physicochemical parameter, can influence particle uptake and its mechanism. As well as size this feature affects the uptake of nanoparticles and biodistribution.[96] In a recent study, Gliga *et al.* showed size-dependent cytotoxicity of AgNPs in human lung epithelial cells. However, in this study, the results indicated that intracellular metal release rather than differences in cellular uptake or intracellular localization was a probable explanation for the perceived differences in cytotoxicity.[97]

Shape Awargal *el al.* evaluated the influence of shape and size of different polyethylene glycol diacrylate based hydrogel nanoparticles. They found that uptake was depending on the geometry of the particle and the cell model used. Based on the *in vitro* results, they hypothesized that intracellular uptake of NPs depends on three main factors, the sedimentation effect, directed by the weight of the NPs, the forces between the NP surface and cell membranes and the cell membrane strain energy necessary for membrane deformation around the nanoparticle. However *in vivo* evaluation is required

to evaluate the *in vivo* relevance of the study.[98] Another established example is carbon nanotubes, which are thought to exert toxicity due to their "needle-like" shape, that allow them to penetrate cell membranes. This may be relevant at least for multi-walled carbon nanotubes. [99] In addition, the differences in shape may affect particle clearance as well, as demonstrated by Barua *et al.* in a recent *in vitro* study, which spherical, disk and rod-shaped polystyrene nano- and microparticles were evaluated for uptake in human cancer cell lines.[100]

When it comes to **surface charge**, in general positive materials give a stronger cytotoxic response compared to neutral or negative charged NPs. This may be correlated with their enhanced cellular uptake. Indeed, positive NPs cause higher disruption of the plasma membrane integrity.[101] Cationic PAMAM dendrimers have been associated with higher cytotoxic responses than non-anionic ones. The cationic response of PAMAMS appears to be generation dependent, which is associated to a different number of amino groups on the surface.[44,102] Controlling and balancing the density of cationic groups on the surface of the nanomaterials can be used to minimize toxicity, which would be beneficial for specific biomedical applications.[103] In general, neutral or negative surface charge nanoparticles showed prolonged blood circulation time and higher EPR, than positive charge nanoparticles, that are more toxic and induce higher inflammatory reaction than anionic or neutral nanoparticles.[104] Another example is illustrated the distinct hydrophilicity/hydrophobicity of graphene oxide (GO), where it was shown that the different surface oxidation states modulate their cellular uptake, gene regulation and toxicological response in HepG2 cells. Overall, the GO appear to be internalized whereas, reduce GO was adsorbed and aggregated at the cell surfaces with lower uptake.[105]

Colloidal stability determines the dispersion stability of the NPs, and it is an important physicochemical characteristic, which directly affects toxicity. Okuda-Shimazaki *et al.* demonstrated the importance of the aggregation state and revealed that larger aggregates of TiO_2 NPs (596 nm) were more cytotoxic than smaller aggregates (166 nm) upon exposure to THP1 cells. [106] In this particular example, the findings suggested that the surface characteristics were more important for cytotoxic effects than the size. Wick *et al.* also illustrated the role of agglomeration on the cytotoxic effect of nanomaterials by using carbon nanotubes as a model nanoparticles.[107] Balasubramanian *et al.* showed the role of the primary size of particles on the biodistribution by exposing rats to airborne Au NP agglomerates of primary size of 7 and 29 nm. They showed that the smaller agglomerates of Au NPs were distributed in more organs than the bigger ones.[108]

In order to evaluate the benefits of using nanomaterials in medical applications we should consider the possible exposure risk and understand their biodistribution. Nanoparticles can cross biological barriers and enter and distribute within cells by different pathways.[109] For that reason, they are considered a primary vehicle for targeted therapies. In the body we can find i) cellular barriers, including cell membrane, endosome, lysosome, and intracellular trafficking, and ii) physiological barriers

including blood, liver, spleen, kidneys, immune system, and the barriers that prevent extravasation of foreign substances from the blood. There is a concern for the risk of nanoparticles to translocate to organs and tissues and to cross different barriers. [3] Understanding the barriers imposed by a biological system is critical for the design of new materials for biomedical application.[110] Several studies have raised concern about the possible translocation of nanoparticles in the body. For instance, Choi *et al.* synthesized a series of near-infrared (NIR) fluorescent nanoparticles to determine how their physicochemical properties affect their biodistributions, clearance and their translocation. Their result showed that nanoparticles with hydrodynamic diameter less than 34 nm with non-cationic surface charge translocate rapidly from lungs to regional lymph nodes in rats following intratracheal instillation. Moreover, nanoparticles with an hydrodynamic diameter less than 6 nm were able to traffic from the lungs to lymph nodes and the bloodstream and cleared by the kidneys.[111] Schleh *et al*. by using different sizes gold nanoparticles with different surface charges (positive and negative), showed that size and surface charge of gold nanomaterials govern absorption across intestinal barriers and accumulation in secondary the organs after oral administration in a healthy rat model.[112]

The rapid clearance of circulating particles during systemic delivery is a major challenge for diagnostic contrast agents and drug delivery systems in nanomedicine. Nanoparticle parameters such as size, shape, elasticity, surface chemistry, and shape are of particular interest since the effect of the delivery vehicles in the biological systems depends directly on these properties.[113]

The evolution of the degradability of the nanomaterials is an important parameter to consider for their toxicity assessment and the future design of improved nanomaterials. Therefore, adverse effects may occur when nanoparticles are accumulated or cannot be biodegraded. Indeed, it is important that the degradation products are non-toxic. In accordance, Kagan *et al*. showed that single-wall carbon nanotubes could undergo biodegradation *in vitro* through human myeloperoxidase (hMPO), an enzyme present in neutrophils and in less degree in macrophages. Importantly, the enzyme degradation of nanotubes diminishes the potential of these nanomaterials to induce pro-inflammatory effects, as demonstrated *in vivo.*[114] Moreover, it is necessary to evaluate systematically the toxicological effects of the intrinsic properties of the nanomaterials.

1.2.3 Nano-bio interactions: the protein corona

The nano-bio interface consists of the dynamic physicochemical interaction, kinetics, and thermodynamic exchanges between the surface of engineered nanomaterial and the biological constituents.[115] When nanoparticles enter in contact with biological fluids they are immediately covered by proteins, forming what is known as "protein corona".[116] The proteins and other biomolecules which are adsorbed on the surface of materials directly affect the interaction between the nanoparticles and biological systems.[117] Indeed, the protein corona critically determines the biological identity of the material. Therefore, a combination of both, i) the intrinsic properties of the materials

and their physicochemical properties, and ii) the biological identity as given by the protein corona, will dictate the biocompatibility, reactivity, biodistribution, and final fate of nanoparticles.[92, 118] However, to be able to understand these biological effects, it is essential to characterize and identify the nature of the protein corona[119]. For that reason, although different studies have attempted to address this issue, more quantitative analysis is required, to provide new knowledge on the possible biological impact.[120] Studies on protein corona formation of a well-defined i*n vitro* system, where the effect of an individual protein was evaluated, indicated that under saturation conditions, the protein corona forms a single monolayer.[121] This suggests that proteins bind on the nanoparticles in specific orientation.[122]

As it has been stated earlier, protein corona formation is the primary occurrence at the interface between nanomaterial surfaces and biological fluids. In general, loosely bound proteins will form the initial soft corona driven by protein-protein interactions.[123] However, with time, a denser corona will be formed,[118] which is strongly attached to the nanomaterial surface and which is formed by high affinity proteins.[116]

Importantly, the corona formation is governed by the intrinsic properties of the nanomaterials, the ratio between nanomaterials and proteins, and the medium composition.[116] Moreover, the formation of the protein corona appears to be a dynamic process[124] that depends on the temperature, which directly influences its composition.[125] There are studies indicating that the formation of the hard corona is an irreversible process.[126] However, it is still unclear whether the protein-NP complexes may fluctuate depending on the NP environment. Importantly, further changes in the hard corona may occur when nanoparticles translocate from one biological compartment to another one.[127] Liu *et al.* recently indicated that when nanoparticles are at the similar range sizes as proteins, the protein corona formation may be a reversible and unstable process as compared with protein formation on larger NPs.[128]

One of the key challenges in nanomedicine is to design nanomaterials with the ability to target to specific tissues and cells.[129] However, the physicochemical parameters that dictate the successful targeting of nanomedicines under *in vivo* conditions are still poorly understood. Indeed, whether the protein corona is able to mask the surface modification of nanoparticles and thus obscures the design of the synthetic identity remains to be further investigated. Salvati *et al.* explored the role of the protein corona formed in fetal bovine or human serum on the targeting capabilities of transferrin conjugated nanoparticles.[130] For this fluorescent silica nanoparticles coated with polyethylene glycol (PEG) and functionalized with human transferrin were synthetized. To this end, A549 cells expressing the transferrin receptor were used as a model system. The result of their study demonstrated that the specific targeting ability of nanoparticles was lost in the presence of serum. Notably, the overall uptake of bare PEG silica nanoparticles (without transferrin functionalization) was higher than that of the target nanoparticles. Whether this situation will occur *in vivo* needs to be further evaluated.[130] The biodistribution of nanoparticles in the body will be significantly dependant on their interaction with plasma proteins.[131,132] Is known that nanoparticle

surface, size, and characteristics determine the binding properties of plasma proteins, as well as their effect in biological systems.[116] Blood plasma coronas are sufficiently stable that they, rather than the nanomaterial surface, will be the surface that the cell may "see.[133] Consequently, the protein corona may have an impact on immune cells and macrophages.[134] Mononuclear phagocyte system (MPS) plays an important role in clearance of nanomaterials. One of the principal roles of phagocytic cells is to recognise and clear foreign materials. For that reason, strategies to modify nanomaterials to moderate their residence time *in vivo* have been suggested. Coating of nanoparticles with polymers such as PEG is a common approach. Indeed, PEGylation presents an important tool to prolong the blood circulation times[135].

There are studies showing the successful outcome of nanomedicines designed with targeting strategies. For instance, Rodriguez *et al.* showed the beneficial use of a "self peptide" CD47, attached to streptavidin-coated polystyrene beads. CD47 is a marker of "self" that is express in cell membranes, that cancer cells employ to inhibit macrophage mediated obliteration. The authors showed that the functionalized nanoparticles successfully modulated their clearance rate *in vivo.* The protein increased the circulation time of nanoparticles by delayed macrophage-mediated clearance and enhanced drug delivery to tumors in mice.[136]

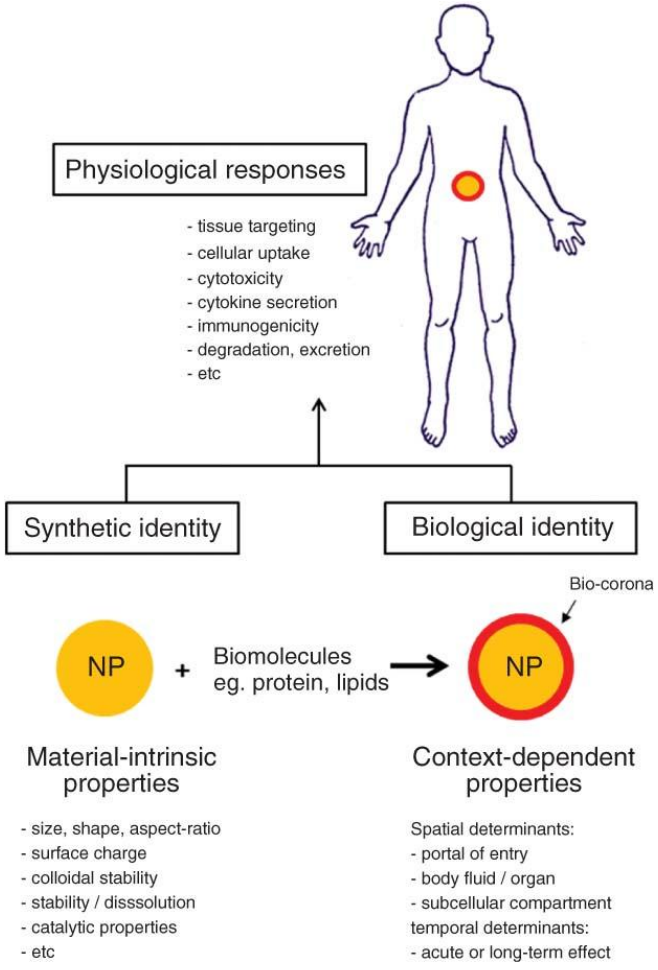

Figure 4: Synthetic and biological identities of nanomaterials. The image is reproduced from Fadeel *et al.*[92] with permission from. © 2013 Wiley Periodicals, Inc

