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BIOCOMPATIBILITY OF SYNTHETIC NANOMATERIALS AND THEIR APPLICATIONS IN GENE DELIVERY

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Stockholm 2012

Cover: TEM image of cellular internalization of silica nanoparticles (photo: Kjell Hultenby).

Published by Karolinska Institutet.

ISBN 978-91-7457-861-4

Printed by

www.reproprint.se
Gårdsvägen 4, 169 70 Solna

To my family

ABSTRACT

Nanomedicine is the use of nanoscale or nanostructured materials in medicine that due to their structure have unique medical effects. Prominent applications of nanomedicine are the use of nanomaterials for the delivery of drugs and nucleic acids (to correct gene defects). Nanomaterials offer several attractive features as delivery vehicles: First, their size in the nano-regime endows them with more desirable pharmacokinetic and biodistribution profiles *in vivo*. Second, they are amenable to diverse chemical engineering that enables loading of a wide range of substances. Third, they can protect therapeutic agents from premature degradation or from inducing undesired side effects.

In this thesis, two types of synthetic nanomaterials, namely silica and polythiophene, were investigated for their biocompatibility and applications in gene delivery.

In Paper I, human red blood cell hemolysis and premyelocytic leukemia HL-60 cell cytotoxicity induced by silica nanoparticles with distinct physicochemical properties were studied, suggesting that silica nanoparticles potentially induce membrane permeability through a universal mechanism of action. Moreover, plasma protected against silica nanoparticle-induced membrane damage primarily by shielding the surface of silica particles.

In Paper II, the cytotoxicity and oxidative stress induced by amorphous silica nanoparticles were compared to nanoparticles with similar size but different chemical compositions. Overexpression of the liver phase II enzyme microsomal glutathione transferase 1 (MGST1) in human breast carcinoma MCF-7 cells reversed the cytotoxicity and oxidative stress induced by some silica nanoparticles but did not protect against the cytotoxic effects induced by zinc oxide nanoparticles.

In Paper III, amino-functionalized silica nanoparticles were used to deliver plasmid DNA (pDNA) into human breast carcinoma MCF-7 cells, with the nonporous particles delivering pDNA at higher efficiency than their mesoporous counterparts (with 2.4 nm pore diameter).

In Paper IV, polythiophene nanoparticles were used as vectors to deliver small interference RNA (siRNA) into human osteosarcoma U2-OS cells and human cervical carcinoma HeLa cells. The cationic polythiophenes were considerably more efficient delivery vectors than their zwitteronic counterparts.

In conclusion, studies to improve the understanding of the biocompatibility and delivery efficiency of nanomaterials, are crucial to assist the rationale design of nanomaterials for delivery applications.

LIST OF PUBLICATIONS

I. Shi J, Hedberg Y, Lundin M, Odnevall Wallinder I, Karlsson HL, Möller L. The hemolytic properties of synthetic nano- and porous- silica particles: the effect of surface properties and the protection by the plasma corona. Acta Biomateriala, 2012; 8: 3478-90.

II. Shi J, Karlsson HL, Johansson K, Gogvadze V, Xiao L, Li J, Burks T, Garcia-Bennett A, Uheida A, Muhammed M, Mathur S, Morgenstern R, Kagan VE, Fadeel B. Microsomal glutathione transferase 1 protects against toxicity induced by silica nanoparticles but not by zinc oxide nanoparticles. ACS Nano, 2012; 6(3): 1925-38.

III. Shi J, Rhode Y, Ersson C, Geny S, Ye F, Muhammed M, Smith CIE, Möller L. Amino-modified nonporous and mesoporous silica nanoparticles as non-viral vectors for the delivery of plasmid DNA. *Manuscript,* 2012.

IV. Lundin P, Viola JR, Moreno PMD, Shi J, Zaghloul EM, Möller L, Smith CIE, El-Andaloussi S. Delivery of small interfering RNA (siRNA) using an amino acid-modified polythiophene. *Submitted,* 2012.

Additional publications:

1. Kagan VE, Konduru NV, Feng W, Allen BL, Conroy J, Volkov Y, Vlasova II, Belikova NA, Yanamala N, Kapralov A, Tyurina YY, Shi J, Kisin ER, Murray AR, Franks J, Stolz D, Gou P, Klein-Seetharaman J, Fadeel B, Star A, Shvedova AA. Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. Nature Nanotechnology, 2010; 5(5): 354-9.

2. Kagan VE, Shi J, Feng W, Shvedova AA, Fadeel B. Fantastic voyage and opportunities of engineered nanomaterials: what are the potential risks of occupational exposures? Journal of Occupational Environmental Medicine, 2010; 52(9): 943-6.

3. Vogt C, Toprak MS, Shi J, Torres NF, Fadeel B, Laurent S, Bridot JL, Müller RN, Muhammed M. Optimised synthetic route for tuneable shell SiO_2@Fe_3O_4 core-shell nanoparticles, in advances in material design for regenerative medicine, drug delivery, and targeting/imaging. Materials Research Society Symposium Proceedings, 2009; 1140: 209-14.

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LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
BET	Bruner, Emmett and Teller method
BSA	Bovine serum albumin
CD	Circular dichroism
CDNB	1-chloro-2,4-dinitrobenzene
CFE	Colony formation efficiency
CLSM	Confocal laser scanning microscopy
CPP	Cell penetrating peptide
CQ	Chloroquine
DCFH-DA	Dichlorofluorescein diacetate
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EPR	Enhanced permeability and retention effect
FACS	Flow cytometry
FITC	Fluorescein isothiocyanate
FPG	Formamidopyrimidine DNA- glycosylase
GFP	Green fluorescent protein
GSH	Glutathione
GST	Glutathione transferase
HaCaTa	Human keratinocytes
HeLa	Human cervical carcinoma cells
HL-60	Human promyelocytic leukemia cells
H_2O_2	Hydrogen peroxide
ICP	Inductively coupled plasma
ICP-MS	Inductively coupled plasma-mass spectrometry
L-02	Human hepatic cells
LAL	Limulus amebocyte lysate
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases

MCF-7	Human breast carcinoma cells
MGST1	Microsomal glutathione transferase 1
mRNA	Messenger RNA
MRP	Multidrug resistance proteins
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NF-κB	Nuclear factor kappa B
NPC	Nuclear pore complex
Nrf2	Nuclear factor like 2
NTA	Nanoparticle tracking analysis
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PEI	Polyethyleneimine
POMT	Poly(3-[(*S*)-5-amino-5-methoxycarboxyl-3-oxapentyl]-2,5-thiophenylene) hydrochloride
POWT	Poly(3-[(S)-5-amino-5-carboxyl-3-oxapentyl]-2,5-thiophene) hydrochloride
RES	Reticuloendothelial system
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
siRNA	Small (short) interfering RNA
TEM	Transmission electron microscopy
TNF	Tumor necrosis factor
TMRE	Tetramethylrhodamine ethyl ester
U2-OS	Human osteosarcoma cells
XPS	X-ray photon electron spectroscopy
XRD	X-ray diffraction

1 NANOMEDICINE: A BRIEF INTRODUCTION

'Nano-' is a prefix derived from the Greek 'νᾶνος' signifying 'dwarf', and refers to a billionth (10^{-9}) in the metric system. Nanomedicine is the use of nanoscale or nanostructured materials in medicine that, due to their size or structure, have unique medical effects [1]. The field of nanomedicine is therefore highly multidisciplinary in nature, integrating knowledge from nanosciences to medical sciences [2].

One of the most prominent applications of nanomaterials in biomedicine is their use for delivery of pharmaceutical agents such as drugs and nucleic acids into the human body. In the year 2003 alone, drug delivery systems accounted for 59% of more than 2,000 patent filings in the arena of nanomedicine [1]. In april 2006, *Nature Materials* estimated that 130 nanotechnology based delivery systems were being developed worldwide [3]. Some examples of nanomaterial-based delivery that already exist on the market are shown in Table 1. Nanomaterials offer several advantages as delivery vectors. First, their small size *per se* allows them to escape the recognition and clearance by the reticuloendothelial system (RES) and to cross biological barriers. This endows them with the capability to alter the pharmacokinetic and biodistribution profiles of therapeutic agents *in vivo* [4]. A certain size range of nanomaterials is also particularly useful since it allows passive accumulation of nanomaterials in tumors by exploiting the characteristic large vasculature and defective lymphatic drainage of tumor tissues, an effect termed enhanced permeation and retention (EPR) [5]. Second, their chemical versatility makes them suitable for loading a wide range of substances enabling multifunctionality [6] (Figure 1). For instance, nanomaterials can be engineered for both diagnostic and therapeutic purposes, holding great promises for personalized medicine [7]. Moreover, appropriate designs can be made to achieve specific functionalities such as active targeting of cells as well as controlled release of therapeutic cargo upon the stimuli of choice (e.g. thermal, pH, enzymatic, photochemical triggered processes), in order to protect therapeutic agents from undesired interactions with the body and maximize their bioavailability at specific target sites over a period of time [8]. In summary, the primary driving forces for nanomaterial-based delivery to meet medical needs are: (a) the ability to improve pharmacokinetic and biodistribution profiles, (b) the amenability to diverse chemical engineering, and (c) the protection of therapeutic agents from undesired reactions.

Table 1. Examples of nanomaterial therapeutics on the market (nanomaterials used for the delivery of pharmaceutical agents) [1].

Therapeutic Agent	Nanomaterial Formulation	Company	Indication
Ambisome	Liposomal Amphotericin B	Gilead, Fujisawa	Fungal infections
Doxil/Caelyx	Liposomal	Ortho Biotech,	Cancer, Kaposi

	doxorubicin	Schering-Plough	sarcoma
Visudyne	Liposomal verteporfin	QLT, Novartis	Age-related macular degeneration
Copaxone	Copolymer of alanine, lysine, glutamic acid and tyrosine	TEVA Pharmaceuticals	Multiple sclerosis
Renagel	Crosslinked poly(allylamine) resin	Genzyme	Chronic kidney disease
Emend	Nanocrystalline aprepitant	Elan Drug Delivery	Antiemetic
Rapamune	Nanocrystalline sirolimus	Elan Drug Delivery	Immuno-suppressant
Triglide	Nanocrystalline fenofibrate	SkyePharma	Lipid regulation
Abraxane	Paclitaxel protein bound nanoparticles	Abraxis BioSciences, AstraZeneca	Cancer

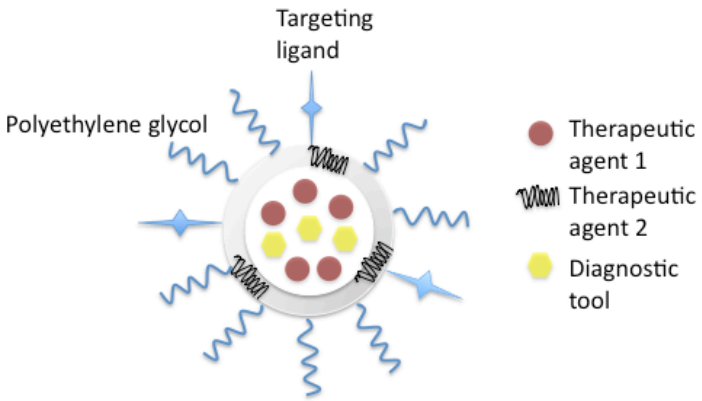


Figure 1. Multifunctional nanoparticles [6].

