Toxicological Hazards of Inhaled Nanoparticles—Potential Implications for Drug Delivery

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Nanoparticles (NP), here defined as particles with a diameter smaller then 100 nm, are increasingly used in different applications, including drug carrier systems and to pass organ barriers such as the blood-brain barrier. On the other hand, a large body of know-how is available regarding toxicological effects of NP after inhalation. More specifically, a number of effects of inhaled NP are attributed to their (i) direct effects on the central nervous system, (ii) their translocation from the lung into the bloodstream, and (iii) their capacity to invoke inflammatory responses in the lung with subsequent systemic effects. This paper gives a brief review on the toxicology of inhaled NP, including general principles and current paradigms to explain the special case of NP in pulmonary toxicology. Since the evidence for health risks of NP after inhalation has been increasing over the last decade, this paper tries to extrapolate these findings and principles observed in inhalation toxicology into recommendations and methods for testing NP for nanocarrier purposes. A large gap is present between research on NP in inhalation toxicology and in nanoscaled drug carrying. This review recommends a closer interaction between both disciplines to gain insight in the role of NP size and properties and their mechanisms of acute and chronic interaction with biological systems.

Keywords: Nanoparticles, Inhalation, Systemic Effects, Drug Delivery, Toxicology.

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1. INTRODUCTION

Nanotechnology is expected to bring a fundamental change in manufacturing in the next few years and will have an enormous impact on Life Sciences, including drug delivery, diagnostics, nutriceuticals and the production of biomaterials.[1, 2] Nanoparticles (<100 nm) play an important role in a number of these applications. The reason why nanoparticles (NP) are attractive for such purposes is based on their important and unique features, such as their surface to mass ratio, which is much larger than that of other particles and materials, allowing for catalytic promotion of reactions, as well as their ability to adsorb and carry other compounds. The reactivity of the surface originates from quantum phenomena and can make NP unpredictable since, immediately after generation, NP may have their surface modified, depending on the presence of reactants and adsorbing compounds, which may instantaneously change with changing compounds and thermodynamic conditions. Therefore, on one hand, NP have a large (functional) surface which is able to bind, adsorb and carry other compounds such as drugs, probes and proteins. On the other hand, NP have a surface that might be chemically more reactive compared to their fine (>100 nm) analogues (Fig. 1).

Apart from their potential use in life sciences and catalysis, NP have been put forward to explain increased mortality and morbidity due to environmental particle exposure. Since the first publication of the so-called Six Cities study[3] that described an association between mortality in six US cities and the annual mean of particulate mass sampled by convention with a 50% cut-off at 2.5 μm ($PM_{2.5}$), numerous epidemiological studies have confirmed and quantified this finding.[4–6] From these studies, it is estimated that per 10 $\mu g/m^3$ increase in the concentration of $PM_{2.5}$, overall mortality increases by 0.9%, while deaths from specific respiratory diseases can increase by as much as 2.7% (Table I). It is by no means clear how exposure to PM, typically as low as 30 $\mu g/m^3$, can produce the health effects observed in epidemiology studies and which attributes of PM mediate these effects. There is ample evidence that a small proportion of the mass but a large proportion of the number of the particles in ambient air are ultrafine in size, i.e., less than 100 nm in diameter (Fig. 2). Numerous toxicological studies have now forwarded these ultrafine particles to be responsible for adverse effects (reviews: 7–10), but so far few human studies have been able to investigate.[11, 12] Since one of the important features of

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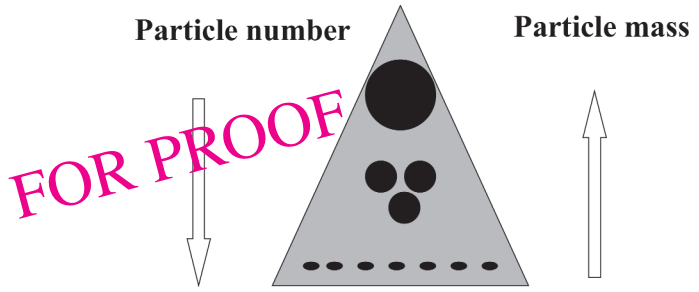

Fig. 1. The paradigm of particle mass and number. At a given mass concentration, the number of particles decreases when the median particle size is bigger. On the other hand, the same mass concentration can be obtained by a large particle number. This paradigm has important toxicological consequences.