Furthermore, it has been shown that phagocytic cells are able to sense and recognize the self-peptide as a "non-eat me" signal. Konduru *et al.* successfully reported the use of SWCNTs coated with a phosphatidylserine as an "eat-me" signal, to make this material recognizable by different phagocytic cells once they are administrated.[137] In addition, Kunjachan *et al.* evaluated the benefits of active versus passive tumour targeting using 10 nm sized polymer drug nanocarriers to target tumor blood vessels. The authors found that long-term levels of target nanoparticles where lower than the control polymers, suggesting the potential of active over passive tumor targeting in short circulation times. However, in the case of long-circulating nanomedicines, targeting effects should not be overemphasized.[138]

There are studies addressing the effect of surface coatings on the protein corona and their role in cell uptake and recognition. For instance, Simberg and co-workers demonstrated that the surface of dextran-coated iron oxide nanoparticles could be recognized by macrophages, despite the plasma corona formation. [139] Furthermore, Kunzmann *et al.* reported that the uptake of SPIONs by primary human immune-competent cells was determined, in part, by the nature of the surface coating[140]. Association of drug delivery systems with blood proteins is considered one of the most critical factors that determine the biodistribution, efficacy, and toxicity of nanoparticles.[141] Lartigue *et al.* investigated the interaction of nanoparticles with blood plasma proteins and if they are concentration dependent. Nanoparticles in contact with low plasma concentrations formed clusters, whereas nanoparticles in contact with higher plasma concentrations were coated and stabilized. Their results demonstrated that differences in coating directly dictated macrophage uptake.[142]
Nanoparticle based drug delivery systems have been developed to improve the efficacy and reduce the systemic toxicity of drugs.[143] Whether the active targeting in relevant fluids will be achieved/ disturb needs to be further investigated.

1.2.4 Systems biology and predictive toxicology of nanomaterials

In 2007 the National Academy of Science proposed the so called "Toxicity Testing in the 21st Century" report[144]. This paradigm was based on the need for new strategies for toxicity assessment, with the aim to shift from descriptive toxicology endpoints to more predictive toxicology approaches by using high throughput *in vitro* screening assays and computation tools, while minimizing animal tests.[145] Collins *et al.* further emphasized the need for a shift from *in vivo* animals experiments to *in vitro* test using lower organisms, high content techniques, and computational modelling.[146] In recent years, increased interest in developing rapid and comprehensive toxicology screening tools to predict the human toxicity in response to compounds has been raised.

High-throughput screening (HTS) is a method for scientific experimentation that comprises the screening of large chemical libraries for activity against biological targets via the use of automation, miniaturized assays and large-scale data analysis. [147] The

use of HTS techniques have emerged as a useful tool to predict effects of nanomaterials, providing faster, repetitive, systematic, and quantitative data of new materials. HTS approaches could aid to rapid assess hazards of different engineered nanomaterials.[77]

Toxicology assessment of nanomaterials is expensive and time-consuming, therefore, additional experimental approaches, such us the in-silico methods have been found to be important models to predict the potential toxicity of new nanomaterials.[6] Quantitative structure–activity relationship (QSAR) method used to predict the physicochemical properties of chemical compounds can be applied to predict metal toxicity of nanoparticles.[148] There are currently only few nano-QSAR modelling studies. George *et al* reported the use of a HTS assay to test the cytotoxicity of metal oxide nanoparticles in cells.[149] In a subsequent study, the authors combined the established HTS assay with in-silico tools to compare *in vitro* hazard to *in vivo* toxicological effects in zebrafish. Their results determined that the method was able to identify hazard and activity relationships through linkage to in-silico analysis tools.[150] Moreover, Puzyn *et al.* established a model to describe the cytotoxicity of 17 different types of metal oxide nanoparticles to Escherichia coli. The authors found that the model was able predicts the toxicity of metal oxide nanoparticles.[148] Furthermore, a biological surface adsorption index (BSAI) was established. The BSAI approach evaluates the adsorption properties of nanoparticles by quantifying the competitive adsorption of a set of small molecule probes onto the nanoparticles.[151] Xia *et al.* using the BSAI approach the determined that the nanomaterials clusters according to their surface adsorption properties.[152]

The "omics" technologies provide information on the whole genome, with the advantage to characterize mostly all members of a family of molecules (such as DNA, RNA, proteins, etc.) in a single analysis, enabling researchers to investigate with a global approach instead of the traditional "one gene at the time" approach.[153] With these new tools, one can address biological enquiries by understanding the biological systems as whole.[154] Toxicogenomics refers to the study of toxicology using techniques that elucidate how genomes respond to a toxicant or compound. Toxicogenomics approaches aim to use the new global "omics" analysis technologies such as transcriptomics, proteomics, metabolomics, lipidomics, and epigenetics, to provide evaluation of cellular molecular changes caused by chemicals and other environmental agents, with the benefit to move away from animal testing to mode-of-action based *in vitro* tests.[155] This technique relies on the computational tools to analyse, process, and interpret the enormous amount of data generated, that all together will provide comprehensive information to elucidate molecular mechanisms and specific cellular pathways that have been affected, and identify patterns or signatures related to a compound or toxicity.

Systems Toxicology is expected to provide information on the dynamic interaction between molecular components of biological systems and how a determined compound

could disturb these interactions.[156] It will provide information about the possible mechanisms, for both, compounds and nanomaterials, which will give new insights useful for risk assessment. (*cf.* Figure 5)

Systems toxicology has four main areas of interest.[156] i) To analyse the data obtained by the high-throughput methods, ii) to represent the relevant mechanisms of action, iii) to identify the biological responses (quantifying the dose-and time response perturbations) and iv) to build and validate the predicted computational model, with the final goal to use the information for risk assessment.

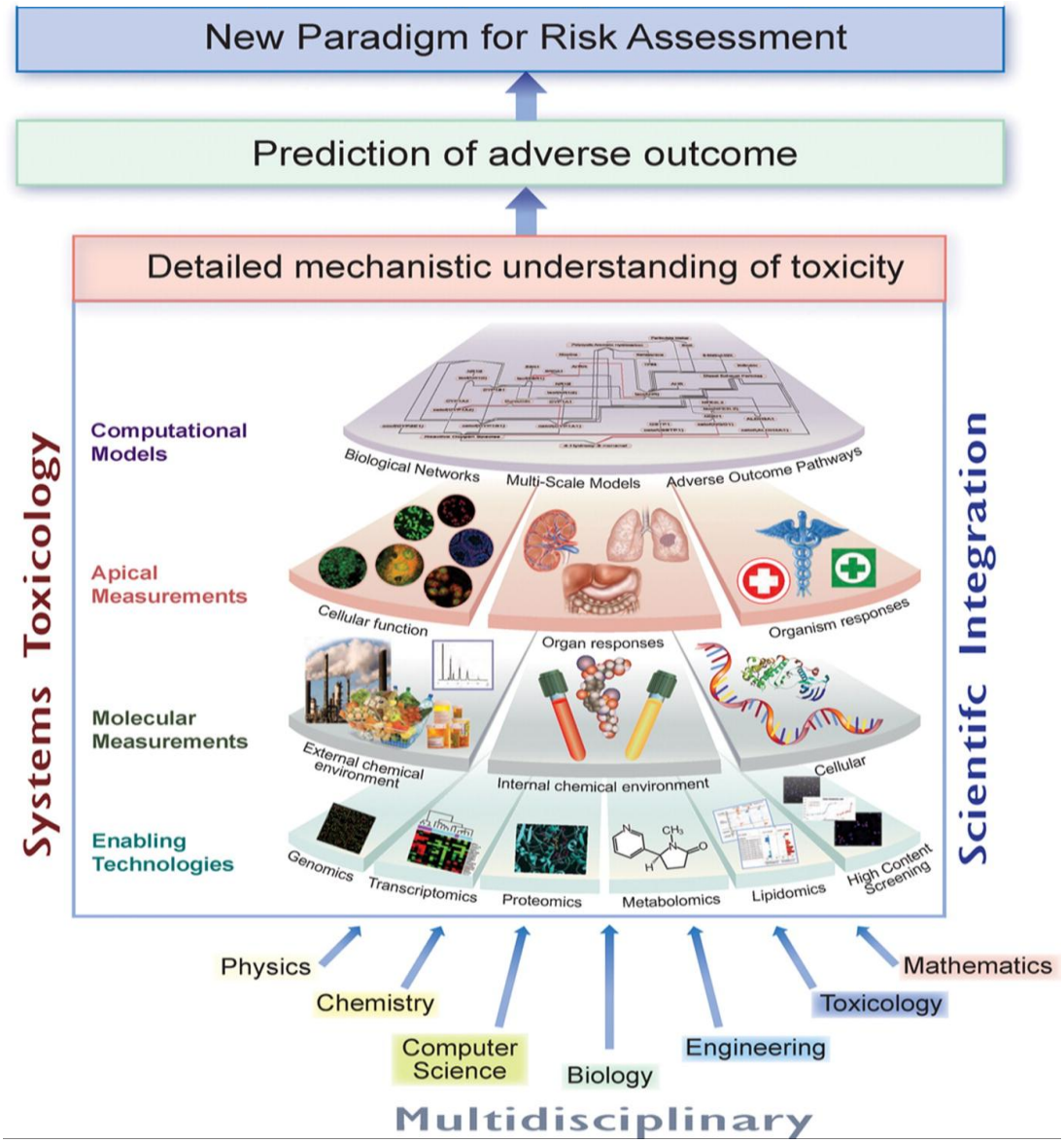

Figure 5: Systems Toxicology. The image is a adapted with permission from Sturla *et al.*[156] Copyright (2014) American Chemical Society.

As recently discussed by Andersen *et al*. one of the primary focus upon the exposure of a compound/chemical substance to cells or tissues is to rather identified biological pathways instead of observing only the toxicity itself.[157] This is also relevant for the toxicity of nanomaterials.