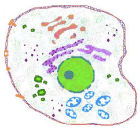
Moreover, a wide range of other biomedical applications of nanomaterials include *in vivo* imaging and diagnostics, regenerative medicine, infection biology, neuroelectronics, biosensors and so on [1, 9-11]. Many of which make use of properties of materials that differ on the nanoscale (as compared to bulk materials of the same composition) owing to surface chemistry and/or quantum effects, giving rise to novel optical, electric, and magnetic properties [1].

This thesis focuses on the biomedical applications of nanomaterials for gene delivery.

2 NANOMATERIALS AND THEIR PHYSICOCHEMICAL PROPERTIES

An introduction to the world of nanosciences started with Richard Feynman's classic talk in 1959 ″There's plenty of room at the bottom – an invitation to enter a new field of physics″ [12]. Nanomaterials are generally defined as materials in size ranging from 1 to 100 nm at least in one dimension, although it has been pointed out that novel size-dependent properties rather than arbitrary size thresholds is a more appropriate definition in some contexts [13]. Therefore the broad definition of nanomaterials encompasses materials from a few nanometers to several micrometers in size. Nanoparticles have all three dimensions in this scale, whereas nanotubes have two dimensions and nanosurfaces have one dimension in this scale. Importantly, nanomaterials can be in the same size range as elements of living cells, including subcellular organelles and biomacromolecules (proteins, lipids, nucleic acids) (Table 2).

Table 2. Nanomaterials are in the same size range as elements of living cells.

	A water molecule is around 0.1 nm in width and length.
	A glucose molecule has a diameter around 1 nm.
	The DNA double helix has a width around 2 nm and one nucleotide unit measures 0.33 nm long [14].
	An antibody is around 10-20 nm in diameter [15].
	Cellular structure and intracellular organelles: the thickness of cell membranes is around 7 nm [16], and the diameter of the nuclear pore is around 50 nm [17]; the nucleus is around 3-10 μm, the mitochondrion 3 μm, and the endosome 200-500 nm in diameter.
	Cells: A typical human red blood cell has a disk diameter of 7-8 μm, a human macrophage is about 20 μm in diameter, and a human egg about 100 μm in diameter.

Synthetic nanomaterials include several important classes of nanomaterials, such as carbon nanotubes, metal nanoparticles, oxide nanoparticles, quantum dots, polymers and liposomes [18]. They can be further engineered to derive a large pool of derivatives. Synthetic nanomaterials have wide applications in nanotechnology and nanomedicine. This thesis focuses on two categories of synthetic nanomaterials: silica nanomaterials and polythiophenes.

2.1 SILICA NANOMATERIALS

Silica is one of the most abundant materials on earth, and occurs in its natural form as quarts sand, rocks, and clays. These primary raw materials are chemically treated to produce direct silica sources, such as sodium silicate, silicon tetrachloride, and alkoxysilane. These are in turn used to produce synthetic silica products, such as silica gel, precipitated silica, silica sol/colloidal silica, and fumed silica [19]. Moreover, the silica surface is populated with Si-OH groups known as silanol groups (some of these silanol groups ionize to $Si\text{-}O^-$ upon contact with water), which can be used to functionalize the surface with a variety of desired modifications [20]. Synthetic and engineered silica nanomaterials have numerous applications in various areas such as electronics, sensor technologies, coatings and additives, and are also of considerable interests for diagnostic and therapeutic applications in medicine [19]. Due to their chemical properties and biocompatibility, they are also commonly applied as surface coatings to other functional materials [21].

Mesoporous silica nanomaterials, a type of silica materials exhibiting porous structures on the mesoscopic scale (2-50 nm), offer attractive properties for loading and releasing large quantities of biomedical agents such as drugs, genes and proteins [22, 23]. Figure 2 shows the mesoporous structures of these materials by means of transmission electron microscopy (TEM). Mesoporous structures are typically synthesized by introducing self-assembling micellar templates to a sol-gel synthesis of silica [24]. The organic micellar templates (e.g. amphiphilic surfactants) can self-assemble into different structures (cubic, hexagonal, cylindrical) and are removed by thermal calcination or solvent extraction after synthesis of silica species, revealing the mesoscale pores supported by a silica wall [24-26]. This results in materials with very high surface area (> 1000 m^2/g) that is advantageous for accommodating large amounts of therapeutic load.

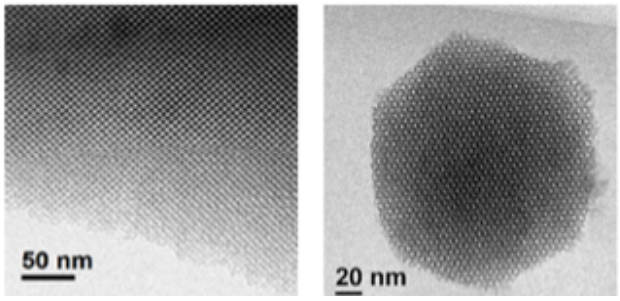


Figure 2. TEM images of common mesoporous structures. *Courtesy: Dr. Alfonso Garcia-Bennett.*

2.2 POLYTHIOPHENES

Polythiophenes constitute an interesting class of synthetic polymer materials, resulting from the polymerization of thiophenes (Figure 3). They can be synthesized chemically or electrochemically [27, 28]. Synthetic polymers have traditionally been regarded as poor

electronic conductors and are often used as insulators. However, polymers can be made electrically conductive when electrons are added or removed from the conjugated π-orbitals via a process called doping. The discovery of conductive polymers was awarded the Nobel Prize in chemistry in 2000 [29]. Moreover, conductivity is not the only interesting property resulting from electron delocalization, the same mechanism also confer optical properties. Polythiophenes are utilized for a number of applications such as conductive films, electrochemical transistors, as well as diagnostic and imaging tools [30, 31].

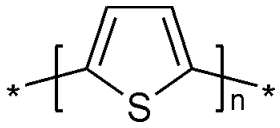


Figure 3. The chemical structure of polythiophenes.

2.3 PHYSICOCHEMICAL PROPERTIES OF NANOMATERIALS IN RELATION TO THEIR PHARMACOKINETIC PROFILES

The behavior of nanomaterials *in vivo* is the result of a combination of many different factors, including their size, surface charge, porosity, shape, mechanical flexibility, biodegradability, and so on. Therefore, the following discussion is only of reference and not of absolute term.

Size.

Particle size has a significant impact on their blood circulation time [32]. Very small particles (< 10 nm) are quickly excreted through the kidneys whereas large particles (> 200 nm) are easily recognized and cleared by phagocytes of the RES. The optimal particle size for intravenous therapeutics is suggested to be around 100 nm owing to their extended blood circulation time, whereas the upper limit would be around 1.5 μm since larger particles are expected to clog capillaries [33]. Due to the EPR effect of tumor tissues, particles ranging from 100-200 nm in size accumulate more readily in solid tumors [34]. Smaller particles are more prone to cross biological barriers, and it has been shown that particles less than 11.7 nm have the potential to cross the tight junctions of the blood brain barrier in rodents [35]. The excretion of injected particles were also shown to be size-dependent, with the 50 nm particles excreting faster than 100 and 200 nm fluorescence labelled silica particles via the urine and bile [36].

Surface charge/hydrophobicity.

Surface charge is an important factor that affects the behavior of nanoparticles. Generally, the RES has better clearance of positively charged particles than negatively charged particles, with neutrally charged particles being the least affected and therefore having the longest blood circulation time [33]. Hydrophobic particles tend to have more

interactions with proteins and cells than their hydrophilic counterparts. A hydrophilic polymer extended surface such as polyethylene glycol (PEG) is therefore often used to shield nanoparticles from immune responses [33].

Porosity.

Materials with pore diameters less than 2 nm are termed microporous, with pore diameters between 2-50 nm are termed mesoporous, and with pore diameters larger than 50 nm are termed macroporous. Mesoporous materials are most useful for biomedical applications since a large proportion of therapeutic agents are within this size range. Porous materials have a significantly higher total surface area but a lower external surface area than their nonporous counterparts, potentially affecting their interactions with biological systems [37]. Intravenous injections in immune-competent mice showed that mesoporous silica nanoparticles exhibited a higher accumulation in the lung than nonporous silica nanoparticles of similar size. These mesoporous nanoparticles were transiently associated with the lung and then redistributed out of this organ without significant internalization [38].

Shape.

Shape also plays a significant role for the biological behaviors of nanomaterials. Particles with different shapes experience distinct hydrodynamic forces in the blood flow. Non-spherical particles (compared to spherical particles) have a higher tendency to move towards the blood vessel walls, referred to as margination effect [33]. Shape is also important during the filtration process through the spleen and kidney, as well as during phagocytosis [33].

Mechanical flexibility.

The rigidity of particles can influence their ability to pass through blood vessels, as well as through the filters of the spleen and kidney. Rigid particles are also taken up to a higher extent by macrophages compared to their soft and flexible counterparts [33].

Biodegradability.

Similar to the size-dependency, nanomaterials biodegraded into small molecular weight components exert different pharmacokinetic profiles. For example, silicic acid, the dissolution product of silica (at high pH), can be efficiently excreted from the human body through urine [39].

3 BIOCOMPATIBILITY ASSESSMENT

It is of vital importance to assess the biocompatibility of nanomaterials before they can be used for medical applications. Moreover, understanding the relationship between the physicochemical properties and the biocompatibility/toxicity of nanomaterials will further assist the rationale design of these materials with improved biocompatibility.