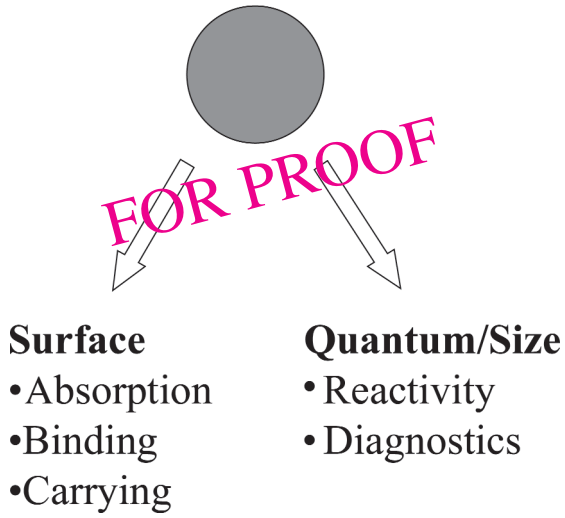

Fig. 2. Properties of ultrafine particles that are suggested to be relevant for their potential biological effects. First of all, Nanoparticles, due to their large surface area, can absorb and bind numerous endogenous components as a non-specific binding. Secondly, due to their small size, many NP have a reactive surface that should be considered to react and inactivate many important mediators and constituents.

nanotechnology is that of multidisciplinarity, this review attempts to bring together the findings on nanoparticles after inhalation with potential therapeutic applications in an effort to understand the particle properties and biological mechanisms that need to be considered before using NP in biomedical applications.

2. THERAPEUTIC APPLICATIONS

An important area of applications of NP includes novel drug delivery techniques, which are quicker and less risky compared to the costs of developing new drugs. A large percentage of ordinary drugs taken orally are destroyed by the stomach or liver, and then distributed throughout the entire body, despite the fact that optimal effects would arise from focusing the drug to the target organ. In addition, this can lead to side-effects in other organs, and some potentially effective drugs are not being used precisely because of (cumulative) side-effects. Targeted drug-delivery by NP has the potential to overcome some of these problems, and render treatment more effective with ensuing cost and safety benefits. The therapeutic potential of currently available drugs is also hampered by micro-kinetics such as local instability issues and difficulties in crossing certain biological barriers such as the *blood-brain barrier* and *placenta*. NP can help to address these problems, and several applications in drug delivery are being developed. Among those, **polymer-drug conjugates** are being explored as drug-delivery devices that use water-soluble polymers such as polyglutamate (PGA), polyethyleneglycol (PEG), dextran or N-(2-hydroxypropyl)metacrylamides (HPMA) to carry drugs into compartments they could never otherwise enter. Unlike low molecular weight drugs which penetrate cells easiliy, the cellular uptake of polymeric pro-drugs is restricted to the endocytic route, which basically means that pro-drugs are delivered to the cellular lysosomes. It is this change in pharmacokinetics that limits rapid access to many tissues and helps to limit side-effects of toxic drugs such as cytostatics. Therefore, this polymer "therapy" has been mostly applied in tumour treatment and has been successful in reducing the cardiac toxicity of adriamycin. The increased plasma residence of polymer conjugates (compared to free drugs) encourages passive accumulation within solid tumor tissue due to increased vascular permeability and the lack of effective tumour drainage. This phenomenon (so-called EPR = Enhanced Permeability and Retention)—first described by Maeda and coworkers[13]—is responsible for higher effective levels of NP-bound drugs in tumors. The last step is the intracellular release of the drug by lysosomal thiol-dependent proteases such as Cathepsin B. Moreover, as with the polymer-drug conjugates, tumour-specific delivery by NP also can be improved by the attachment of ligands. However, only one tumor-specific ligand has been produced.[14]

Table I. Effect of ambient particle exposure on mortality in risk groups (all ages).