There are only limited system biology studies on nanomaterials. In an elegant example of the use of proteomics and bioinformatics assessment by GO-based annotation, Tsai *et al.*[51] revealed that gold nanoparticles can cause cell death through the induction of endoplasmic reticulum (ER) stress in the human myeloid leukemia-derived cell line K562. In a recent study Chatterjee *et al.* evaluated the effects of two commonly used graphene nanosheets, graphene oxide (GO) and reduced graphene oxide (rGO) in human hepatoma HepG2 cells using global gene expression and pathway analysis. Their results showed that the distinct oxidation states of grapheme regulated their nano-bio-interaction effect, which was observed in the cytotoxicity studies and, in the global gene expression data. Indeed, omics approaches indicated that GO trigger the TGFβ1 mediated signalling pathway whereas rGO might cause host–pathogen interaction and innate immune response through TLR4–NFkB pathway. [105]

ToxCast, a project of the National Center for Computational Toxicology at the United States Environmental Protection Agency was created to develop methods to predict human toxicity of chemicals utilizing *in vitro* assays, computational chemistry, high-throughput screening (HTS) and various toxicogenomic approaches.[158] A number of different reports attempted to implement the same paradigm on nanomaterial risk assessment. Intelligent testing strategy (ITS) allows the risks of nanomaterials to be performed in an accurate, effective and efficient manner.[159,160] The ITS-NANO project was launched in 2012 to allow development of an ITS for nanomaterials safety.[160] This approach has been used in other ongoing European Union projects including FP7-MARINA (see www.nanosafeycluster.eu). Overall, Systems Biology and HTS-based predictive toxicology approaches have been suggested as emerging methods to evaluate the bio-nano interaction between nanoparticles and living organisms.[161] Therefore, in the coming years we expect to implement system biology as a tool to identify possible hazards of nanomaterials.

2 AIM OF THE THESIS

The overall aim of the thesis project is to understand the potential hazards effects of engineered nanomaterials and to evaluate their interactions with biological systems, in particular, assessment of potential immunotoxicity in order to develop optimal nanomaterials for biomedical applications. The specific aims of the study are:

- to evaluate the biocompatibility and stability of a library of library of hydroxyl functional bis-MPA dendrimers on immune cells (primary human monocyte derived macrophages and cell lines)

- to evaluate the effects of non-acutely cytotoxic PAMAM dendrimers in human bronchial epithelial cells using systems biology approaches

- to synthesize novel superparamagnetic iron oxide nanoparticles and evaluate the biocompatibility in primary human monocyte derived macrophages to be used in biomedical application such contrast agent for magnetic resonance imaging

- to study the biocompatibility in primary human macrophages and targeted uptake of iron oxide nanoparticles with different surface coatings in ovarian cancer cells.

3 MATERIALS AND METHODS

In this thesis, different methodologies have been used to efficiently characterize the nanomaterials and evaluate their toxicity impact on different cellular models, including primary immune competent cells. Detailed information regarding synthesis of the materials and methods used are described in the paper I-IV. The methods used can be divided in to two sections; synthesis and characterization of the nanomaterials and *in vitro* toxicity assessment. For material synthesis and characterization different instrumentation has been used for; i) synthesis ii) determination of particle size, iii) surface are and charge, iv) determination of chemical composition. For *in vitro* studies we utilized; i) microscopy techniques, such us TEM, ii) Cytotoxicity assays, including; viability, necrosis, mitochondrial function iii) uptake studies, iv) different cellular assays, including cell cycle, transfection, protein corona, cytokine detection, protein corona iv) bioinformatics analyses.

Detailed description of the materials and methods used in our studies can be found in the publications and manuscripts included in the thesis. The sections below provide an overview of the methods used.

3.1 MATERIAL SYNTHESIS

3.1.1 Dendrimers

Polyester bis MPA dendrimer

Bis-MPA, TMP and hydroxyl functional aliphatic ester dendrimers of generation 1 to 5, and carboxylic functionalized 4th generation Bis-MPA dendrimers were provided by Polymer Factory Sweden AB, Stockholm, Sweden. Dendrons were synthesized as described as previously reported.[35 162] (Paper I).

PAMAMs dendrimers

PAMAMs dendrimers were purchased from Dendritech Inc., Midland, MI.

3.1.2 Fluorescent labeling of dendrimers

In order to monitor the internalization of dendrimers in cells, PAMAM-NH_2 and PAMAM-OH were labelled with the fluorescent dyes, FITC and 5-DTAF, respectively, according to procedures previously reported. [22 23] Briefly, the labelled dendrimer solutions were dialyzed against distilled water through sterilized and rinsed membrane tubing for 24-48 h. Upon subsequent lyophilization, labelled dendrimers were obtained as yellow powders. The material was stored at -20ºC until further use.

3.1.3 Magnetic nanoparticles

Synthesis of the DHAA-Fe_3O_4 nanoparticles

For the synthesis of DHAA-Fe_3O_4 nanoparticles, 0.1 M $Fe(OH)_3$ colloid solution was first prepared by adding $FeCl_3 \cdot 6H_2O$ aqueous solution into 0.45 M $NaHCO_3$ solution, and stirred for 30 min. Then, vitamin C water solution in a molar ratio to Fe^{3+} of 1:6 was added progressively. The mixture was further stirred for another 10 min, and then transferred into a steel-lined Teflon autoclave with a volume capability of 50 mL. The autoclave was kept at 150 ^{o}C for 4 h. After the synthesis, the particles were washed with water and ethanol for 3 times and collected by centrifugation. The nanoparticles were then easily re-dispersed in water, PBS buffer and cell culture media by ultrasonication.[163] (Paper IV).

Synthesis of Fe_3O_4-SiO_2-FA and Fe_3O_4-PEG-FA nanoparticles

Fe_3O_4-PEG nanoparticles were synthesized following a slightly modified solvothermal process previously reported by Yan *et al.*[20] Experimental details are provided in the paper IV. Core shell nanostructures were synthesized using a variation of the well-known Stöber process. The synthetic pathway for Fe_3O_4-SiO_2-FA particles and Fe_3O_4-PEG-FA particles, and experimental details are provided in (Paper IV).

3.2 MATERIAL CHARACTERIZATION

3.2.1 Particle size and morphology

High Resolution Transmission Electron and Scanning Electron Microscope

High Resolution Transmission Electron Microscope (HR-TEM) and Scanning Electron Microscope (SEM) were used for imaging of the nanoparticles. The morphology, size and size distribution of the iron oxide cores and iron oxide nanoparticles were investigated using these techniques. For sample preparation refer to the original articles (Paper III and IV), Briefly, In paper IV, the SEM images were obtained on a Zeiss NEON 40 Microscope and the TEM images on a Philips CM300 electron microscope accelerating a voltage of 120 kV, or the High Resolution (HR)-TEM instrument JEOL JEM 2100F (HR) with the acceleration voltage maintained at 200 kV.

3.2.2 Surface area and charge

Dynamic light scattering and zeta potential

Dynamic light scattering (DLS) studies were undertaken to assess the hydrodynamic properties of the suspensions. The experiments were performed on a Malvern Zetasizer NanoZS instrument using a He-Ne laser at 633 nm. In paper II, PAMAMs were dispersed in phosphate buffered saline solution (PBS), deionized water and keratinocyte serum-free medium, prior to DLS evaluation. In Paper III, the measurements were performed in deionized (DI) water and in RPMI + 1% penicillin/streptomycin + 10% FBS cell culture medium. In Paper IV, the

measurements were performed in McCoy's 5A medium cell culture medium with (+) and without (-) 10% fetal bovine serum non heat inactivated or in DI water using a concentration of 10 µg/ml NPs at 25°C. Size was determined by triple measurements with 10 scans in each measurement and averages are reported (Paper II, III and IV).

Z potential

Zeta potential (ζP) is the electrical potential created between the charged surface groups of the particle and its surrounding medium, and provides information on the particle surface charge, an important parameter to evaluate in terms of material characterization since it will provide information on the interaction between nanoparticle and biological entities such as cells surface, proteins, etc. [164] A Malvern Zetasizer NanoZS instrument was operated with a He-Ne laser at 633 nm and 25 °C. Zeta potential measurements were performed at 25°C on the NanoZS. In paper II, PAMAMs were dispersed in phosphate buffered saline solution (PBS), deionized water and keratinocyte serum-free medium. In Paper IV, the measurements were performed in McCoy's 5A medium cell culture medium with (+) and without (-) 10% fetal bovine serum non hit inactivated or in deionized (DI) water using a concentration of 10 µg/ml NPs at 25°C (Paper II, III and IV).

3.2.3 Chemical composition

MALDI-TOF MS

Characterization of the polyester dendrimers was conducted using Matrix Assisted Desorption Ionization Time of Flight (MALDI-TOF), one of the most common mass spectrometry techniques used for the characterization of dendrimers. Briefly, MALDI-TOF-MS is used to obtain non-fragmented mass data of high-molecular-weight compounds.[32] The MALDI-TOF MS spectrum acquisitions were conducted on a Bruker UltraFlex MALDI-TOF MS with SCOUT-MTP Ion Source equipped with a N_2-laser (337nm), a gridless ion source and reflector design. The detector mass range was set to 500-5000 Da in order to exclude high intensity peaks from the lower mass range matrix fragments. The laser intensity was set to the lowest value possible to acquire high resolution spectra. The obtained spectra were analyzed with FlexAnalysis Bruker Daltonics, Bremen, version 2.2 (Paper I).

NMR spectroscopy

Nuclear magnetic resonance (NMR) is one of the most frequently used techniques for the analysis of small molecules, polymers and dendrimers, including ^{1}H NMR and ^{13}C NMR. [165] Briefly, experiments were performed on a Bruker Avance 400 MHz NMR instrument. ^{1}H NMR spectra were acquired with a spectral window of 20 ppm, an acquisition time of 4 seconds, and a relaxation delay of 1 second. ^{13}C NMR spectra were acquired with a spectral window of 240 ppm, an acquisition time of 0.7 seconds, a relaxation delay of 2 seconds. The residual solvent peak was used as internal standard (Paper I).

Assessment of dendrimer degradation

In order to evaluate the stability of polyester dendrimers, The TMP-G4-OH dendrimer was dissolved in phosphate-citrate buffer of constant ionic strength (I=0.15 M) at various pH (pH 4.5, 5.5, 6.5, 7.5). For comparison, the TMP-G4-OH dendrimer was dissolved in 10 mL deionized water. The solutions were kept at 8°C, 20°C or 37°C and aliquots were taken at specific times for analysis by MALDI-TOF MS (Paper I).

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a characterization technique that provides information on particle surface. FT-IR spectroscopy includes the absorption, emission, reflection, or photoacoustic spectrum achieved by Fourier transform of an optical interferogram.[166] In paper IV FT-IR spectra were measured with a PerkinElmer FTIR spectrometer in a range of 400-4000 cm^{-1}. (Paper III and IV).

X-ray powder diffraction

X-ray powder diffraction (XRD) was used to characterization of iron oxide nanoparticles. XRD can provide information on crystal structure, crystallite size, and strain, x-ray diffraction patterns. [167](Paper III and IV). In Paper IV the phase composition of the core material was characterized by X-ray diffraction using a STOE-STADI MP with Cu $K_{\alpha 1}$ (λ = 1.5406 Å) radiation.

Thermogravimetric analyses (TGA)

In paper IV The mass of the conjugated FA was stated by thermal gravimetric analysis (TGA) using a TGA Q50 (IV Q50 (TA Instruments) instrument in a flowing air atmosphere with a heating rate of 10°/min. The UV-Vis spectra were recorded in water at room temperature on a Lambda 950 (Perkin Elmer) spectrophotometer (paper III and IV).

Ultraviolet-visible spectroscopy

The ultraviolet-visible spectroscopy technique measure the attenuation of a beam after it passes through a sample. In paper IV, the UV-vis spectra were recorded in water at room temperature on Lambda 950 (Perkin Elmer) spectrophotometer.

Determination of iron content

Total Fe was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) on a Varian Liberty 220ICP. The suspension was prepared by combining a 0.2 mL aliquot of the sample with 0.5 mL 8.8 N analytical grade HCl, and 1 mL deionised water. The mixture was heated to boiling and then immediately removed from the heat and allowed to cool to room temperature. The volume was adjusted to 50 ml for spectrometric analysis (Paper III).

Elemental analysis (EA)

In paper IV, to determine the elemental surface composition elemental analysis (EA) was performed by a CHNS EuroEA 3000 Analyzer from Hekatech.