3.1 TARGET ORGAN BIOCOMPATIBILITY/TOXICITY

Common routes of administration for nanomaterial-based delivery systems are through systemic injection, inhalation, or oral absorption. Systemic injection results in direct exposure to the circulation system, whereas inhalation or orally administered agents may also end up in the blood stream owing to the ability of nanoparticles to cross biological barriers [40]. Indeed, it has been shown that following inhalation, nanoparticles are capable of crossing the alveolar-capillary barrier and entering the bloodstream, especially in the presence of inflammation as it increases the permeability of the endothelium [41]. Similarly, nanoparticles can enter the circulation and subsequently be distributed to other tissues/organs following gastrointestinal absorption [42]. Therefore it is of primary importance to understand the blood biocompatibility (red blood cell hemolysis, blood coagulation, interactions with white blood cells, serum biochemistry) of nanomaterials.

In studies with mice, it was shown that nanoparticles are taken up extensively by the liver and spleen, where they are passively entrapped in the fenestrations of the endothelium of these organs [38]. Physical sequestration accumulates particles in these organs, such as liver, the powerhouse of biotransformation and immune clearance [43]. Enzymatic reactions (e.g. Phase I and II) in the liver may result in detoxification or aggravated hepatoxicity [43]. Therefore, the impact of liver enzymes constitutes an interesting aspect for the biocompatibility/toxicity investigations of nanomaterials as drug and gene delivery systems.

For a more comprehensive assessment of the biocompatibility/toxicity of nanomaterials, please refer to an excellent review by Zhao and Castranova [44].

3.2 MECHANISMS OF POTENTIAL CYTOTOXIC EFFECTS

As the saying goes, ‘the dose makes the poison’. In biocompatibility/toxicity evaluations, it is important to investigate dose-response relationships as well as high dose scenarios where toxic responses are revealed, as these can be used to determine appropriate dosages and acceptable limits [45]. It is also important to keep in mind that, the same substance may have different mechanisms of action depending on the magnitude of the exposure [46].

The imbalance between reactive oxygen species (ROS) and antioxidant defense termed oxidative stress has been proposed to be the dominant paradigm for potential nanoparticle-induced toxicity at the cellular level [47], although not all studies confirm this general notion [48]. ROS can be generated from the reactive surface of some nanoparticles (e.g. the photocatalytic properties of TiO_2 nanoparticles) [49], the mitochondria as the main intracellular ROS generating source of eukaryotic organisms, and/or the multi-component enzyme NADPH oxidase as the main ROS generating machinery of phagocytic cells against foreign invaders [50]. Depending on the level of oxidative stress, cellular responses may vary from adaptation and damage repair to senescence and cell death (Figure 4). At low levels of oxidative stress, the cell or organism adapts by up-regulating their defence systems. Increased levels of oxidative stress may switch mitotic cells into senescence cells that can survive for longer periods. Failure to cope with such oxidative stress may cause cells to die through apoptosis, which protects surrounding tissues from further damage. Under more severe conditions of oxidative stress, cells may undergo necrotic cell death exposing surrounding tissues to further inflammatory responses [46]. Important pathways involved in the regulation of oxidative stress include mitogen-activated protein kinases (MAPK), nuclear factor kappa B (NF-κB), and nuclear factor like 2 (Nrf2) signalling pathways. Lipids, proteins and DNA are primary cellular targets of oxidative stress. Furthermore, damages from oxidative stress have implications in aging, cardiovascular diseases, neurodegenerative diseases, cancer, and so on [51].

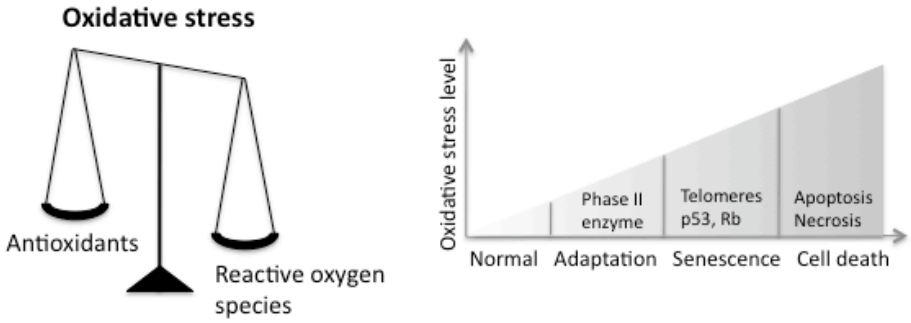


Figure 4. The hierarchical model of oxidative stress. Adapted from Meng et al., 2009 [52].

On the other hand, other mechanisms of nanoparticle-induced toxicity such as nanoparticle-induced inflammation and/or genotoxicity have also been observed [53, 54]. The mechanisms of nanoparticle-induced oxidative stress, and whether oxidative stress is the primary cause of cellular destruction or rather stem from the injury triggered by other mechanisms, remain to be interesting areas of investigation [55].

3.3 BIOLOGICAL DEFENSE SYSTEMS

Evolutionary forces have shaped biological systems with a multitude of physical and chemical defense systems. Here, two of these defense systems, blood plasma/serum and liver enzymes, are highlighted.

3.3.1 Blood plasma/serum

Blood plasma is the blood fluid that holds blood cells in suspension. It contributes to about 55% of the total volume of whole blood. Blood serum is blood plasma without clotting factors such as fibrinogen. Blood plasma/serum contains glucose, electrolytes, hormones, antigens, and thousands of different proteins (whose abundance varies by twelve orders of magnitude [56]), many of which serve important functions to defend the body against potential dangers. For example, metallothioneins sequester heavy metals through their cysteine residues [57]; albumins exhibit important antioxidant properties [58].

This thesis focuses on the so-called plasma/serum 'corona' over particle surfaces. Upon contact with biological fluids such as blood plasma/serum, particles are immediately coated by the adsorption of biomolecules such as proteins and lipids, forming a 'corona' over the particle surface [59]. The biological corona has been suggested to be determined by the size and surface properties of the original particle surface [60]. A quantitative approach to characterize surface adsorption energy included parameters such as hydrophobicity, hydrogen bonds, polarity/polarizability, and lone-pair electrons, to simulate the interaction forces of nanomaterials in biological systems [61]. Studies have also shown that the corona can be loosely divided into two components: a long-lived 'hard' corona, with a durable coating of high affinity proteins bound for at least a few hours over the bare nanoparticle surface; and a short-lived 'soft' corona with typically short exchange times and loosely bound proteins [56, 60]. Examples of the hard corona proteins include albumin, apolipoproteins, glycoproteins, plasminogens, fibrinogens, and complement factors [56]. The protein corona is a dynamic phenomenon: proteins in the corona not only exchange with proteins in the biological fluids in a static environment [59]; the protein corona also evolves when particles navigate in the body and pass from one biological fluid to another [62]. The surface of nanoparticles is therefore modified by a dynamic layer of biological factors, which affect their recognition, behavior, and toxicity [63].

3.3.2 Liver enzymes

Liver is the most important organ for the detoxification of xenobiotics by enzymes. Phase I enzymes, particularly cytochrome P450, catalyze the oxidative and reductive reactions of xenobiotics. Many products of phase I reactions then become substrates of phase II enzymes, which catalyze conjugation reactions to convert their substrates into more polar products in order to facilitate their excretion through the urine and bile [43].

Microsomal glutathione transferase 1 (MGST1), a phase II enzyme extensively studied for its ability to detoxify substances of both endogenous and exogenous origin, is highlighted here [64]. In cells, it is primarily located in the endoplasmic reticulum and the outer mitochondrial membranes [65]. The structure of MGST1 is a homotrimer, each subunit with a molecular weight of 17.3kDa and a binding site for glutathione (GSH) [66]. Its active site is located at the residue cysteine 49, where covalent binding to GSH induces conformational changes and thereby activates the enzyme [67]. MGST1 has

broad substrate specificity, as the enzyme has been shown to be activated by N-ethylmaleimide (NEM) [67], trypsin [68], radiation [69], heat [70], and oxidative stress [71, 72]. MGST1 displays both glutathione transferase and glutathione peroxidase activities. Using its glutathione transferase activity, MGST1 catalyzes the conjugation of GSH to its electrophilic hydrophobic substrate and converts it into more polar metabolites [64]. The reaction is the first out of four steps in the mercapturic acid pathway [73]. These GSH-conjugates are then transported out of the cells via transmembrane multidrug resistance proteins (MRP) and subsequently excreted out of the body [74]. Using its glutathione peroxidase activity, MGST1 catalyzes the GSH dependent reduction of lipophilic hydroperoxides and lipid hydroperoxides [67, 75, 76] (Equation 1).

Glutathione peroxidase activity: $2GSH + ROOH \rightarrow GSSG + ROH + H_2O$ (1)

The glutathione peroxidase activity of MGST1 plays an important role in the context of oxidative stress. MGST1 has been shown to be activated by oxidative stress both on the transcriptional level as well as by protein modification [64]. It can protect cells against lipid peroxidation by displaying its glutathione peroxidase activity towards lipid hydroperoxides and lipid ozonides [75, 76]. It can also protect against downstream products of lipid peroxidation by conjugation of their toxic end products, e.g 4-hydroxyalk-2-enals [76]. Indeed, MGST1 has been shown to protect against injury from oxidative stress in HEK293 cells [77], MCF7 cells [78, 79], and retinal pigment epithelium [77]. Interestingly, an increase in the expression of MGST1 has been observed with aging [80], chronic obstructive pulmonary disease [81], and various tumors [82-86], all of which appear to be associated with increased oxidative stress. Up-regulation of MGST1 mRNA and protein synthesis has been suggested to be an early stage biomarker of various diseases associated with oxidative stress [86-88].

4 GENE DELIVERY

Gene therapy is the therapeutic approach aiming at the permanent, or transient, correction of a gene defect by intracellular delivery of nucleic acids. Major therapeutic targets for gene therapy include cancer, monogenic hereditary diseases, infectious diseases and respiratory diseases [89-93]. However, delivery issues remain one of the most important bottlenecks in the development of gene therapy [93]. Gene vaccination is another application of gene delivery, where the introduction of antigen encoding genes into target cells triggers cellular and humoral (antibody) immune responses [94].