Condition	Mortality (% change per 10 $\mu g/m^3$ PM10)	Reference
Respiratory disease (all causes)	0.9% (0.2–1.3)	4
Asthma	1.2% (0.2–2.3)	70
Pneumonia	2.7% (1.5–3.9)	71
COPD (3.3–5.0)	1.5% (1.0–1.9)	72
	1.7% (0.1–3.3)	71
Cancer (lung)	8.0% (1.0–16.0)	5
Cardiovascular (all)	1.1% (0.9–1.3)	72
Ischaemic heart disease	1.6% (1.0–2.2)	65
Congestive heart failure	0.8% (0.5–1.5)	73

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Other approaches have used **polymeric NP** to overcome the multidrug resistance of cancers and, also, to carry anticancer drugs such as adriamycin across the blood-brain barrier. This latter application is mediated by particle binding to the ApoE-receptor, which shows the level of specificity that such interactions and translocations can attain.[15] These particles can be manufactured with biodegradable polymers such as polycyanoacrylates. Since not all drugs bind to these polymers, the employment of other polymers such as gelatin, serum albumin, polylactic acid (PLA) and polylactic-co-polyglycolic acid (PLGA) may enable similar effects.

3. PARTICLE TOXICOLOGY IN THE LUNGS—GENERAL PRINCIPLES

This section pertains to the inhalation exposure of particles, which is relevant in environmental exposure to NP and occupational production of nanomaterials. Particle toxicology has been the subject of a number of reviews, considering the appropriate methodology for exposure, *in vitro* versus *in vivo* dose comparisons[16] and its historical development.[17] For the interpretation of inhaled particle effects, five Ds have to be taken into account, i.e.: (1) Dose, (2) Deposition, (3) Dimension, (4) Durability and (5) Defense. First of all, the dose at a specific site (in the lungs) determines the potential toxicity. This deposited dose is, of course, dependent on the concentration and the dimensions of the particle. Interestingly, the deposition probability of NP increases steeply in the respiratory tract as particle size decreases. Moreover, a major fraction of it will be deposited on the fragile epithelial structures of the terminal airways and gas exchange region.[18] If a particle is neither soluble nor degradable in the lung, it has a high durability and there will be rapid local accumulation upon sustained exposure. The lung, however, has extensive defense systems such as mucociliary clearance (upper airways) and macrophage clearance (lower airways, alveoli) to remove deposited particles. Although the above concept is simple, most of these parameters are interrelated and dimension—as in the case of fibers—may have profound effects on defense and, thereby, chronic dose. Long (>20 μm) fibers are not taken up by alveolar macrophages, and therefore have a longer half-life in the lung compared to the same material with shorter fibers and, consequently, a lower toxic potency. In addition, particle transport by macrophages from the alveolar region towards the larynx is slow in humans, even under normal conditions, thus, eliminating only about a third of the deposited particles in the lung periphery; i.e., the other two thirds accumulate in the lungs without clearance unless they are biodegradable and cleared by other mechanisms.[19] If particles are reactive or present at sufficient dose, macrophages and epithelial cells can be activated or damaged, leading to inflammation, which drives most pathogenic effects of particles.

3.1. Nanoparticles—A Special Case

Although the deposition of uF in the lungs follows largely the same distribution as fine particles, the underlying mechanisms are different. Nanoparticles (<100 nm) have a size dimension that makes them less subject to gravity and turbidometric forces and, therefore, their deposition occurs mostly by diffusion.[18] In addition, their size causes them to interact with other potential targets rather than conventional fine particles. Defense is also inefficient, since their small size may prevent recognition by macrophages, and sometimes surfaces have been designed to behave as "stealth " particles, remaining unrecognised by phagocytosing cells.[20] Maybe as a result of their low recognition, NP have posed research for new problems, since they can migrate to body compartments away from their application or deposition sites. In particular, because of their low uptake by macrophages, NP will be taken up by endothelial cells and they have access to cells in the epithelium, the interstitium and the vascular walls. This is nicely illustrated in Fig. 3, showing the uptake of uF TiO_2 in epithelial cells both after *in vitro* and *in vivo* exposure. Wherever they are deposited or translocated the large surfaces of NP can carry and absorb many endogenous substances (proteins, enzymes). These surfaces are sometimes chemically very active and, therefore, expected to react with numerous molecules such as anti-oxidants or proteins.[21] Over the past decade, NP have shown some typical responses in animal models, including inflammation and carcinogenicity. Human (epidemiological) studies have suggested that NP can also induce or aggravate systemic effects.[22, 23] Results of these numerous reports were presented at the Society of Toxicology meeting. Science magazine launched an editorial headed "Nanomaterials show signs of toxicity"[24] and the EPA has recently launched a STAR program to stimulate research on the effects of nanomaterials. In the following paragraphs, the pulmonary and systemic effects of NP will be briefly reviewed. A schematic summary of key studies on toxicological effects of NP is given in Table II.