3T MR-imaging and relaxometry

In paper III, MR-imaging and MR-relaxometry at 3T was performed in a clinical whole-body MR scanner (PHILIPS Achieva, the Netherlands) using a knee coil (SENSE-flex-M, PHILIPS, the Netherlands) at room temperature. Briefly, DHAA-Fe_3O_4 water suspensions were diluted in deionized water at Fe concentrations ranging from 0.00017 to 1.25 mM. For MR measurements 0.3 mL/well of the diluted solution was filled in custom made phantoms. Furthermore, T1-, T2- and T2*-weighted images were acquired. For details, we refer to paper III.

3.3 CELL MODELS

3.3.1 Macrophages isolation and cell culture

Macrophages are professional phagocytic cells and serve as a first line of defence against foreign intrusion. Peripheral blood mononuclear cells (PBMC) were prepared from buffy coats obtained from healthy blood donors by density gradient centrifugation using Lymphoprep as described before. [168] Briefly, monocytes were positively selected based on CD14 expression using CD14 MicroBeads. To obtain HMDM, $CD14^+$ monocytes were cultured in complete RPMI-1640 medium, supplemented with 50 ng/mL recombinant macrophage colony-stimulating factor (M-CSF) for 3 days. (Paper I-IV).

3.3.2 Primary human bronchial epithelial cells

The respiratory tract opens the external environmental directly into the airways. Indeed, bronchial epithelial cells are in first line of defence against infections and one of their main function is to recruit neutrophils by chemotaxis as an initial response to the entry of microbes. [169] The establishment of the PBEC is described in detail in previous publications.[170] Briefly, cells were cultured in 80 cm^2 plastic flasks at a density of (1–2) $\times 10^6$ in keratinocyte serum-free medium, supplemented with epidermal growth factor bovine pituitary extract and penicillin/streptomycin. The cultures were kept at 37 °C in a humidified atmosphere of 5% CO_2 in air, and medium was changed every second day. At confluence, the cells were detached by exposure to trypsin/EDTA solution and reseeded in the desired plates and grown to 80% confluence. (Paper II).

3.3.3 Cell line culture and differentiation

HeLa cells

HeLa, is the first continuous human cells line derived from a cervical cancer that was established in 1951[171] from the cancer patient Henrietta Lacks. HeLa is a common cell model system used in scientific research. Cells were maintained in DMEM supplemented with 10% FBS, 1 mM Na-pyruvate, 100 U/mL penicillin, and 100

μg/mL streptomycin at 37°C in 5% CO_2. Cells were counted and maintained in a logarithmic growth phase for all experiments (Paper I and IV).

THP.1cells

THP.1 is a human monocytic leukemia cell line established form the blood of a boy with acute monocytic leukemia in 1980.[172] Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM Na-pyruvate, 5.0×10^{-5} M β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. To induce differentiation into macrophage-like cells, $5.0x10^5$ cells/mL were stimulated with 150 nM PMA for 3 days at 37°C in 5% CO_2. Cells were counted and maintained in a logarithmic growth phase for all experiments (Paper I).

A549 cells

A549 originates from a human carcinoma and was established in 1972 [173]and is one of the most common *in vitro* model for a type II pulmonary epithelial cell found in the alveoli of the lung. Cells were maintained in DMEM supplemented with 10% FBS, 1 mM Na-pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2. Cells were counted and maintained in a logarithmic growth phase for all experiments (Paper I).

SKOV-3 cells

SKOV-3 is a human ovarian carcinoma cells established in 1973 from a 64 year old female with an ovarian tumour. Cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. Cells were counted and maintained in a logarithmic growth phase for all experiments (Paper IV).

3.4 CYTOTOXICITY ASSESSMENT

3.4.1 Limulus Amebocyte Lysate (LAL) test

Previous studies have highlighted the importance preparing endotoxin-free nanoparticles for biomedical applications,[174] for that reason, nanomaterials were controlled for endotoxin contamination prior to incubation with cells. The chromogenic LAL test was used, that detect and measure the bacterial endotoxins in the samples such as Lipopolysaccharides (LPS). LPS is known to elicit immune response in animals and immune-competent cells. Briefly, the assay is based on the measurements of yellow colour, produced by the enzyme reaction, developed upon cleavage of the chromophore, p-nitroanyline, which is proportional to the amount of endotoxin concentration in the sample. The unknown concentrations of endotoxin in the samples are obtained in relation to a standard curve. The US Food and Drug Administration (FDA) mandated limits of acceptance for chromogenic LAL assays are 0.5 EU/mL). [175] [176] (Paper I-IV).

3.4.2 MTT assay

Cell viability was assessed based on the mitochondrial conversion of 3-(4, 5-dimethyldiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide (MTT). MTT is reduced by dehydrogenase enzymes to insoluble formazan in the mitochondria of the living cells. [177] Briefly, cells were seeded in 96-well plates and exposed to nanoparticles at the doses and time-points indicated in complete (10% FBS) or serum-free medium. Then, the supernatant was removed and cells were incubated with 100 µL of MTT solution (0.5 mg/mL) for 3 h at 37°C. The formazan (dark purple) is then solubilized with 50µl of dimethyl sulfoxide (DMSO), and the concentration determined by UV-vis at 570 nm. The percentage of mitochondrial function was calculated relative to untreated cells (*cf.* Papers I-II,IV).

3.4.3 Alamar blue assay

Alamar Blue assay was used to assess cell viability based on the reduction potential of metabolically active cells[178]. The assay utilizes Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide), a non-fluorescent blue indicator dye, that is converted to highly red fluorescent resorufin via the reduction reactions of metabolically active cells. Briefly, cells were seeded in 96 well plates and exposed to specific stimuli at the indicated time-points. At the end of the treatment period, 10 µl of AlamarBlue® reagent was added and incubated for 2-4 hours at 37ºC. The fluorescence was measured at 560 nm excitation and 590 nm emission wavelengths. Since the amount of fluorescence produced is proportional to the number of living cells, the metabolic activity (cell viability) of treated cells was expressed as percentage versus the control (Paper II).

3.4.4 LDH assay

Cell viability was assessed base on the measurement of the release of lactate dehydrogenase (LDH), [179], a cytoplasmic enzyme used as a marker of loss of plasma membrane integrity. Cells were seeded in 96-well plates and exposed to nanomaterials at the doses and time-points indicated. Then, 50 µL of supernatants were assayed for LDH activity following the manufacturer's protocol. Results are presented as % Cytotoxicity = Experimental LDH / Maximum LDH release and related to untreated cells (Paper I-IV).

3.5 CELLULAR UPTAKE OF NANOPARTICLES

3.5.1 Transmission electron microscopy

TEM was used to evaluate interaction of nanoparticles with cells. TEM is able to provide information of morphology of the cell upon nanomaterials exposure, and to visualize nanomaterial (both primary particles and agglomerates) and their intracellular localization. In paper IV, internalization of folic acid-conjugated iron oxide nanoparticles was assessed by TEM analysis. Briefly, HMDMs were exposed to 50 µg/mL of nanoparticles and SKOV-3 cells to 10 µg/ml. At the indicated time-points, cells were harvested and samples were analyzed and visualized according to standard procedures.

3.5.2 Inductively coupled plasma-mass spectrometry (ICP-MS)

ICP-MS is a high specificity and sensitive method that can detect analytes at a very low concentrations, (part per trillion). For this reason, is an excellent sensitive quantification tool to evaluate the cellular uptake of nanoparticles upon proper digestion. However, we should take in account that ICP-MS cannot differentiate between adsorbed or internalized nanoparticles.[180] Quantification of the cellular content of iron was performed by ICP-MS, Briefly, $1.0x10^5$ SKOV-3 cells were seeded in FA-free medium and exposed the next day for 2 h to 10 μg/mL of nanoparticles in FA-free medium. Cells were then washed with PBS, counted and centrifuged at 1200 rpm for 5 min. Cell pellets were kept at -80 °C until further analysis. Measurement of iron content was performed using ICP-MS as described previously[5] (Paper IV).

3.5.3 Confocal microscopy

Confocal microscopy was employed to monitor and visualize cellular internalization of FITC labelled PAMAMs. Briefly, PBEC and A549 were seeded in 24-well plates containing cover slips and allowed to attach for 24 h and were then incubated in the presence of labelled PAMAMs (0.1μM) in KSMF medium and (1μM) in DMEM medium respectively, for the desired time at 37 ºC. To determine the co-localization of PAMAMs with subcellular compartments, PBEC were incubated with labelled PAMAMs as indicated. Thirty min before fixation, Lysotracker (1 μM) or MitoTracker Deep Red FM (150 nM) was added. Slides were fixed with 4% formaldehyde, and mounted with DAPI-containing medium and visualized using a ZEISS LSM510META microscope (Paper II).

3.5.4 Flow cytometry analysis of particle uptake

The uptake of fluorescently labeled NPs can generally be quantified by flow cytometric analysis.[181] [182] To study the internalization of FITC labelled PAMAMs, PBECs were exposed to the fluorescently labelled PAMAMs (0.1 μM) for the indicated time-points. Cells were then washed with PBS and incubated with trypan blue (250 μg/ml) (to quench extracellular fluorescence) for 5 min. Cells were then washed, centrifuged at 800 rpm for 5 min, and washed again. Flow cytometry was performed using a FACScan equipped with a 488 nm argon laser (Becton Dickinson, San Jose, CA) operating with CellQuestPro software (Becton Dickinson) (Paper II).

3.6 MEASUREMENT OF CYTOKINES

3.6.1 ELISA

Nanomaterial exposure could modulate normal immune function, for instance interacting with cells of the immune system, including phagocytes, that represents one of the first lines of defence against foreign organism and particles. Consequently, release of cytokines could occur.[132] Cytokine secretion was studied as a functional end-point in HMDM cells upon exposure to nanoparticles. Indeed, secretion of TNF-α, a

pro-inflammatory cytokine known to be released from activated HMDM was evaluated by the enzyme-linked immunosorbent assay (ELISA), an enzymatic assay that uses antigen – antibody reaction. Briefly, culture supernatants from HMDM were collected following nanomaterial exposure and kept at -80 ºC until cytokine analysis. TNF-α release was measured according to the manufacturer's instruction. The absorbance was measured at 405 nm. Results were expressed as pg/mL of released cytokine using cells from different healthy blood donors.

3.6.2 ELISpot

Macrophage secretion of cytokines was assessed by enzyme-linked immunosorbent spot (ELISpot), a common assay to monitor immune responses in humans and animals. ELISpot assay measures cytokines secreted by cells at the single cell level. Briefly, cells are cultivated on a 96 well plate coated with a desired capture antibody. Upon stimulus, cells could secret cytokines, which successively will be captured by the specific antibodies on the surface. Indeed, to capture anti-cytokine antibodies for macrophage inflammatory protein (MIP)-1β, tumour necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, IL-10 and IL-12 were diluted in sterile PBS to a concentration of 15 μg/mL and added to the plates. After incubation overnight at +4°C, unbound antibodies were removed by washing with sterile PBS followed by blocking for 30 min with complete cell culture medium. 500 or 750 hundred HMDM cells in 100 μL cell culture medium were added and co-incubated with nanoparticles at desired doses and time points. Cells were washed out with PBS and the plates were incubated for 2 h at room temperature (RT) with 100 μL/well of biotinylated antibodies to MIP-1β, TNF-α, GM-CSF, IL-6, IL-10 or IL-12 diluted to 1 μg/mL in PBS with 0.5% FBS. Then, the plates were washed in PBS and incubated for 1 h at RT with streptavidin–alkaline phosphatase diluted 1:1000 in PBS with 0.5% FBS. Finally, the plates were developed with fresh filter BCIP/NBT substrate solution at RT until distinct spots emerged. The colour development was stopped by repeated washings with tap water. After drying, the spots were counted with an ELISpot reader using the AID ELISPOT software.[168]

3.7 SIRNA TRANSFECTION

3.7.1 siRNA transfection

siRNAs are simply internalized into cells with a siRNA transfection reagent. The success of gene silencing will depend on the correct delivery of siRNA. Transfection of SKOV-3 cells using specific siRNA against FOLR1 (Folic acid receptor alpha) was performed with Transfection Reagent DharmaFECT1 according to the manufacturer's protocol. A scrambled sequence was used as a negative control (Paper IV).