4.1 GENE DELIVERY VECTORS

Initial delivery of genes exploits the natural mechanisms of viruses as delivery vehicles. Despite the higher delivery efficiency of viral vectors, they often suffer from toxicity and immunogenicity-related issues [95]. Non-viral vectors are emerging as safer alternatives to viral vectors. Major research efforts are directed towards understanding the mechanisms associated with the enhancement of gene delivery efficiency [95, 96] as well as the development of safe and efficient novel gene delivery vectors [97].

Classical non-viral vectors include lipids, cationic polymers and cell penetrating peptides, whereas more recent applications explore the use of nanomaterials such as silica nanoparticles, gold nanoparticles, magnetic nanoparticles, and carbon nanotubes for gene delivery [93, 98]. Endogenous nano-size vesicles, so called exosomes, have also been explored as delivery vehicles [99]. Moreover, combined approaches are being investigated in the pursuit of multifunctional platforms to improve their performance in targeting and efficiency [100, 101].

In general, delivery vectors shall be able to carry out the following steps: (1) form stable complexes with nucleic acids, (2) enter target cells by endocytosis-mediated uptake, (3) escape the endosomes to reach the cells' cytoplasm, (4) in certain cases, such as delivery of DNA, the complexes or the released nucleic acids enter the cells' nucleus, and (5) execute targeted and efficient gene regulation [29]. These are depicted in Figure 5 and explained in more details in the following sections.

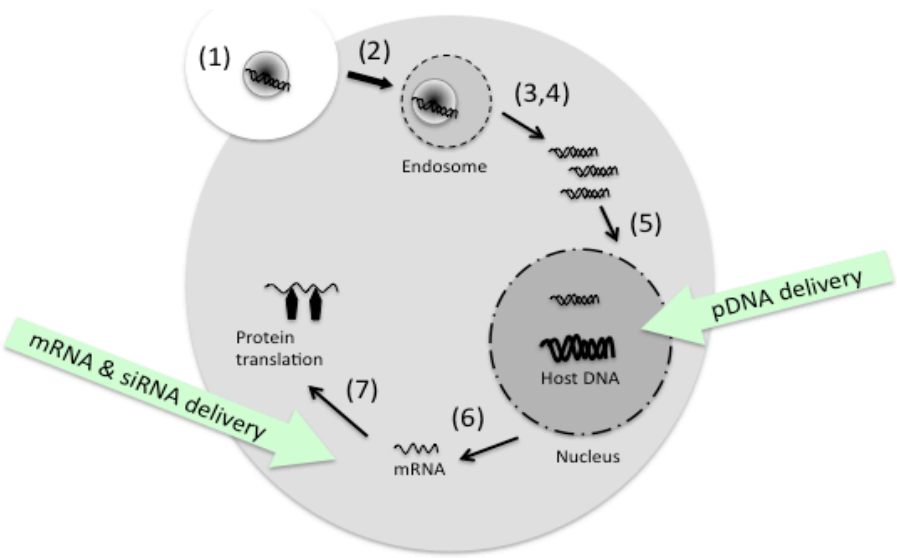


Figure 5. Critical steps in gene delivery: (1) formation of stable complexes between the delivery vectors and oligonucleotides; (2) endocytosis mediated uptake of the stable complexes; (3) endosomal escape; (4) oligonucleotides release; (5) nuclear uptake of oligonucleotides and their subsequent replication with host DNA; (6) transcription to mRNA; (7) translation to protein.

4.2 INTRACELLULAR UPTAKE AND TRAFFICKING

Complex formation.

Gene delivery vectors often bear cationic charges to enable electrostatic interactions with anionic nucleic acids. Following interactions with the vectors, the extended nucleic acids are reversibly converted into compact particles, known as nucleic acid condensation [102]. Thermodynamic analysis suggested that multivalent cations present on the vector associate with the anionic nucleic acid phosphate groups, which causes local bending of the nucleic acid (forming rods and toroid-like structures) and results in a reduction of entropy [103]. This process often makes nucleic acids more stable and resistant towards degradation by serum nucleases [104].

Endocytosis.

Endocytosis refers to the cellular uptake of macromolecules and solutes into membrane-bound vesicles derived by the invagination and pinching off of pieces of the cell plasma membrane. In non-phagocytic cells, there are at least four different pathways: clathrin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, and clathrin/caveolin-independent endocytosis. They differ in the

composition and size of the membrane vesicle, as well as the fate of the internalized particles. Most of these pathways can involve receptor-ligand interactions. [105].

The 'trojan horse' of delivery.

For cationic lipid-based vehicles, nucleic acids are thought to be released from endosomes into the cytoplasm through exchange and fusion between lipids in the liposome and the endosomal membrane [106]. A different model has been proposed for the endosomal escape of cationic polymer-based vehicles, such as polyethylenimine (PEI). According to the 'proton sponge hypothesis', the unsaturated amino groups on these vectors sequester protons, and protons are therefore continuously pumped into the endosome promoting passive entry of chloride ions and subsequent osmotic swelling and endosome rupture [107]. Disassembly of nucleic acids from the delivery vectors can occur after endosomal release in the cytosol or in the nucleus [108-110]. For novel vectors, such as inorganic nanoparticles, the mechanisms of cellular uptake and endosomal escape are important areas of investigation.

Nuclear transport.

The transport of certain vector-nucleic acid complexes or released nucleic acids across the nuclear envelope occurs through the nuclear pores. While very small particles can freely diffuse through the nuclear pore, larger molecules enter the nucleus through a nuclear pore complex (NPC) that can be enlarged to about 55 nm in diameter [17]. Studies indicate that DNA can traverse the NPC by itself in a process driven by nucleotide triphosphate hydrolysis and/or energy released upon binding to nuclear components [111, 112]. Moreover, nuclear localization signals are common strategies used to facilitate nuclear delivery [113].

4.3 GENE REGULATION

Gene regulation is the process that cells and viruses use to regulate the expression of genes into gene products. The regulation of gene expression by exogenous delivery of nucleic acids includes plasmid DNA (pDNA), small interference RNA (siRNA), antisense oligonucleotides, splice correction oligonucleotides, and so on. Delivery of pDNA and siRNA represents two complementary approaches to restore or silence a specific cellular function [114]. The completion of the human genome sequencing in 2001 [115, 116], enabled groundbreaking progress for gene regulation.

Circular double-stranded pDNA molecules are to be introduced into the cell nucleus. Besides the therapeutic gene(s), pDNA may also contain other sequences such as promoter/enhancer elements. For example, tissue-specific promoter sequences can be used to restrict the gene expression to specific target tissues [117].

Double-stranded RNA sequences of 21-24 nucleotides, known as siRNA, are introduced into the cell cytoplasm to allow sequence-specific gene silencing. In the

cytosol, siRNA binds to a protein complex termed the RNA-induced silencing complex (RISC), which mediates the unwinding of the siRNA duplex to bind to the target mRNA [118].

Luciferase and green fluorescent protein (GFP) are often used as reporter genes for the proof of principle of gene regulation due to their sensitivity and ease of detection. However, restoring or silencing of functional genes is the main purpose of gene therapy. The major types of genes targeted in gene therapy clinical trials are listed in Table 3.

Table 3. Types of genes regulated in gene therapy clinical trials in 2007 [119].

Gene types	Example	Percentage	Number
Antigen	ALVAC-HIV	20.3%	266
Cytokine	IL-2	18.9%	247
Tumor suppressor	p53	12%	157
Growth factor	GM-CSF	8.2%	107
Suicide	Survivin-T34A	8.2%	107
Deficiency	SCID-X1	7.9%	103
Receptor	TCR	5.1%	67
Marker	CD4+	4.1%	54
Replication inhibitor	Ribozyme	3.7%	48
Other	P-glycoprotein	11.5%	153

5 PRESENT INVESTIGATIONS

5.1 AIMS OF THE THESIS

The overall objective of this thesis is to investigate the biocompatibility of synthetic nanomaterials of medical relevance and to explore their applications in gene delivery. The specific aims in papers I-IV are:

- I: to study the blood cell toxicity/biocompatibility of silica nanoparticles, as well as plasma protection mechanisms;

- II: to study the cytotoxicity/biocompatibility and oxidative stress induced by synthetic nanoparticles, as well as protection mechanisms by the liver phase II detoxification enzyme MGST1;

- III: to explore the applications of amino-modified silica nanoparticles as vectors for the delivery of pDNA;

- IV: to explore the applications of amino acid-modified polythiophenes as vectors for the delivery of siRNA.

5.2 METHODOLOGY

Cell models and methods used in papers I-IV are described in detail in the respective '*Materials and methods*' sections. Below follows an overview of each cell model and method with references to the paper(s) in which they are used:

Cell models:

In Paper I, red blood cells freshly isolated from human volunteers and HL-60 human promyelocytic leukemia cells were used to study the ability of silica nanoparticles to induce permeability in biological membranes (hemolysis and cytotoxicity).

In Paper II, MCF-7 human breast carcinoma cells, with and without overexpression of rat MGST1, were used as model systems to investigate whether MGST1 could protect against the cytotoxicity of SiO_2, TiO_2, CeO_2, and ZnO nanoparticles. Human breast cells rather than hepatocytes were used, because these MCF-7 human breast carcinoma cells have low expression of MGST1 as well as cytosolic glutathione transferases (GSTs). The sense cells were stably transfected with a vector that contains rat MGST1, and the antisense cells with the antisense orientation of rat MGST1. The overexpression level of rat MGST1 in sense cells is ten times less than the expression level in the liver [120].

In Paper III, MCF-7 human breast carcinoma cells were used to investigate the cellular delivery of pDNA using silica nanoparticles as vectors.

In Paper IV, the human osteosarcoma cells U2-OS, either wild-type or stably transfected with a luciferase-encoding plasmid, were used to investigate the cellular delivery of luciferase siRNA using polythiophenes as vectors. Human cervical carcinoma HeLa cells were used for live-cell fluorescence microscopy, as the U2-OS cells contain a GFP construct that could interfere with the absorption and fluorescence emission of poly(3-[(*S*)-5-amino-5-methoxycarboxyl-3-oxapentyl]-2,5-thiophenylene hydrochloride) (POMT).

Methods:

Bicinchoninic acid (BCA) protein assay [I,II]	A biochemical assay for determining the concentration of protein in solution.
Brunauer, Emmet and Teller method (BET) [I]	Calculates the surface areas of solids by physical adsorption of gas molecules.
C_{11}-BODIPY$^{581/591}$ [II]	A fluorescent probe of lipid peroxidation.