4. PULMONARY EFFECTS OF NANOPARTICLES

4.1. Pulmonary Inflammation

The toxicological profile of NP has only emerged during the past decade. An early key study demonstrated that ultrafine TiO_2 (20 nm) caused more inflammation in rat lungs than exposure to the same airborne mass concentration of fine TiO_2 (250 nm).[25] Until then, TiO_2 had been considered a non-toxic dust and, indeed, had served as an inert control dust in many studies on the toxicology of particles. Therefore, this report was highly influential in highlighting that a material that was low in toxicity in the form

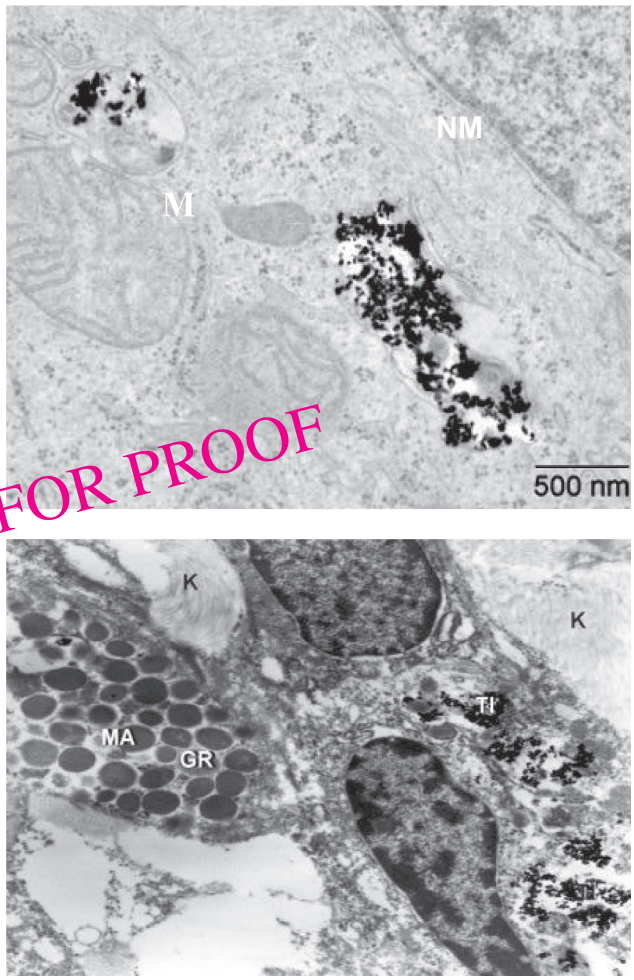

Fig. 3. Transmission electron microscopic images of uptake of ultrafine TiO_2 particles after short term incubation of A549 epithelial cells (upper panel) and in rat lung two years after a single instillation of a 30 mg dose (lower panel). The upper panel shows aggregates and primary particles in lysosomes near mitochondria (M) and the nuclear membrane (NM). The lower panel shows particles (Ti) in an interstitial macrophage with an adjacent mast cell (MA) in beginning degranulation, illustrated by the dense granula (GR). Magnification of the lower panel is 12,800. Images are courtesy of *Dr. Doris Höhr* (upper panel) and *Prof. Wolfgang Drommer* (lower panel).

Table II. Important findings on the biological activity and key publications in the toxicity of Nanoparticles between 1990 and now.