3.7.2 Flow cytometric assessment of receptor expression

Surface expression of FR-α on SKOV-3 was evaluated by indirect immunostaining using the anti-folate receptor antibody (2 μg/ml) followed by Alexa488 conjugated anti-mouse secondary antibody. Furthermore, non-specific fluorescence was monitored

using an isotype-matched control antibody followed by labelling with the secondary antibody. Flow cytometry analyses were performed using a FACScan operating with CellQuestPro software (Becton Dickson) (Paper IV).

3.8 CELL CYCLE ANALYSIS

PBECs and A549 cells were seeded at a density of 12 and 5.0 $x10^4$ cells/mL, respectively in a 12-well plate and exposed to PAMAMs. After harvesting and washing with cold PBS, the cells were re-suspended in 500 μL Triton X-100 staining buffer to permeabilize the cells. Then, propidium iodide was added and cells were analyzed by flow cytometry within 30 min on a FACSCalibur (Becton Dickinson) operating with CellQuestPro software. MultiCycle AV (De Novo Software) was used for cell cycle analysis (Paper II).

3.9 "OMICS" ANALYSES

3.9.1 Transcriptomics

mRNA sequencing data acquisition and pre-processing

PBECs were exposed to PAMAMs and total RNA was extracted and purified from the cells and the samples were kept at -80°C until further use. The mRNA Seq of polyadenylated transcripts from total RNA (samples are purified by poly-A enrichment before library creation using Illumina Tru-seq RNA) was used to profile nanoparticle treated and control samples at the Science for Life Laboratory, Stockholm, Sweden.

mRNA sequencing data analysis

Counts from RNA sequencing, mapped to Ensemble gene identifiers (hg19), were processed and further analyzed using the R statistical programming language version 3.0.2 (2013-09-25) and Bioconductor version 2.13 packages edgeR_3.4.1 and limma_3.18.4. Only genes that were differentially expressed between PAMAM-NH_2 and control as well as PAMAM-NH_2 and PAMAM-OH were further analyzed. In addition, donor-specific effect ratios were calculated by normalizing filtered counts, offsetting the normalized pseudo-counts by 0.5 and log2 transforming them. Genes were filtered by requiring that from the PAMAM-NH_2 *vs*. controls and *vs*. PAMAM-OH, 5/6 (ratio) had to be in in the same direction.

3.9.2 Bioinformatic assessment

Gene Ontology enrichment analysis

Gene Ontology (GO) categories describe how gene products behave in a cellular context: biological process: molecular function and intracellular compartment. GO enrichment analysis was performed with R/Bioconductor package topGO_2.14.0 using annotations from GO.db_2.10.1 and org.Hs.eg.db_2.10.1. Only gene categories which had a significance of $p<0.01$ and at least three significant genes are reported.

Pathway and upstream regulator analysis

The regulatory networks and upstream regular analysis were generated using the Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com, Redwood City, CA, application version 220217, content version 16542223). Based on the information data generated from millions of IPA tool generates molecular networks from the selected/interest genes.

3.10 ASSESSMENT OF BIO-CORONA

3.10.1 Isolation of the hard protein corona

The preparation of nanoparticle protein corona formation consists of three main steps: i) preparation of the biological fluid (plasma or serum) ii) Incubation of NPs with biological fluid, iii) Separation of NP–protein corona complexes from excess of fluid by and washes to eliminate loosely bound proteins " soft corona"[183]. In Paper IV, the hard corona proteins recovered from the iron folic acid conjugated iron oxide nanoparticles incubated with different % of FBS were compared by separation using SDS-PAGE gel electrophoresis. Briefly, to induce the formation of the protein corona at the nanoparticle surface, NPs were mixed with different FBS concentrations (1:10 volume ratio) for 1 h at 37ºC. After incubation, the samples were centrifuged to pellet the nanoparticle protein complexes. The supernatant was discarded and the pellet was washed with PBS. Finally, after the last centrifugation, the pellet was suspended carefully with 100 μL of PBS buffer. Then, samples were incubated for 5min to 100 C to denature the proteins, cooled to room temperature, and loaded into a gel electrophoresis until the proteins neared. The gels were stained on the same day using the silver staining kit (Paper IV).

4 RESULTS

4.1 PAPER I. STABILITY AND BIOCOMPATIBILITY OF A LIBRARY OF POLYESTER DENDRIMERS IN COMPARISON TO POLYAMIDOAMINE DENDRIMERS

Dendrimers are polymer materials that could be design in a control manner to obtain specific, size, shape, density, branches, and surface functionality. Taken together this make these polymer structures optimal candidates for therapeutic applications. In Paper I, we investigated the stability and biocompatibility of different polyester dendrimers based on 2,2-bis(methylol)propionic acid (bis-MPA) in human cancer cells and primary human macrophages and compare them to polyamidoamine (PAMAMs) dendrimers. Dendrimers were synthesized as reported and thoroughly characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), proton and carbon nuclear magnetic resonance (^{1}H, ^{13}C NMR) spectroscopy (^{13}C NMR).

In order to investigate the biocompatibility of the library of hydroxyl functional bis-MPA dendrimers, first we evaluated the mitochondrial function and plasma membrane integrity after dendrimer exposure to human cervical cancer (HeLa) cells and acute monocytic leukemia cells (THP.1) differentiated into macrophage-like cells, and our results showed good biocompatibility at the time and dose used. Then, we evaluated their effects in primary human monocyte derived macrophages (HMDM) and compared with commercial PAMAMs with two different surface functionalization, amine and hydroxyl end groups respectively for comparison. Our findings demonstrated excellent biocompatibility for the entire hydroxyl functional bis-MPA dendrimer library to HMDMs, whereas the cationic, but not the neutral PAMAMs exerted dose-dependent cytotoxicity.

Nanoparticles may have an impact on the functional responses of immune cells[168], for that reson, we investigated whether dendrimers trigger cytokine release in HMDM by using the ELISpot assay. For that, we exposed dendrimers, dendrons to HMDM at doses of 10 μM for 24 h. Our results showed that no significant release of the pro inflammatory cytokines, TNF-α, IL-1β, IL-6, or GM-CSF, a growth factor for granulocytes and monocytes, or MIP-1β, a chemoattractant for monocytes and other immune cells, was detected in macrophages exposed either to bis-MPA dendrimers or PAMAMs, however LPS (0.1μg/mL) that was used as a positive control in the experiment, induce cytokines secretion in HMDM.

In addition, the degradation rate of TMP-G4-OH dendrimer as a function of pH, temperature, and time was evaluated by MALDI-TOF MS. The stability was evaluated at the following parameters; pH 4.5, 5.5, 6.5 and 7.5 and at 8°C, 20°C and 37°C, and all preparations were prepared at a 100 μM and an ionic strength of 0.15 M. Our results showed that the bis-MPA dendrimers were degradable materials; indeed, TMP-G4-OH

dendrimers degraded more rapidly at physiological pH (7.5) when compared to acidic pH (4.5) (where no degradation was observed up to 40 days), demonstrating that the stability of the materials increases at low pH.

In summary, we suggest that due to their excellent biocompatibility found in our *in vitro* studies, the hydroxyl functional bis-MPA dendrimers are promising materials for prospect biomedical applications. However, further *in vivo* validation is required to confirm these findings.

4.2 PAPER II. NEXT GENERATION SEQUENCING REVEALS LOW-DOSE EFFECTS OF DENDRIMERS IN PRIMARY HUMAN BRONCHIAL EPITHELIAL CELLS: A SYSTEMS BIOLOGY STUDY

While the toxicity of engineered nanomaterials are currently being extensively investigated using different *in vitro* systems, up today, there are few studies addressing the effects of low doses of materials. To this end, we evaluated the effect of non-acutely cytotoxic doses of PAMAM dendrimers in primary human bronchial epithelial cells using a systems biology approach to predict the effects of these materials. Specifically, we evaluated the toxicogenomic profile of dendrimers by exposing primary human bronchial epithelial cells to Generation 4 PAMAM dendrimers with hydroxyl (-OH) or amino (-NH_2) terminating groups using next generation sequencing (RNA-Seq). PBEC were exposed to PAMAMs at doses that did not elicit acute cytotoxicity. To this end, dose-response studies evaluating cell vitality and plasma membrane damage were conducted. Notably, PAMAM-NH_2, but not PAMAM-OH elicited dose- and time-dependent cytotoxicity in PBECs. Based on our results, we selected non-cytotoxic doses (at 48 h) for subsequent studies (0.1 μM). Next, we evaluated the internalization of fluorescently labelled PAMAM dendrimers by PBECs using flow cytometry and confocal microscopy. Our results showed that PAMAM-NH_2 were more readily internalized when compared with PAMAM-OH and cellular uptake appeared to ensure through an active mechanism, as demonstrated by the reduction of uptake upon incubation with Cytochalasin D, an inhibitor of actin cytoskeleton polymerization. Furthermore, dendrimers were internalized and localized in lysosomes and not found either mitochondria or cell nuclei.

Then, PBECs from three different human donors were exposed to not acutely cytotoxic doses of PAMAMs or medium alone for 48 h and subjected to whole gene transcription. The RNA-Seq data revealed that PAMAM-OH did not elicit any transcriptional responses while PAMAM-NH_2 induced changes in gene transcription at the doses and time used. Overall, our statistical analyses indicate increased expression of 61 genes and decreased expression of 152 genes. In order to better understand and classify the transcriptional changes observed in PBEC upon exposure of PAMAM-NH_2, bioinformatics tools were employed. Gene Ontology (GO) enrichment analysis was performed, markedly, the GO category for ‘cell division’ encompassing 47/152 genes ($p<10^{-22}$) was the most strongly down-regulated category. Notably, GO

categories with *increased* activities involved extracellular matrix disassembly, positive regulation of cell migration, and immune responses. Furthermore, Ingenuity Pathway Analysis of putative upstream regulators proposed that several genes were transcriptional targets of p53 and NF-kappaB.

We therefore evaluated the effects of PAMAMs on cell cycle progression in PBEC since appear to be the most strongly down-regulated category. We found that PAMAM-NH_2 impaired the cell cycle, with retention of cells in S-phase. Then, we investigated whether higher doses of PAMAM-OH could effect to cell cycle. Even when PBECs were exposed to higher doses or for a longer time (72h), PAMAM-OH did not cause cell cycle arrest. Thus, suggesting that cell cycle arrest observed is related to surface functionalization of the PAMAM dendrimers. The upstream regulator analysis indicated that several of cell cycle-related genes were associated to NF-κB activation, then, we evaluated the role of NF-κB in cell progression. For that, PBEC were pre-incubated with a non-cytotoxic dose of Bay-117082 an inhibitor of NF-κB. Notably, Bay-117082 restored cell cycle progression in PBECs exposed to PAMAM-NH_2 as predicated by the upstream analyses.