Circular dichroism (CD) spectroscopy [IV]	The measurement of differential absorption of circularly polarized light exhibits optically active chiral molecules.
1-chloro-2,4-dinitrobenzene (CDNB) assay [II]	A spectrophotometric assay to measure GST activity.
Chloroquine (CQ) [IV]	Leads to swelling and bursting of endosomes.
Colony formation efficiency (CFE) assay [II,III]	Measures cell colonies as index of long-term viability or proliferation ability.
Confocal laser scanning microscopy (CLSM) [IV]	Optical imaging technique enabling scanning through cells.
Dichlorofluorescein diacetate (DCFH-DA) [I,II]	A fluorescence probe that measures hydrogen peroxide (H_2O_2) production.
3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [II,III]	A colorimetric assay that measures the activity of a mitochondrial enzyme which is crucial for cell viability.
Dynamic light scattering (DLS) [I,II,III]	Measures the size distribution of small particles in suspension by means of light scattering.
Fluorescein isothiocyanate (FITC) labelling of nanoparticles [II]	The conjugation of fluorochrome for tracking of nanoparticles.
Formamidopyrimidine DNA-glycosylase (FPG)-comet assay [II]	Detection of oxidative DNA damage using a gel electrophoresis based assay.
Flow cytometry (FACS) [II]	Laser based analysis of cells in flow using fluorochrome conjugated antibodies.
Fluorescence microscopy analysis [II]	Optical microscope that uses fluorescence to generate images.
Gel retardation assay [III]	Affinity electrophoresis to study nucleic acid interactions with other substances.
Hemolysis assay [I,IV]	Assay for the rupture of red blood cells.

Inductively coupled plasma (ICP) analysis [II]	Detection of metal and non-metal ions by electromagnetic induction.
Lactate dehydrogenase (LDH) assay [I,II]	A colorimetric assay for the release of LDH as measure of membrane integrity.
Limulus Amebocyte Lysate (LAL) endochrome assay [II]	Enzyme based test to detect lipopolysaccharide (LPS) in solution.
Luciferase assay [III,IV]	A reporter assay to assess gene regulation activity in transfected cells.
Mitochondrial respiration [II]	Measurement of oxygen concentration as a function of mitochondrial respiration.
MitoSOX™ [II]	A fluorescent probe that measures mitochondrial superoxide production.
Newport Green™ DCF [II]	A fluorescent probe indicating the presence of metal ions.
Nanoparticle tracking analysis (NTA) [IV]	Combines laser light scattering microscopy with a charge-couple device camera for particle sizing in solution.
Scanning electron microscopy (SEM) [I]	Provides images of a sample surface by scanning it with a high-energy beam of electrons.
Statistical analyses [I,II,III,IV]	Data analyses using methods of probability theory.
Surface modification [I]	Acid/base treatment of silica surface to enable modification of silanol groups.
Tetramethylrhodamine ethyl ester (TMRE) [II]	A fluorescent dye that measures mitochondrial membrane potential.
ThioGlo® [II]	A fluorescent dye that measures active thiols.
Transmission electron microscopy (TEM) [I,II,III]	Microscopic technique using a beam of electrons instead of light.

Trypan blue exclusion [I]	Dye exclusion test to measure cell membrane integrity.
Western blot analysis [II]	Gel electrophoretic separation of proteins and subsequent transfer to membranes for antibody detection.
X-ray diffraction (XRD) [I]	Tool to investigate structures on the atomic scale.
X-ray photon electron spectroscopy [I]	Spectroscopic technique that measures the elemental composition and electronic state of the elements within a material.
Zeta-potential [I,II,III]	Measures the electrokinetic potential in colloidal systems.

5.3 RESULTS

Paper I. The hemolytic properties of synthetic nano- and porous- silica particles: the effect of surface properties and the protection by the plasma corona.

In Paper I, the hemolytic properties of amorphous silica nanoparticles with primary sizes of 7-14 nm (hydrophilic versus hydrophobic), 5-15 nm, 20 nm, and 50 nm, and model meso/macroporous silica particles with pore diameters of 40 nm and 170 nm were investigated. A crystalline silica sample (0.5-10 μm) was included for benchmarking purposes. The results showed that the temperature and chosen solution could affect the hemolytic properties of silica particles, emphasizing the importance of hemolysis testing at physiological conditions. Although no single parameter (such as size, surface charge, total surface area) alone was observed to correlate significantly with hemolysis, surface modification experiments clearly demonstrate that surface properties are linked to the hemolytic activities of these particles. Moreover, hydrophobic modified particles completely inhibited the hemolytic activity of pristine hydrophilic particles. Furthermore, a significant correlation was observed between the hemolytic profile of red blood cells and the cytotoxicity profile of human promyelocytic leukemia HL-60 cells induced by nano- and porous- silica particles, suggesting that silica particles potentially induce membrane permeability through a universal mechanism of action. Importantly, the generated results suggest that the protective effect of plasma towards silica nanoparticle-induced hemolysis as well as cytotoxicity is primarily due to the protein/lipid corona shielding the silica particle surface rather than the functional activities of plasma (Figure 6).

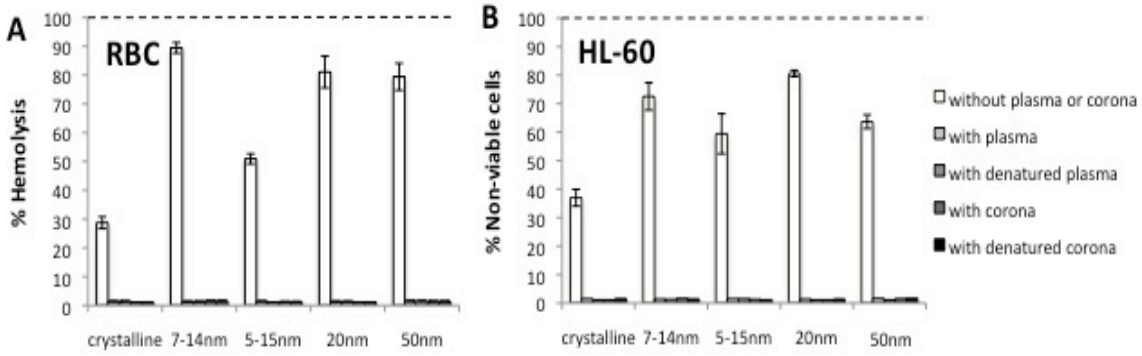


Figure 6. Effect of heat-denatured plasma or plasma corona on hemolysis and cytotoxicity induced by silica particles: (A) hemolysis and (B) cytotoxicity induced by 2 mg/mL silica particles. n=3-4. All values were significantly different ($p<0.001$) from those without plasma or corona.

Paper II. Microsomal glutathione transferase 1 protects against toxicity induced by silica nanoparticles but not by zinc oxide nanoparticles.

In Paper II, the cytotoxicity and oxidative stress induced by TiO_2 (rutile/anatase), CeO_2, SiO_2 (amorphous) and ZnO nanoparticles of similar size (primary size less than 30 nm), was evaluated in human breast carcinoma MCF-7 cells with or without overexpression of MGST1. In the absence of serum, SiO_2 and ZnO nanoparticles caused dose- and time-dependent toxicity whereas no obvious cytotoxic effects were induced by TiO_2 and CeO_2 nanoparticles. Four additional SiO_2 nanoparticles were tested and three out of four also showed pronounced cytotoxic effects. Notably, overexpression of MGST1 reversed the cytotoxicity of two of the SiO_2 nanoparticles tested but did not protect cells against ZnO-induced cytotoxic effects (Figure 7), suggesting different underlying mechanisms of action for the different nanoparticles. Moreover, the cytotoxicity of SiO_2 nanoparticles was dramatically reduced whereas that of ZnO nanoparticles was only slightly reduced in the presence of serum, further suggesting different interactions between serum and the different nanoparticles. The results suggest a prominent role of lipid peroxidation in SiO_2 nanoparticle-induced cellular damage, and the role of zinc ion dissolution for ZnO nanoparticle-induced cellular damage.

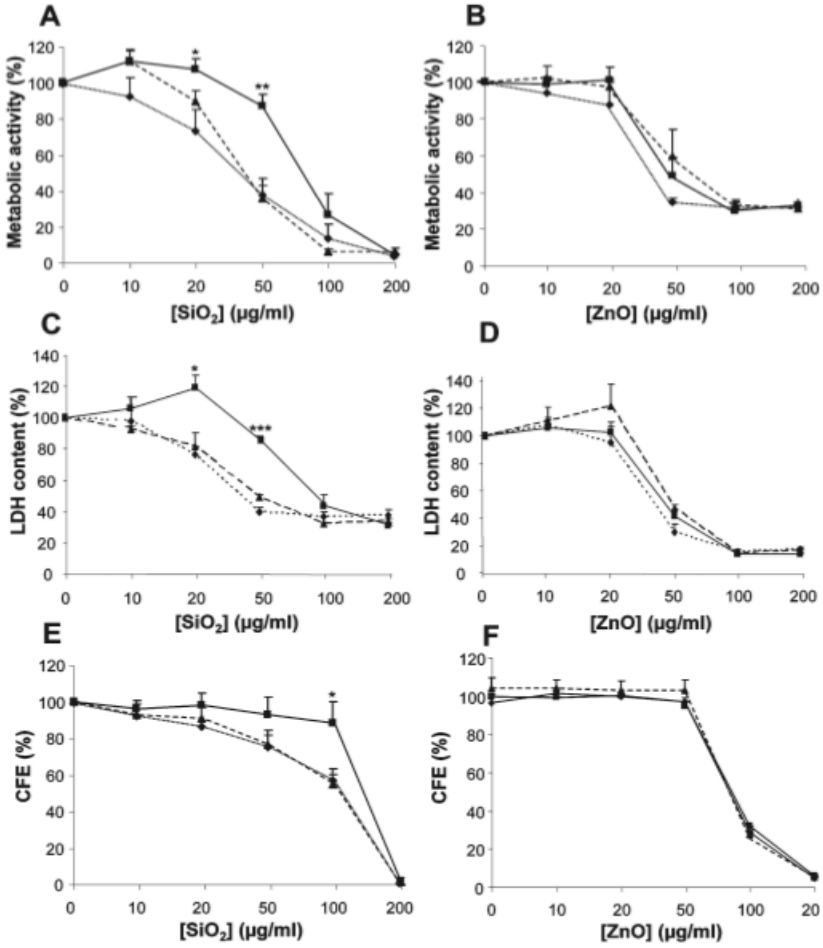


Figure 7. MGST1 protects against SiO_2 nanoparticle-induced cytotoxicity but not ZnO nanoparticle-induced cytotoxicity. MGST1 protection against nanoparticle-induced

cytotoxicity at 24 h was assessed using MTT assay for assessment of metabolic activity (A, B), LDH assay to monitor cell membrane damage (C, D), and CFE assay to monitor the late effects of particle exposure (24 h exposure, followed by a further 7 day incubation) (E, F). MGST1 overexpressing cells are indicated by filled squares and solid line, antisense transfected cells by triangles and dashed line, and MCF-7 wild-type cells by diamonds and dotted line. The results are expressed as mean values ± standard deviations (n = 3-4); *<0.05, **<0.01, ***<0.01.