To this end, we evaluated whether PAMAM-NH_2 are able to induce cell cycle arrest in another cell system, the human lung adenocarcinoma epithelial cell line A549. Notably, our biocompatibility studies revealed that A549 cells were distinctly less sensitive than the primary bronchial epithelial cells**.** Based on our cell dose response viability studies, we select non-cytotoxic dose (1 μM) to evaluate potential effects of PAMAMs on cell cycle progression upon exposure for 48 and 72 h. Our results showed that PAMAMs did not induce cell cycle arrest in A549. However, Resveratrol, known to cause accumulation of cells in S-phase, triggered retention of A549 cells in S-phase.

In summary, the integrative approach presented in paper II, based on whole transcriptome sequencing using RNA-Seq and computation tools, have enabled the discovery and prediction mechanisms underlying cellular responses of non-acutely cytotoxic doses of PAMAMs to PBEC. The prediction was confirmed by in vitro cell-based assays, demonstrating the benefits of system biology approaches in toxicology and risk assessment of nanomaterials.

4.3 PAPER III. WATER-SOLUBLE SUPERPARAMAGNETIC MAGNETITE NANOPARTICLES WITH BIOCOMPATIBLE COATING FOR ENHANCED MAGNETIC RESONANCE IMAGING

The aim of the study was to synthesize novel ultra-small superparamagnetic iron oxide nanoparticles (USIRONs) that could be used as negative contrast agents for MRI and to investigate possible toxic effects in primary human macrophages. USIRONs were synthesized by a simple hydrothermal method and were prepared by the chemical reduction of $Fe(OH)_3$ colloid, by using vitamin C (a natural nutrient) as reducing agent under mild hydrothermal condition. This synthesis, provides a control and slow

condensation of the iron hydroxides that allows to obtain SUIRONs with superior physiochemical properties as compared with the traditional co-precipitation methods.[184] Indeed, the oxidized Vitamin C, the dehydroascorbic acid (DHAA), also acts as a stabilizer and allows the synthesis of water soluble iron oxide nanoparticles by a simple one-step method.

Overall, USIRONs exhibit an average crystalline core size of (5.1 ± 0.5 nm). XRD measurements confirmed the nature of the iron oxide phase (cubic Fe_3O_4) and the TEM images illustrated that those nanoparticles have spherical shape and narrow size distribution. The hydrodynamic conditions were studied by DLS, and found that the average hydrodynamic sizes was 41 nm, bigger that the core size determined by XRD, TEM and magnetometry. Additionally, nanoparticles present a relatively monodisperse distribution with a PDI value of 0.12. Importantly, size, PDI and scattered intensity appear to be stable over time at different pH ranges. At the end, FT-IR was used to confirm the chemical transformation of vitamin C to DHAA.

A comprehensive magnetic characterization was performed on USIRONs. The results showed that DHAA-Fe_3O_4 nanoparticles presented superparamagnetic properties with a saturation magnetization value of 47 emu g^{-1}, a value higher than other described iron oxide nanoparticles of similar size prepared by the solvothermal and high-temperature decomposition processes[185]. Furthermore, phantom experiments on the contrast agent (clinical 3 T MRI scanner) of DHAA-Fe_3O_4 suspensions revealed a relaxivity value ratio was r_2/r_1 of 36.4 (with r_1= 5 s^{-1} mM^{-1} and r_2= 182 s^{-1} mM-1), a value greater than clinically approved agents. Strong contrast was observed by T_2 and T_2*-weighted images giving evidence of the potential use of DHAA-Fe_3O_4 nanoparticles for biological applications, for instance, as a negative contrast agents for MRI.

In order to evaluate the biocompatibility of the USIRONs, primary human monocyte-derived macrophages were selected as a relevant model system, because when nanoparticles are intravenous administrated they may come in contact with immune competent cells. HMDM were exposed to different doses of DHAA-Fe_3O_4 nanoparticles for desired dose and time, and cell vitality was assessed by the lactate dehydrogenase (LDH) release assay. Our results showed that cell viability of primary human macrophages exposed to USIRONs for 24 h was not affected as compared with ZnO used as a positive control. We further assessed the effect of DHAA-Fe_3O_4 on cytokine secretion (TNF-α) upon exposure to HMDM, and no cytokine release was observed in cells following incubation with USIRONs.

In summary, we effectively synthetized a biocompatible DHAA-Fe_3O_4 nanoparticle with narrow size distribution and long term colloidal stability with excellent superparamagnetic properties, that altogether, make these materials promising candidates for future medical application such as negative contrast agents.

4.4 PAPER IV. TARGETED UPTAKE OF FOLIC ACID-FUNCTIONALIZED IRON OXIDE NANOPARTICLES BY OVARIAN CANCER CELLS IN THE PRESENCE BUT NOT IN THE ABSENCE OF SERUM

Understanding the interaction of nanomaterials with proteins in a physiological environment is of considerable importance. In the present study, we synthesized folic acid (FA)-conjugated iron oxide nanoparticles with two different intermediate coatings, inorganic silica (SiO_2) and organic polyethylene-glycol (PEG), respectively, and assessed biocompatibility and targeting efficiency using primary human macrophages and ovarian cancer cells. Indeed, we decided to investigate whether FA functionalization of NPs would lead to specific uptake by ovarian cancer cells, which were shown to express high levels of the folate receptor (FR)-α.

Magnetic iron oxide nanoparticles (Fe_3O_4-PEG) with narrow size distribution and an average primary particle size of 18.4 nm were synthesized by solvothermal decomposition of iron salt in aqueous solution. The TEM and XRD measurements confirmed that phase structure of the iron oxide. Next, NPs were coated with either SiO_2 or PEG. To form SiO_2 nanoparticles, a ligand exchange with PVP in isopropanol was required. For the subsequent functionalization of core-shell nanoparticles with FA, amino-propyl-tri-methoxy silane was used as the coupling agent. The TEM images and the FT-IR analyses evaluated the patterns of the core and shell phases and the silane linkers on the nanoparticles respectively, confirming the successful reactions. Finally, functionalization with FA was achieved by coupling the terminal amino groups of the Fe_3O_4-SiO_2-NH_2 nanoparticles previously obtained with FA using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling agent. To form Fe_3O_4-PEG-FA, Fe_3O_4-PEG particles were coupled directly to FA using a condensation reaction. The characterization data revealed that the primary particle sizes were 20 nm for Fe_3O_4-PEG-FA and 80 nm for Fe_3O_4-SiO_2-FA nanoparticles.

Next, we evaluated their biocompatibility in primary human macrophages; overall, nanoparticles were shown to be non-cytotoxic at the time and doses used, as revealed by the mitochondrial function studies and TEM images. Subsequent, folate receptor alpha-mediated cellular uptake was investigated using ovarian cancer cell lines (SKOV-3), which were shown to express high levels of the receptor. Nanoparticles were exposed to 10 μg/mL for 2h. The data indicated that the reduction of folate receptor-alpha expression (using specific siRNA) resulted in a significant decrease in the internalization of the Fe_3O_4-SiO_2-FA nanoparticles in ovarian cancer cells, whereas the uptake of Fe_3O_4-PEG-FA nanoparticles was not affected as evaluated by ICP-MS and TEM images. Notably, specific FA targeted uptake was only detected in the presence of serum proteins.

PEG modification usually disrupts the protein adsorption; this could be the reason for that specific FR-α-mediated cellular uptake was observed only for Fe_3O_4-SiO_2-FA nanoparticle. For that reason, we investigated the protein adsorption capability between

of the two nanoparticles to get a better understanding of their cellular uptake mechanism. For that, we evaluated whether the protein corona could be formed for both nanoparticles; Fe_3O_4-SiO_2-FA and Fe_3O_4-PEG-FA were incubated with 10% or 55% FBS or in PBS alone for 1 h at 37°C and proteins recovered from the 'hard' corona were visualized by SDS/PAGE. Under these conditions, we found that the Fe_3O_4-PEG-FA particles adsorbed serum proteins more abundantly than the Fe_3O_4-SiO_2-FA particles.

To conclude, we successfully synthesized for Fe_3O_4-SiO_2-FA and Fe_3O_4-SiO_2-FA core shell nanostructures using a variation of the well-known Stöber process with a narrow size distribution, presenting crystalline magnetic core. Interestingly we found that serum is required for folate dependent targeting to ovarian cancer cells. Due to their targeting capability and in vitro biocompatibility, Fe_3O_4-SiO_2-FA nanoparticles are promising candidates for biomedical applications.

5 GENERAL DISCUSSION

5.1 BIOCOMPATIBILITY OF NANOMATERIALS: TOWARDS PREDICTIVE TOXICITY TESTING

Nanotechnology, nanotoxicology and nanomedicine are complementary disciplines that aim to provide future novel and superior medicines.[76] However, for the successful development and implementation of new design materials, it is essential to understand how the material intrinsic properties are linked to the physiological responses.[92]

In order to increase our knowledge of how physicochemical parameters of nanoparticles affect biological systems, implementation of systematic and mechanistic *in vitro* studies is needed.[186] In particular, it is important to assess how the intrinsic properties of the ENM (that will dictate their biological "identity" affects in immune system, which comprises the first line of defence again foreign materials[187]. Thus, part of the work presented here emphasizes the importance and the relevance of using primary human cells for the toxicity assessment of ENM. Primary cells maintain their phenotype *in vitro* and therefore a suitable model to closely mimic the *in vivo* situation. However, cell lines were also used, in particular for the assessment of targeting efficiency, and for reproducible systematic screening assays.

Macrophages are one of the principal cells of the immune system, and represent the first line of defence against foreign materials including nanomaterials. Macrophages play important key roles in host defence, comprising phagocytosis, antigen presentation and immunomodulation. Notably, recent studies suggested that macrophages are mature differentiated cells with possible self-renewal capacity (local proliferation), similar to the capacity adopted by the stem cells.[188] It will be important to determine the general mechanism of nanoparticle induce immunotoxicity, including i) recognition of nanoparticles thorough surface receptors, for instance Toll-like receptors, ii) macrophages dysfunction, iii) inflammation responses such us induction of proinflamatory cytokines, chemokines and adhesions molecules and iv) genotoxicity, such generation of oxygen radicals.[187] The effects of nanoparticles on macrophages are still largely unknown. In this thesis, we carefully evaluated the possible immunotoxicity of dendrimers and SPIONs in primary human derived macrophages, as key cells of the innate immune system. The airway epithelium is the primary barrier for inhaled particles and compounds into the body, including particulate matter and airborne chemicals.[189] For that reason, in paper II, primary human bronchial epithelial cells (PBEC) were selected as a model system as these cells play an important function in the innate immune defence against inhaled pathogens and particulates,[190] and are involved in the recruitment of neutrophils by chemotaxis[169] and in the cell clearance in the airways.[191]

Engineered nanomaterials present a great potential for a wide range of medical applications. In the thesis, it was attempted to evaluate how different physicochemical

properties nanomaterials affect their biocompatibility, for their use in medicines. The nanomaterials evaluated are dendrimers and SPIONs, two different types of nanomaterials with great potential as drug delivery systems and imaging agents (MRI) respectively.

In paper I, we evaluated the biocompatibility and stability of an entire library of bis-MPA polyester dendrimers. This included the building blocks, core structures, as well as PAMAMs. Different methodologies have been used to efficiently characterize these nanomaterials and evaluate their possible cytotoxic impact on different cell lines and primary human macrophages. Overall, polyester dendrimers studied in this work exhibit excellent biocompatibility at the doses and incubation times used.