Paper III. Amino-modified silica nanoparticles as non-viral vectors for the delivery of plasmid DNA.

In Paper III, the applications of amino-functionalized silica nanoparticles for gene delivery are investigated. In this study, amino-modified silica nanoparticles of primary size 20-50 nm were used to successfully deliver luciferase-encoding pDNA into human breast carcinoma MCF-7 cells, as confirmed by an increase in luciferase gene expression. The delivery efficiency was higher using amino-modified nonporous silica particles as compared to amino-modified mesoporous silica particles (pore diameter of 2.4 nm), with similar size and loading of amino groups (wt%) (Figure 8). Moreover, the delivery efficiency was higher in the presence of serum than in the absence of serum. The binding of pDNA to amino-modified silica nanoparticles was confirmed with a gel retardation assay, and TEM images revealed the intracellular localization of these particle-DNA complexes to be in membrane-enclosed vesicles. Particle vectors alone as well as particle-DNA complexes showed good biocompatibility, with the nonporous particles/particle-DNA complexes slightly more toxic than their mesoporous counterparts. And both particles/particle-DNA complexes were slightly more toxic in the absence of serum than in the presence of serum.

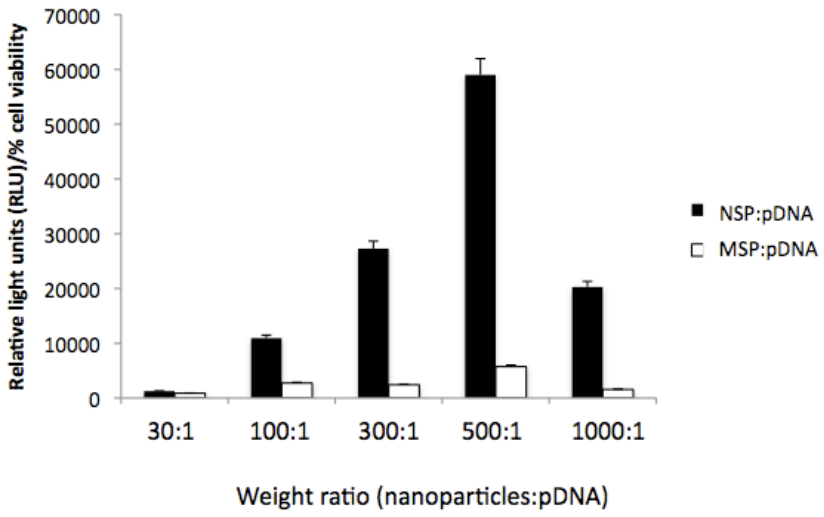


Figure 8. Delivery of luciferase expressing plasmid using amino-functionalized nonporous and mesoporous silica particles in MCF-7 cells in the presence of serum. n=3. *<0.05, **<0.01, ***<0.001.

Paper IV. Delivery of small interfering RNA using an amino acid-modified polythiophene.

In Paper IV, the polythiophenes (namely POMT and POWT) were used as vectors for the cellular delivery of siRNA. Human osteosarcoma U2-OS cells, wildtype or stably transfected with a luciferase-encoding plasmid, were used to confirm the delivery of anti-luciferase siRNA upon non-covalent complex formation with polythiophenes. Notably, the cationic POMT was highly efficient in the delivery of siRNA whereas its zwitteronic analogue POWT was considerably less efficient, underscoring the importance of polymer cationicity in the delivery efficiency of the vector. Figure 9 demonstrates the successful delivery of anti-luciferase siRNA using POMT. Furthermore, mechanistic and biocompatibility studies were performed for POMT. Pre-incubation of siRNA:POMT at 4°C substantially reduced delivery efficiency, implying that the siRNA:POMT complexes triggered energy-dependent uptake into mammalian cells. Pre-incubation of siRNA:POMT with chloroquine (which prevents endosomal acidification) did not enhance delivery efficiency, suggesting that endosomal escape was not a limiting factor in the delivery process. Circular dichroism spectroscopy indicated that POMT maintained a helical conformation even after complexation with siRNA, a feature that could potentially explain their efficient cellular internalization and endosomal escape. Moreover, HeLa cells were used to probe co-localization of Cy5-labeled siRNA and the autofluorescent POMT by live-cell fluorescence microscopy. The results suggested potential co-localization of Cy5-siRNA and POMT directly after transfection, which decreased after 24 h. Biocompatibility studies showed that siRNA:POMT complexes displayed negligible hemolysis of red blood cells (medical acceptance level is less than 5%) up to 24 h.

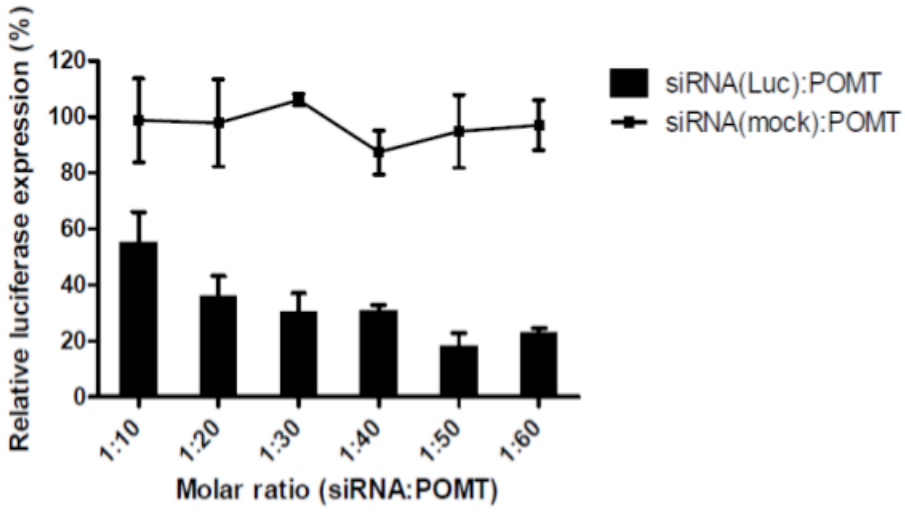


Figure 9. The optimal molar ratio for delivery of anti-luciferase siRNA, assayed at an siRNA concentration of 50 nM, was 1:50 siRNA:POMT. An unrelated siRNA at the same concentration did not induce any significant silencing at any molar ratio.

5.4 GENERAL DISCUSSION

Physical, chemical and biological differences between conventional drug/gene and nanomedicine therapeutics.

As mentioned in previous sections, nanomaterials offer a number of advantages as delivery vectors. Some physical, chemical and biological differences between conventional drug/gene pharmaceuticals and nanomedicine therapeutics are highlighted in Table 4. In Paper III and Paper IV, it was clearly shown that the pDNA or siRNA *per se* would not be able to execute its effect without the delivery vectors. The Papers (I-IV) in this thesis aim to further investigate the safety and efficacy of nanomaterials as delivery vectors, as well as factors affecting their behaviors.

Table 4. Comparison between conventional drug/gene and nanomedicine therapeutics.

Characteristics	Drugs	Genes	Nanomedicines
Synthesis	Chemical synthesis	Isolated from plant/animals or synthesized by means of genetic engineering	Formation of complexes between drugs/genes and nanovectors
Molecular weight or particle size	Low molecular weight, less than 1 nm	High molecular weight, usually a few nanometers	High molecular weight, usually around 1-100 nm
Physical and chemical characteristics	Characteristics of well-defined small molecular weight chemicals	Complex physicochemical characteristics (e.g. tertiary structure)	Characteristics of material science and particle science, including size, shape, mechanical properties, etc
Interactions with cells	Typically diffusion once inside the cell cytoplasm	Typically degraded by cellular enzymes	Typically confined intracellular location
Interactions with the human body	Poor pharmacokinetics often lead to major side effects	Typically degraded by serum enzymes	Improved pharmacokinetics

Physicochemical properties of nanomaterials in relation to their biocompatibility and gene delivery efficiency.

The work in this thesis emphasizes the basic understanding of the physicochemical properties of nanomaterials in relation to their biocompatibility and gene delivery efficiency. Although there is no clear consensus in the literature, some patterns are emerging. However, a larger sample size or meta-analysis would be necessary for deriving meaningful conclusions from statistical analyses of correlations between their physicochemical properties and biological endpoints. Moreover, the physicochemical properties of nanomaterials are interdependent (for example, synthesis of well-defined nanoparticles with different sizes also results in different surface charges) [121], therefore computer simulations would be needed to fully appreciate such complex relationships.

Chemical composition and crystallinity.

Currently, most nano-formulations that already exist on the market for *in vivo* delivery and imaging purposes are lipid and liposome based nanocomposites, polymers and iron oxide nanoparticles [1]. Indeed, chemical composition is among the determining factors for the biocompatibility of nanomaterials for biomedical applications. In Papers I-III, the use of silica nanomaterials as biocompatible nanomaterials for biomedical applications was investigated. In Paper II, amorphous silica nanoparticles were also compared to cerium oxide, titanium oxide, and zinc oxide nanoparticles of similar size. Results from Paper II and others suggest that amorphous silica is considerably more biocompatible compared to many other materials such as zinc oxide, zirconia [122], etc. It is noteworthy that the crystalline form of silica is rather toxic and not suitable for biomedical applications [122, 123]. In Paper IV, the novel utilities of polythiophenes for gene delivery in biomedicine are explored. The toxicity of polythiophenes is not well understood, however, it was shown that polythiophene conductive polymers improve the biocompatibility of electrodes on primary mouse neurons [124]. Therefore, chemical composition and crystallinity has a strong impact on the biocompatibility of nanomaterials. Silica and polythiophene nanomaterials are potentially interesting materials for biomedical applications, with mesoporous silica nanoparticles entering the stage of preclinical development [125]. Other potential platforms include gold, magnetic nanoparticles, and carbon nanotubes [1, 18].

Size.

There is substantial concern of a higher toxic potential at the nanolevel compared to the microlevel [126], due to the higher proportion of atoms exposed at the surface of nanomaterials (compared to bulk materials of the same composition) as well as the ability of smaller particles to penetrate deeper into the body. In Paper I, the biocompatibility of silica nanomaterials with different size, surface charge, total surface area, hydrophobicity, and porosity were compared. These results, although inconclusive, suggest that smaller size particles seem to be more hemolytic and cytotoxic than larger ones at the same mass dose. Similarly, other studies found size-

dependent toxicity of amorphous silica particles *in vitro* and *in vivo*, with the smaller particles being more toxic. For example, smaller particles compared to larger ones were shown to be more cytotoxic in various cells by the MTT and LDH assays [121, 127-129], induce more apoptosis in human keratinocytes HaCaTa cells as detected by the annexin V-propidium iodide assay [130], and induce more oxidative stress (ROS generation, lipid peroxidation and GSH depletion) in human hepatic L-02 cells [131]. Mice intravenously injected with 75 nm silica particles induced liver injury at 30 mg/kg body weight, whereas 311 and 830 nm particles had no effect at 100 mg/kg [132]. Feeding of mice for 10 weeks (total fed amount of 140 g/kg mice) with 30 nm silica nanoparticles induced higher levels of alanine aminotransferase (ALT) and fatty liver patterns compared to those of 30 μm silica microparticles (with similar liver retainment) [133]. Smaller polymer nanoparticles of 45 nm also showed higher cytotoxicity compared to larger 90 nm particles in terms of ROS production, adenosine-5'-triphosphate (ATP) depletion, tumor necrosis factor (TNF)-α release as well as the reduction of mitochondrial membrane potential in different cells [134]. Interestingly, it was reported that certain specific sizes can be substantially toxic, i.e. gold nanoclusters of 1.4 nm are remarkably more toxic than marginally smaller or larger gold nanoparticles potentially due to their interactions with the major grooves of DNA [135].

Higher delivery efficiency *in vivo* is generally attributed to nanoparticles with a diameter around 100 nm, which are capable of circulating in the plasma for a few hours rather than seconds to minutes for smaller or larger particles [4]. In addition to plasma circulation time that is a critical prerequisite for delivery, other factors such as cellular uptake are also important in governing the delivery efficiency of nanoparticle vectors. Size-restrictions affect cellular uptake via different mechanisms of endocytosis (clathrin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, and clathrin/caveolin-independent endocytosis) [33, 105]. Nabiev et al. reported that the cell's active transport machinery delivered nonfunctionalized nanocrystals to different regions of the cell in a size-specific manner [136]. He et al. showed that the availability of particles to be internalized is better for the smaller particles among particle sizes of 190, 420, and 1220 nm in various cells [129]. Lu et al. showed by confocal laser scanning microscopy and ICP-MS that cellular uptake in human cervical HeLa cells was optimal for silica particles of 50 nm compared to 30, 110, 170 and 280 nm [137]. Aoyama and co-workers demonstrated an optimal diameter around 50 nm for the cellular uptake of calix[4]-resorcarene-coated macrocyclic glycocluster amphiphiles or quantum dots [138]. Chan and co-workers also reported 40-50 nm diameter to be optimal for cellular internalization of pristine and protein-coated gold nanoparticles [139, 140]. Theoretical models converge on similar conclusions that particles ought to have a minimum diameter between 40 and 60 nm in order to achieve effective cellular uptake [141]. Therefore, a delivery system has an optimal physical size in the nanometer range that facilitates their cellular binding and uptake (while also depending on other parameters), at least in non-phagocytic cells. On the other hand, it was suggested that larger particles are also able to enhance gene delivery in cell culture systems *in vitro*, which might be explained by the concentration of nucleic acids at the surface of cultured cells as a result of gravity [142].

Surface charge.

A positively charged surface is generally more toxic than a negatively charged surface, due to its potential interactions with many negatively charged biological molecules (such as glycolipids and nucleic acids) [143]. However, Slowing et al. showed that when amorphous silica particles were functionalized with carboxylic acid, their zeta-potential was similar (from -45.9 to -47.3 mV) but hemolysis was inhibited. This indicates that in the case of silica, hemolysis is specific to the silica surface despite the negative surface charge. The results in Paper I further points to the specific effects of surface silanol groups on the hemolytic and cytotoxic properties of silica particles. Isoda et al. found that intravenously administered amino group or carboxyl group modified silica nanoparticles were much less toxic than unmodified particles as shown by the level of liver injury (serum alanine aminotransferase level, liver hydroxyproline content, fibrosis) in mice [144]. These *in vivo* findings are also in line with the specific silica surface induced toxicity. For many other types of nanomaterials, such as polymers, higher positive charges are generally correlated with higher toxicity [145-147].

Delivery vectors often carry positive charge to enable ionic complexation with nucleic acids. In Paper IV, it was demonstrated that the delivery efficiency of the cationic polythiophene was much higher than the zwitteronic polythiophenes. Cellular binding and uptake can be achieved either via non-specific adsorptive endocytosis (by providing excess positive surface charge) or specifically via receptor-mediated endocytosis [148, 149]. On the other hand, the strength of the ionic interactions between the delivery vectors and the nucleic acids can be a limiting factor later during the disassembly of the complexes [150]. In terms of *in vivo* delivery efficiency, the nanoparticle-nucleic acid complex is most desirable to be near neutral in order to avoid non-specific interactions with blood components, extracellular matrix and non-target cells or tissues *in vivo*.

Porosity.

Porosity may have an important role in determining the toxicity of nanoparticles. Slowing et al. suggested that mesoporous silica particles have reduced hemolytic activity (compared to nonporous silica particles) which correlates to their lower external surface area as a result of their porous structures [37]. Similarly, lower hemolysis and cytotoxicity were generally observed for porous silica particles in Paper I, Paper III as well as a study by Rabolli et al. [121] in different cell types. However, more studies need to be performed to confirm this relationship.

Gao et al. demonstrated pore-size dependent drug release rate and therefore anticancer activity using mesoporous silica nanoparticles in drug sensitive and drug resistant MCF-7 cell lines [151]. Na et al. showed pore-size dependent delivery of siRNA *in vitro* and *in vivo* using mesoporous silica nanoparticles, particles with larger pores (23 nm) being more efficient than those with smaller pores (2 nm) [152]. In Paper III, nonporous

silica nanoparticles were shown to have superior delivery efficiency compared to mesoporous silica nanoparticles with pore diameters of 2.4 nm. Several reasons could account for this observation: these mesoporous silica nanoparticles with 2.4 nm pore diameter have small pore spaces that could not be efficiently explored for the accommodation of cargo; the different distribution of functional groups over the surface of mesoporous and nonporous silica particles may subsequently affect their binding to nucleic acids as well as aggregation state; there might be less cellular association of mesoporous compared to nonporous silica nanoparticles as shown in a quantitative study using ICP-MS [153]. Therefore, the dimensions of the pores could have a strong impact on the delivery efficiency of porous particles.

The effect of plasma/serum.

The effect of plasma/serum on nanoparticle behavior as well as their interactions with biological systems (particularly cytotoxicity and gene delivery efficiency) was examined in Papers I-III.

In Paper I, the presence of a biological corona over silica particles was confirmed by means of X-ray photon electron spectroscopy (XPS). In Paper I, it was demonstrated that the plasma/serum corona is primarily composed of proteins, but lipids may also be involved. The zeta-potential of plasma corona coated particles tends to be fairly similar (-20±5 mV) despite the very different zeta-potential of pristine particles (-10 to -50 mV). Monopoli et al. showed that the zeta-potential of 50 and 200 nm silica particles was modified by plasma corona (approx. from -25 to -10 mV), but the zeta-potential did not vary further with increasing concentrations of plasma (from 3% to 80%) [56]. In Paper II, it was shown that the serum corona reduced the aggregation of nanoparticles (SiO_2, TiO_2, CeO_2, ZnO) and in some cases (e.g. ZnO) enhanced their dissolution. Gualtieri et al. showed that 0.1% bovine serum albumin (BSA) reduced aggregation of silica nanoparticles [154] whereas studies by Monopoli et al. and Drescher et al. observed higher aggregation of silica and polystyrene nanoparticles in the presence of plasma/serum [56, 155]. It was also shown that interactions of polymer-nucleic acid complexes with plasma proteins such as albumin leads to aggregation [156, 157].

Interestingly, the coating of a pathogen with serum components is a mark for ingestion and destruction, a process termed opsonization, often resulting in phagocytosis and clearance from the circulation [158]. Similarly, plasma/serum protein coating over polymer nanoparticles accelerated their removal by phagocytic cells [157, 159]. Moreover, reduced cytotoxicity has been observed for nanoparticles in the presence of albumin [160]. It is however questionable whether the reduced toxicity is due to the antioxidant activities of albumin or the coating of albumin over the reactive surface of these nanoparticles. Indeed, it was shown in Paper I-III that the presence of plasma/serum abolished or delayed the toxicity of pristine silica nanoparticles, amino-functionalized silica nanoparticles and ZnO nanoparticles. In Paper I, further evidence was presented that the plasma corona coating of the silica surface protected silica nanoparticles against hemolysis and cytotoxicity. The human plasma/serum may thus serve the

function to mediate the *in vivo* distribution and excretion of nanoparticles and reduce their toxic effects in the systemic circulation.

On the other hand, reduced blood circulation time following the *in vivo* interactions of nanoparticle formulations with plasma proteins also impairs their delivery efficiency [157]. Moreover, blood plasma/serum is also abundant in nucleic acid-degrading enzymes that can lead to a substantial loss of therapeutic effect [161]. Therefore, research efforts are made towards using hydrophilic polymers (e.g. PEG) to shield nanoparticles from intensive interactions with blood proteins as well as searching for serum resistant formulations for delivery. For example, Lehto et al. showed that the delivery efficiency of a stearylated cell-penetrating peptide transportan 10 was maintained in the presence of serum proteins mimicking *in vivo* conditions [162]. Silica particles provide promising serum resistant features for *in vivo* applications [152], although some discrepancy exists in our study and the literature. In Paper III, amino-functionalized silica particles displayed higher delivery efficiency for pDNA in MCF-7 cells in the presence of 10% serum than in the absence of serum, whereas Na et al. observed marginally lower delivery efficiency for siRNA in human cervical carcinoma HeLa cells with 10% serum than without serum [152]. Nevertheless, Xiao et al. confirmed the protection of DNA by mesoporous silica particles from serum nucleases [163].