In general, the successful translocation of dendrimers from laboratory bench to the clinic, will depend on their biocompatibility, reasonably fast renal elimination, and degradation rate.[192] In addition, the efficacy of drug delivery systems will be also relying on their efficiency to deliver their cargo to the site of interest. In this context, the possible degradation of nanoparticles in lysosomes should be taken in account.[193] There are studies showing that PAMAM dendrimers enter cells by endocytosis [194]. Within cells, the pH values of subcellular compartments may differ. Thus, the acidity increases sequentially from endocytic vesicles to early endosomes (pH=6.5), late endosomes, and lysosomes (pH<5.5). [195] For that reason, in Paper I, we evaluated the stability of dendrimers *in vitro* at different pH values and incubation times by using MALDI TOF MS. We verified that hydroxyl functionalized bis-MPA dendrimers are degradable materials. Specifically, TMP-G4-OH dendrimers degrade faster at physiological pH (7.5) when compared to acidic pH (4.5). This demonstrates that the stability of the dendrimers increases at low pH.

Nanotoxicology aims to identify potential health effects and mechanism of action of nanomaterials. However, careful understanding of nanomaterial physicochemical needed properties is desired for the future use of this material in medicine. Thus, extensive and systematic toxicological studies are needed it to build a comprehensive risk assessment of nanomaterials. For instance, Li *et al.*[196] evaluated the role of surface functionalization and size of CNT materials, which were all obtained from the same pristine batch. A library of CNTs including carboxyl (-COOH,), polyethylene glycol (-PEG), hexane diamine (-NH_2), sidewall amine (-sw-NH_2), and polyethyleneimine (-PEI) functionalized multiwall carbon nanotubes (MWCNTs) was synthetized and well characterized. The *in vitro* studies were conducted in epithelial cells and macrophages. The study demonstrated the important role of surface charge in both toxicity, and cellular response. Indeed, anionic functionalization (-COOH and -PEG) decreased the pulmonary fibrogenic potential compared to as-prepared APMWCNTs. In addition the positive charge of cationic functionalization (-PEI) induced more pulmonary fibrosis. However, neutral and weak cationic functionalization (-NH_2 and sw-NH_2) showed intermediary effects.[196] Furthermore, Li *et al.* recently synthesised a library of 10 rare earth oxide (REOs) NPs with an average size of 18–60 nm and studied their biological transformation in an acidic lysosomal fluid, as well as their impact in triggering lung

fibrosis. With this systematic approach, both *in vitro* and *in vivo*, the authors demonstrated that upon macrophage and lysosomal processing, REO nanoparticles undergo dissolution, leading to NLRP3 inflammasome activation, whereas pre-treatment of REO NPs with phosphates in a neutral pH prevented biological adverse effects.[197] These systematic studies were able to reveal the detailed mechanism of toxicity induced by these nanomaterials.

In paper I, we evaluated a library of polyester bis-MPA dendrimers, to allow a systematic and comprehensive toxicity study. However, due to their excellent biocompatibility, no further mechanism of toxicity was contemplated. In addition, in paper II, we investigated the effect of surface charge on the toxicity response of non-acutely cytotoxic doses of PAMAMs to primary human bronchial epithelial cells. We observed that positively charged PAMAMs exhibit higher toxicity and higher uptake than the neutral PAMAMs, as previous reported also with other model systems.[198 199]

5.2 SYSTEMS BIOLOGY APPROACHES FOR NANOMEDICINE AND NANOSAFETY

Rapid growth in the field of nanotechnology involving novel synthesis of materials has emerged in the past few years due to the fact, that the novel physicochemical properties arising at the nano scale make ENM very attractive and promising for a wide range of applications. However, the increasing use of ENM rise the need to assessed the potential impact on human health and environmental. The effects of these new materials in human health are not well understood, as previously emphasized, nanotoxicology studies will give us references and guidelines to build and design new safe nanomaterials. Nevertheless, efficient and mechanistic toxicology tests are required to compare and predict the new paradigm for assessing the potential risk of these materials. System biology approaches together with *in vitro* high throughput screening (HTS) techniques will facilitate the development of new nanomaterials, by providing early prediction and mechanism information of cell interactions before moving to animal testing. The field of system biology is at an early stage but the goal is to use this approach and techniques to provide prediction of toxicity. With the implementation of these new tools, it will be possible to obtain toxicity data of thousands of nanomaterials, reduce the high time and cost of the animal testing: and facilitate development of safe nanomaterials.

The potential impact of the nanomaterials on gene transcription has attracted considerable interest.[200 201] However, studies addressing the effect of non-cytotoxic doses of ENM on gene expression profiling are rare. Although the toxicity of PAMAM dendrimers have been extensively reported, due to their potential use as nanomedicines, including; drug delivery, diagnosis and therapy,[202] the molecular-level interaction mechanism and the cell pathways involved still not clear. We therefore, in Paper II, investigated the effects of non-cytotoxic doses of PAMAM dendrimers on gene expression in PBEC by using RNA-Seq and bioinformatics tools to identify biological

outcomes of these materials. Indeed, using system biology approach, we showed that PAMAM-NH_2 and not PAMAM-OH prompted changes in gene expression profile as reveal by RNA-Seq and bioinformatics analyses. With the use of computation tools; GO and upstream regulator analyses, we predicted that these changes were induced through p53 and/or NF- κB dependent manner. Because GO analysis suggested that the genes that were affected upon exposure to PAMAM-NH_2 primarily represented the cell cycle category, we assessed the cell cycle in PBECs exposed to PAMAMs dendrimers. Notably, low-dose exposure of cells to cationic PAMAMs, but not to PAMAM-OH, for 48 h triggered cell cycle arrest, with retention of the cells in S-phase as anticipated by the of the systems biology-based predictions. Remarkably, Kim *et al.* reported that amino functionalized polystyrene nanoparticles induced cell cycle arrest in A549 cells was p53-independent. Therefore, it remains possible that different nanoparticles induce cell cycle arrest through different mechanisms.[203]

Nanotoxicological studies are often based on the assessment of acute cytotoxicity, often using unrealistically high doses of materials. For future implementation of nanomedicine, more subtle effects occurring at low doses need to be addressed to better understand the toxicity of these nanomaterials. In Paper II, we have defined a "low" dose as a dose that does not cause acute cytotoxicity in the cell model used, PBEC. Notably, in Paper II, we were able to identify up regulation of gene related to immune systems such as TNF and MMP-9 and MMP-3, upon exposure of low doses of PAMAM.NH_2 to PBEC. Interestingly, in a recent study of transcriptomic responses in zebrafish embryos, Oliveira *et al.* showed that PAMAM-NH_2 (generation 3 and 4) caused transcriptional changes in zebrafish embryos at sub-lethal doses, consistent with the activation of the innate immune response. Specifically, PAMAMs induced expression of genes that are related to the innate immune response to bacteria including *tnfb, irg1l, mmp9* and *mmp13*.[48]

To date, few studies to date have applied system biology approach on nanomaterial-exposed cells. In a recent study, Lucafo *et al.*[49] evaluated the gene expression profile of human MCF-7 breast carcinoma cell line exposed to fullerenes using RNA-Seq technology. The bioinformatics analysis was followed by Connectivity Map (CM) analysis. CM identified similarities between the transcriptional signatures produced by fullerenes and those produced by other compounds with known modes of action. With this tool, the authors indicated that the gene expression pattern of fullerene-treated cells was similar to those induced by selective inhibitors of mammalian target of rapamycin (mTOR) signalling. Notably, in Paper II, when CM analysis was performed on our RNA-Seq data obtained in PBECs exposed to low doses of PAMAMs, we observed that the gene expression profile of PAMAM-NH_2 matched those of several compounds known to cause S-phase arrest (unpublished observation).

Toxicogenomics will move the traditional toxicology through the global observation of genomic responses to chemicals or substances[204] and improve our understanding of mechanisms of toxicity.[205] The combined application of the enabling omics

technologies with bioinformatics tools, will allow us to obtain more predictive information of mechanism of action of nanomaterials, as demonstrated in Paper II.

5.3 THE BIOLOGICAL IDENTITY OF NANOMATERIALS AND ITS BIOLOGICAL IMPLICATIONS

SPIONs are FDA approved nanomaterials for biological applications, such as contrast agents.[206] However, the success of the new improved SPIONs is only accomplished if they present both, superior physicochemical properties and excellent biocompatibility. The main purpose of Paper III and IV was to synthetize new iron oxide nanoparticles with different surface coating, and to evaluate their effect on biocompatibility and mode of action of these materials at the nano-bio interface.

The preparation of water dispersible iron oxide nanoparticles with proper surface coatings poses a challenge to nanomaterials synthesis. Therefore, in Paper III, stable aqueous USIRONs were successful synthetized by chemical reduction of $Fe(OH)_3$ using vitamin C as reducing agent. USIRONs presented excellent biocompatibility in HMDM, favourable colloidal properties, small hydrodynamic size, high r_2 values, and desired relaxivity ratios for the enhanced contrast of T_2- and T_2*- weighted images. Overall, DHAA-Fe_3O_4 nanoparticles as described are estimated to be a promising candidate for negative contrast applications, such as MRI.

Indeed, we emphasized the use of primary human macrophages in our studies, as important immune competent cells[188,207] representing a more relevant *in vitro* model system than transformed cell lines. However, due to the promising target capabilities of the iron oxide nanoparticles synthesized in Paper IV, we used cancer cell lines to evaluate their targeting efficiency.

In a physiological environment nanoparticles will be rapidly cover by proteins. The intrinsic properties of the nanomaterial will dictate the selectivity of the absorbed proteins on the surface, forming the so-called "biological identity" of the nanomaterials that will determine the interaction of the nanomaterials with living systems. The role and the effects of both, composition and dynamics of the protein corona in biological systems remain to be understood. It is known, that protein corona formation on the surface of nanomaterials is a dynamic process. However, the interaction that drives the proteins to assemble in biological scenarios is still not clear. Although several studies have attempted to address this issue, different conclusion could be drawn. This may be due to the different model used, including a variety of nanoparticles, biological media; experimental conditions, and techniques used. However, more systematic and quantitative characterization data of the protein corona could be obtained by simple analytical models to be able to provide comparative studies, to better understand the role of protein corona in the bio-nano interface.[120]

Active targeting aims to distribute drugs to the site of interest while minimizing the exposure to other tissues. It is suggested that surface functionalization of nanomaterials could be designed to enhance targeting properties. Gogary *et al.* synthetized FA-PEG-PLGA vehicle nanoparticles able to passively and actively targeted folate receptors expressed on tumor cells. For that, various types of modified PLGA NPs were synthetized to evaluate the effect of PEG and FA in targeting capabilities of NPs. Uptake studies in HeLa cells showed that the FA conjugates enhanced the cellular uptake as compare with PEG-PLGA NPs. However, *in vivo* studies reporting FA-targeted PLGA NPs encapsulating quercetin for cancer targeting and therapy reveals that uptake is enhanced by take place through both passive and active mechanism. [208]

The biological identity of the nanomaterial will directly affect the interaction with biological systems. In this thesis, we synthetized two FA-functionalized iron oxide nanoparticles with different surface coatings; Fe_3O_4-PEG-FA and Fe_3O_4-SiO_2-FA and evaluated their biocompatibility and the importance of particle coating for targeted uptake in ovarian cancer cells overexpressing folate receptor. It is important to note, that overall, only target uptake was achieved only for Fe_3O_4-SiO_2-FA and not for the Fe_3O_4-PEG-FA and only in the presence of serum. Notably, we founded that both NPs are internalized in cells with or without silencing of *FOLR1* receptor, suggesting that several uptake pathways may contribute to the internalization of nanoparticles. Our data suggested that the differences in targeting capability of the two nanoparticles could be related to differences in protein adsorption and/or agglomeration behaviour in the presence of serum. Although, PEGylation of the surface of nanoparticles is known to reduce protein adsorption and cell adhesion,[126] our studies indicated that Fe_3O_4-PEG-FA NPs adsorb higher amount of protein compared with Fe_3O_4-SiO_2-FA NPs. Based on these results, we speculated that the targeting ligand of the Fe_3O_4-PEG-FA nanoparticles was not accessible for binding to the FR-α receptor on the ovarian cells due to the specific protein adsorption on the surface of the particles or that the ligand was trapped within PEG matrix and was not able to be recognized by the cells. Notably, the same studies were conducted on HeLa cells, which also express FA receptor. However, we could not find target specific uptake upon exposure of both nanoparticles, for that reason, we could suggested that the targeting capabilities of nanomaterials may not be only definite to a specific nanoparticle, but also cell type–specific. Finally, we highlight, that due to their targeting capability and *in vitro* excellent biocompatibility, Fe_3O_4-SiO_2-FA nanoparticles are promising materials for medical imaging.