***In vitro* vs. *in vivo*.**

Although numerous studies have used cell models to investigate the biocompatibility of nanomaterials and their applications for gene delivery, it is questionable how much knowledge from *in vitro* studies can be readily transferred to *in vivo* situations [164]. First, *in vitro* systems lack the complexities of *in vivo* pharmacokinetics, physiological structures, and systemic responses. Second, particles, unlike small molecules, do not necessarily evenly distribute in fluids. On the contrary, they may exhibit distinct behaviors in body fluids and cell cultures [142]. Third, cellular phenotypes (such as their repertoire of expressed receptors) may show significant variations in *in vitro* cell cultures [165]. Nevertheless, *in vitro* studies may still prove to be useful in nanomedical research for identifying similar patterns of biologic activity and understanding the mechanisms of action [166].

In addition to the *in vivo* approach for the administration of therapeutic nucleic acid formulations, the *ex vivo* approach first delivers the genetic material into cells grown *in vitro* (usually autologous cells from the same patient) and then introduce those transfected cells into the patient [167].

5.5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Nanotechnologies hold great promises for numerous biomedical and diagnosis applications. Although nanomaterials have the potential to revolutionize the field of pharmaceutics and nucleic acid delivery [168], intensive research efforts are needed to develop safe, targeted, efficient delivery vectors. Importantly, studies to improve the understanding of their biocompatibility/toxicity and mechanisms of delivery are crucial to assist the rationale design of nanomaterials for delivery applications.

In order to ensure the safety of using synthetic nanomaterials for delivery applications, thorough toxicology assessments linked to their physicochemical properties would be required. One of the primary concerns for future investigations is whether they cause cardiovascular adverse effects. It is noteworthy that small molecular drugs tend to cause more cardiovascular toxicity than hepatoxicity, especially in the long term [169]. Interestingly, epidemiology studies of air pollution found fine particles (0.1-2.5 μm) associated with respiratory diseases and ultrafine particles (0.01-0.1 μm) associated with respiratory-cardiovascular diseases [170]. It was suggested that nano-sized particles induce human vascular endothelial cell cytotoxic injury, inflammatory responses, and inhibition of cell growth, potentially causing cardiovascular diseases [170, 171]. Radomski et al. reported nanoparticle-induced human platelet aggregation *in vitro* and rat vascular thrombosis *in vivo* [172], leading to possible systemic and cardiovascular risks. Moreover, it is important to identify which characteristic physicochemical properties may potentially cause cardiovascular toxicity. For example, surface charge is an important factor for the activation of the complement system and coagulation pathways [173].

In the future, investigations directed towards the engineering of synthetic nanomaterials for gene delivery applications would be of considerable interests. For example, nanoparticles could be functionalized with PEG to better escape immune recognition and/or functionalized with targeting ligands for the active recognition of specific cells (e.g. targeting of folate receptors on tumor cells [174]). Nanoparticles could be combined with cell penetrating peptides for enhanced delivery efficiency. Moreover, it would be of interests to investigate the mechanisms of delivery. Energy depletion and pharmacology inhibitors can be used to probe the mechanisms of cellular uptake, whereas *in vitro* liposome leakage assay can be used to mimic the process of endosomal escape. Last but not least, restoring or silencing of functional genes (e.g. tumor suppressor genes or oncogenes, respectively), as well as combined drug and gene delivery (e.g. to overcome drug resistance), can be investigated for specific therapeutic purposes.

6 ACKNOWLEDGEMENTS

I would like to express my gratitude to the excellent research environment at Karolinska Institutet, together with the multidisciplinary collaborative opportunities with various institutions. The work in this thesis was funded by the European Union 6th and 7th Framework Program ECNIS and NANOMMUNE, and the PhD grant from the Board of Postgraduate Studies at Karolinska Institutet is also acknowledged. I would particularly like to thank those who have inspired and encouraged me during the past few years:

Professor ***Lennart Möller***, my supervisor, for your trust and support. Your vast knowledge has always inspired me with interests and your mental strength inspired me with courage. ***Hanna Karlsson and Clara Ersson***, my co-supervisors, for being brilliant and enthusiastic young investigators, and at the same time for your understanding and friendship.

Professor ***Bengt Fadeel***, for introducing me into this exciting field at the interface of nano-bio research, and I have always admired your dedication for science. Professor ***Valerian Kagan***, for always challenging me on the critical appraisal of scientific research, which are among the most valuable lessons during my doctoral training. ***Hjälmar Brismar***, for showing me confocal microscopic techniques and for being my mentor.

My collaborators: Professor ***Edvard Smith, Samir El-Andaloussi, Per Lundin, Joana Viola, Pedro Moreno, Sylvain Geny, Eman Zaghloul*** for collaborations on such interesting projects, and for sharing your expertise in the field of gene therapy. Professor ***Inger Odnevall Wallinder, Maria Lundin, Yolanda Hedberg*** for your competence in surface and corrosion chemistry as well as the highly enjoyable teamwork. Professor ***Ralf Morgenstern, Vladimir Gogvadze, Katarina Johansson*** for your deep knowledge in oxidative stress, and persistance to dig into difficult questions. Professor ***Mamoun Muhammed, Abdusalam Uheida, Muhamet Toprak, Terrance Burks, Fei Ye, Carmen Vogt, Mohsin Saleemi, Jian Qin, Andrea Fornara, Natalia Kupferschmidt, Rambabu Atluri, Alfonso Garcia-Bennett,*** Professor ***Sanjay Mathur, Lisong Xiao, and Jiangtian Li,*** for sharing valuable materials, techniques, and expertise in material sciences and nanotechnology. Professor ***Anna Shvedova, Aki Maeda, Jianfei Jiang, Zhentai Huang, Weihong Feng, Xi Chen, Natalia Belikova, Sandor Alexej, Nagarjun Konduru, Alexander Kapralov, Yulia Tyurina, Naveena Yanamala, Irina Vlasova,*** for teaching me a vast array of techniques, for broadening my insights with computer modeling, and for all the inspiring discussions during my extended visit at the University of Pittsburgh. Professor ***Karl Tryggvason and Juha Ojala***, for ongoing collaborations with interesting mouse knockout models.

Professor ***Annika Scheynius, Susanne Gabrielsson, Britta Andersson, Helen Vallhov, Tina Thurnherr***, for all interesting discussions at the NanoNet and Nanommune consortium. Assoc. Professor ***Andreas Nyström***, for your fantastic courses on Nanomedicine and Cancer Nanomedicine in the Swedish archipelago. ***Kjell Hultenby***, for the excellence in transmission electron microscopy. ***Junliang Sun and Hong Chen***, for electron microscopic images and inspiring discussions. ***Bertrand Faure*** for the expertise on the ZetaSizer. ***Chunyan Zhao and Hui Gao***, for your expert input as well as friendship.

Professor ***Eckardt Treuter*** and Assoc. Professor ***Lena Mäller***, for the rotation opportunities and interesting projects in your group. It was a valuable experience to broaden my insights in related fields of biomedicine.

Lena Nirland, Monica Ahlberg, Marie Franzén for being so helpful with doctoral education matters, ***Eva Norlander*** for writing me visa letters, and ***Inger Moge*** for help with ordering.

My wonderful collegues ***Johanna Kain, Pontus Cronholm, Therese Bergström, Ylva Rodhe,*** and ***Hanna Zandré*** for being so considerate and fun collegues. Former collegues ***Erika Witasp, Astrid Ottosson-Wadlund, Neus Feliu Torres, Christine Chang, Marjolein Lauwen, Shouting Zhang, Siriporn Dao Jitkaew, Marion Landry, Xiaoli Feng, Giulio Preta, Andrea Kunzmann, Linda Swedin, Laura Hermanns, Oras Mistafa, Vanina Dahlström Heuser, Åse Mattsson, Rebecca Cederlund, Annika Wallberg, Anita Popko-Scibor, Magnus Hansson, Jie Zhang, Zhiwei Ye, Ali Imran, Aram Ghalali, Dong Yang,*** for all your help and great company.

My friends from Forskarskolan: ***Annelie Strålfors, Jeanette Grundström, Karin Sundström, Karin Larsson, Masako Harada, Eva Daskalaki, Ingrid Olsson, Camilla Sundborger, Christine Öberg, Olof Allnér, Cristine Skogastierna, Sofia Unnerstale****,* for all Swedish and non-Swedish fun — midsummer and crayfish parties, games and class trips, love coaching and more! ***Karolina Lindberg***, for being so graceful and caring, and for watching horror movies at your place. ***Karin Edvarsson***, always bringing sunshine and often challenging conventions. ***Anna Ehrlund, Lise-Lotta Vedin, Karolin Guldevall, Lovisa Lundholm, Pauliina Damdimopoulou, Milica Pütnik, Amena Archér****,* for girls fun. ***Marion Korach-André, Peter Swoboda, Patrick Müller, Pia Forsberg****,* for nice talks and company. ***Sebastian Lewandowska****,* for salsa dancing. ***Xi He, Ziyue Sun, Dali Zong, Yuan Xue, Qing Cheng, Jinfeng Shen, Yu Li, Xin Li, Mingmei Shang, Xiaoda Wang, Hao Mo, Xiaofeng Zheng, Ying Huang, Jing Chu****,* for delicious food and memorable time. ***Barbara Mascialino, Cédric and Jan Fiez-Vandal, Christine Oswald, Gabor Petranyi, Nicolas Ventecléf, Simon Sundling, Davide Valentino, Neda Farzad, Neda Keshavarz*** for your warm-heartedness and hospitability. ***Mateutsz Mojsiejuk*** and ***Christian Thorén,*** for always bringing so much laughter and passion to indoor climbing. ***Helle Johansson***, for our philosophical conversations and fun trips together. And ***Lars Gezelius***, for your enduring professonal inspirations!

My family, for unconditional love and support. And ***my Swiss friends,*** for continuous friendship.

Felix, for telling me about statistical mechanics and self-avoiding random walks. Words cannot express what you mean to me, only a mathematical formula can: ☺

$$F(x) \approx (Maths + Sailing + \sum_{x=0}^{\infty} Traveling(x)) \times Love \times Support$$

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