The protein corona may alter the properties of the nanoparticle, thus need to be taken in consideration when study the biological interactions the nature of the nanoparticle surface is investigated.[116] Walczyk *et al.* suggested that protein coronas rather than the nanomaterial surface are likely to be what the cell sees.[133] However, it is unclear whether cell could also recognize the surface of the nanomaterial. In some scenarios, cell receptors may have preferences for the bare surface of nanoparticle. Simberg *et al.* showed that despite protein corona formation of SPIONs surface could be available for recognition by macrophages.[139] To this end, the design future nanomedicines, it is

essential to understand the biological identity of the nanomaterials, especially for surface modified nanoparticles.

Notably, a recent study by Salvati *et al.* indicated that excessive binding of serum proteins may impair selective, ligand-mediated uptake of nanoparticles.[130] This effect may explain the results found for Fe_3O_4-PEG-FA, which appear to bind more proteins than Fe_3O_4-SiO_2-FA.

Nanoparticles are potentially useful for targeted drug delivery.[1] Recent studies support the effective use of active targeting of nanoparticles, not only in animal models, but also in human cancer patients. Davis *et al.* conducted the first in-human phase I clinical trial involving the systemic administration of siRNA to cancer patients using a targeted, nanoparticle delivery system. By using transferrin antigen to target cancer cells overexpressing the corresponding receptor, the authors demonstrated the dose-dependent accumulation of targeted nanoparticles in human tumours, and their consequently effective specific gene inhibition by a RNAi mechanism of action.[209] Furthermore, Hrkach *et al.* showed the beneficial use of a prostate-specific targeted polymeric nanoparticle containing the chemotherapeutic docetaxel for the treatment of patients with solid tumors.[210] In a recent study, Pridgen *et al.* demonstrated that nanoparticles targeted to the neonatal Fc receptor (FcRn), which mediates the transport of immunoglobulin G antibodies across epithelial barriers, are efficiently transported across the intestinal epithelium using both *in vitro* and *in vivo* models. The specificity of the *in vivo* targeting was studied using FcRn knockout mice. Their results indicated that the nanoparticle transport was higher for target nanoparticles.[211]

Interestingly, Caracciolo *et al.* showed that nanoparticles can acquire a targeting capability through the formation of a protein corona. Indeed, thus, lipid nanoparticles incubated in plasma were coated with vitronectin that promoted uptake in cancer cells expressing high levels of the vitronectin receptor.[212]

Remarkably, the biological identity of nanomaterials "protein corona" could induce adverse effects in cells, such us inflammatory response. These possible effects should be taken in consideration for the future design of nanomedicines. Deng *et al.* demonstrated that the hard protein corona, formed on the surface of negative charge gold NPs induced unfolding of fibrinogen proteins, that were able to be recognized by cells though Mac 1 receptors, and induce activation of an inflammatory process.[213] Moreover, Dobrovolskaia *et al.* investigated the role of nanoparticle surface properties and incubation times on the protein coronas formation using human plasma. For that, a series of 30 nm citrate stabilized gold nanoparticles with various molecular weights PEG were used and compared with bare NPs. Interestingly, their results showed that protein composition was not affected by either incubation time or PEG length, but the total amount of proteins was governed by the molecular weight of PEG coating. Protein binding was decreased with increased PEG molecular weight. The main conclusion of the study emphasized that corona formation did not predict hemocompatibility of NPs.[214]

The goal of achieving therapeutic efficiency of siRNA in the target tissue still remains a challenge. siRNA requires targeted delivery to be able to interact with the RNA machinery within the cells by passing through a variety of barriers. Sahay *et al.* evaluated the delivery of siRNA using lipid nanoparticles made by complexation of siRNA with the cationic lipid C12-200. Notably, about 70% of the internalized siRNA underwent exocytosis from HeLa cells through way out of LNPs from late endosomes/lysosomes. This previously unrecognized pathway suggested that new approaches for retaining siRNA inside cells are needed.[215]

If we are able to understand and control these phenomena, in the near future, we may be able to design specific nanomaterials with desired intrinsic properties to be able to dictate the protein corona formation and harness it for various bio-medical applications.

In conclusion, the application of nanotechnology in medicine is under development and nanomedicine is expected to provide new keys for unsettled clinical problems, and for that reason exhaustive toxicology studies should be performed in order to design smart and safe nanomaterials.[75] Our studies have emphasized the importance of:

- Using primary human cells as a model system to closely mimic the *in vivo* situation
- Considering the effects of low doses of nanoparticles on cells
- Applying system biology approaches in nanotoxicology research
- Evaluating the effects of the protein corona on nanomaterials and how different psychochemical proprieties such as size, surface charge, nanomaterial composition and the biological fluid determine its composition and targeting capabilities
- Evaluating the intrinsic properties and the biological identity of NPs and their impact on biocompatibility, uptake, biodistribution and final fate of materials.

Overall, our studies contribute to the development of new nanomaterials and their applications, which may facilitate clinical translation of nanomedicines. However, further studies should be directed to assess both the intrinsic properties and the biological identity of NPs and their impact on biocompatibility, uptake, biodistribution and *in vivo* fate.

6 CONCLUSIONS

The following specific conclusions can be drawn from the presented studies (Paper I-IV).

Paper I

- A library of hydroxyl functionalized bis-MPA dendrimers was effectively synthetized and characterized
- Hydroxyl functionalized bis-MPA dendrimers present excellent biocompatibility in primary human monocyte-derived macrophages and cell lines, whereas the cationic, but not the neutral PAMAMs exerted dose-dependent cytotoxicity in HMDM
- Bis-MPA dendrimers are degradable materials and their stability increases at low pH
- Due to their excellent biocompatibility, the hydroxyl functionalized bis-MPA dendrimers are promising materials for biomedical applications

Paper II

- Biocompatibility studies showed that PAMAM-NH_2, but not PAMAM-OH elicited dose- and time-dependent cytotoxicity in PBECs
- PAMAMs are localized in the lysosomes but not in the nucleus and appear to be internalized through an active mechanism
- RNA-Seq analyses revealed changes of gene expression upon exposure of PBEC to non-acute cytotoxic doses of PAMAM-NH_2 dendrimers
- Gene ontology categories that are induced, are indicative of genes affecting cell cycle and division genes
- Upstream regulator analysis indicated modulation of NF-kappaB and p53 related targets
- Low doses of PAMAM-NH_2 affected cell cycle in PBEC, by arresting cells in S-phase in an NF-kappaB dependent manner
- Whole transcriptome sequencing using RNA-Seq and computation tools, enabled to predict the uncover mechanisms of cellular responses of non-acutely cytotoxic doses of PAMAMs to PBEC

Paper III

- Successful synthesis of novel ultra-small superparamagnetic iron oxide nanoparticles (USIRONs) was described by a simple one-step method
- USIRONs exhibit an average crystalline core size of 5.1 nm and superparamagnetic properties with a saturation magnetization value of 47 emu g^{-1}
- Phantom experiments revealed that the relaxivity value of USIRONs r_2/r_1 was 36.4, which is larger than clinically approved agents

- USIRONs are biocompatible materials as shown by the cytotoxicity studies in primary HMDM.
- USIRONs are promising candidates for future medical application such as negative contrast agents in MRI

Paper IV

- Effective synthesis of folic acid (FA)-conjugated iron oxide nanoparticles with two different intermediate coatings, inorganic silica (SiO_2) and organic polyethylene-glycol (PEG), using a variation of the well-known Stöber process was conducted
- Fe_3O_4-PEG-FA and Fe_3O_4-SiO_2-FA exhibit average sizes of 20 and 80 nm respectively
- Nanoparticles were shown to be non-cytotoxic to primary HMDM.
- Folate receptor alpha-mediated cellular uptake in ovarian cancer cell lines (SKOV-3) revealed that targeted uptake is only achieved for Fe_3O_4-SiO_2-FA nanoparticles in the presence of serum
- Due to their biocompatibility and targeting abilities, Fe_3O_4-SiO_2-FA nanoparticles are promising candidates for biomedical applications

7 FUTURE PERSPECTIVES

Advances in nanotechnology have given rise to the rapid development of novel applications in biomedicine, such as diagnostics, imaging and targeted drug delivery. However, their potential in biological scenarios is still not completely understood.

Nanotoxicology is an interdisciplinary science, which requires the interaction between different disciplines, including material sciences and toxicological sciences. Nanotoxicology will provide us references and guidelines to build and design safe nanomaterials with specific properties, which will be available to achieve the current targets or specific medical approaches. The integration and combination of the different scientific competences will be essential for the successful design of future nanomedicines.

We aimed to provide new knowledge about the interaction between engineered nanoparticles and primary human macrophages and cancer cells, in order to improve the design of these nanomaterials for biomedical applications. Novel, multi-functional nanomaterials for simultaneous drug delivery and imaging, using different imaging modalities are under development. Advances in this area will provide new knowledge of diseases such as cancer. Therefore, it is important to establish model systems for the evaluation of biological interactions and potential toxicological effects of such materials.

Today, a proper risk assessment of nanomaterials is lacking. In order to map and characterise mechanistic pathways and molecular changes upon exposure to nanomaterials to human and health, additional approaches are required. Systems biology approaches combined with classical toxicity endpoint could help to identify in a global perspective, possible harmful effects of nanomaterials. Indeed comprehensive systematic investigations are essential to understand how nanoparticles (with specific physicochemical properties) interact with biological structures. Further studies in this area are needed.

Indeed, we still do not completely understand the internalization process of nanoparticle in cells, and which are the relevant parameters that dictate the uptake mechanism and final fate in biological systems. Probably, they are governed by the intrinsic and the biological identity of nanomaterials. However, the exact factors are still undefined. If we could provide answers to this important question, we may be able to understand the efficacy of targeted delivery *versus* passive targeting. The effects of the protein corona on the targeting capabilities and toxicity response still remain an issue. Future studies should address the impact of the surface-adsorbed biomolecules of nanomaterials in biological systems. If we are able to understand this phenomenon, we may be able to design nanomaterial to modulate the "protein corona" formation. This fact may help us to design and obtain selective delivery of nanomedicines with high specificity.

Finally, omics approaches could be used as a tool to identify perturbations of cellular functions in response to nanomaterials, including responses not detected by conventional screening assays. New predictive nanotoxicology approaches, based on systems biology approaches may facilitate the identification of novel end-points of toxicity.

In this thesis, we presented a system biology approach based on whole transcriptome sequencing coupled with computational methods to reveal key pathways involved in cellular responses to PAMAMs. Our studies demonstrated the feasibility of applying transcriptomics approaches to understand the effects of nanoparticles on cells. Therefore, to define and understand the possible hazard of nanomaterial, follow up studies using this methodology will be of particular interest. This is in line with the 21st Century Toxicology Paradigm, which emphasizes predictive, mechanism-based toxicology.

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