Description of finding	References
NP TiO_2 cause pulmonary inflammation.	25,42
Later studies show that inflammation is mediated by surface area dose.	8,9
NP cause more lung tumors than fine particles in rat chronic studies. Effect is surface area mediated.	37,38, 40,74
NP inhibit macrophage phagocytosis, mobility and killing.	29,30,31,75
NP affect immune response to common allergens.	36,76
NP are related to lung function decline in asthmatics.	77
NP cause oxidative stress *in vivo* and *in vitro*, by inflammatory action and generation of surface radicals.	8,75,76
NP exposure adversely affects cardiac function and vascular homeostasis.	59, 60,61–65
NP have access to systemic circulation upon inhalation.	49,48,55,50,52
NP can affect blood coagulation in human and animal models.	23,10,58, 62,63
NP interfere with Ca-transport and cause increased binding of proinflammatory transcription factor NF-kB.	28,8
NP can affect mitochondrial function.	78

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of fine particles could still be toxic in the form of ultrafine particles. Later studies have demonstrated that the pulmonary inflammation, usually measured as the number of neutrophilic granulocytes (PMN) in bronchoalveloar lavage (BAL), is related to the instilled or inhaled surface area of particles,[8] although at similar surface areas some ultrafines seem to be more inflammatory than others.[26, 27] Among mechanisms by which NP could cause an enhanced inflammatory response, direct effects have been reported on alveolar macrophages such as inward leaching of Ca^{2+},[28] impairment of phagocytosis[29, 30] and cytoskeletal changes.[31]

The immediate effects that occur after NP deposition on the respiratory epithelium are not fully understood. Cells in contact with NP such as macrophages, epithelial cells and neutrophilic granulocytes, which are activated and known to synthesize compounds referred to as *reactive oxygen species* (including OH•, NO, hydrogen peroxide, superoxide), have evolved to kill invading microbes.[32] Within hours, cytokines and chemokines are synthesized and secreted into the affected area. These molecules are mediators that interact with specific receptors on the surfaces of many cell types and result in activation of local cells as well as those in the blood and other tissues. As a result, cells are attracted from the bloodstream and enter the fluid filled interstitial spaces, where they can attack the foreign material. Consequently, particle-induced cell activation events in the airways frequently result in an *inflammatory response*. This response includes both the activation of cells in the epithelium (including the production of the "pro-inflammatory" and reactive oxygen species (ROS) described above), and the activation and migration of cells (particularly, neutrophilic granulocytes and a related cell type, eosinophilic granulocytes) from the blood into the airways.

Epithelial and nerve cells may also contribute to airway inflammation by producing pharmacologically active compounds such as capsacein.[33, 34] In this *neurogenic inflammation*, stimulation of sensory nerve endings releases neurotransmitters which may affect many types of white blood cells in the lung, as well as epithelial and smooth muscle cells. Inflammatory cytokines synthesized by white blood

cells may also affect the nerve cells. Persistent or high inflammatory response may damage the epithelial cell layer at the surface of the tissue and other cells (such as macrophages), which can result in tissue damage and loss of function. Another potential consequence of exposure to NP may be reduced capacity to defend against microorganisms[35] or, in contradiction, an augmentation of allergic immune response to common allergens.[36] Thus, particle deposition on the respiratory epithelium can set off a cascade of events in many different cells, potentially resulting in diverse changes in tissues and organs at sites progressively further away from the initial stimulus. These defense mechanisms are normal responses in healthy individuals, but they may lead to deleterious changes in susceptible hosts or in normals if exposure is prolonged. Such changes may be rapid and temporary and may resolve quickly; but depending on the level and pattern of exposure and the agent to which the host is exposed, the changes may persist. It is not clear whether or how such changes are relevant to the development of NP-induced adverse health effects at low levels of exposure. These changes are thought to have a greater impact on individuals whose respiratory, cardiac or vascular tissues have been previously compromised. One possible consequence of damage to the airways is that the individual may become more susceptible to respiratory infections if exposed to viruses or bacteria (discussed in 35). A second possible consequence is that it may decrease respiratory function in a person whose airways are already damaged by conditions such as bronchitis or asthma. As a result, the symptoms of asthma, for example, may be exacerbated.

4.2. Pulmonary Carcinogenicity

The ability of low toxic poorly soluble particulates (PSP) such as carbon black and titanium dioxide (TiO_2) to induce chronic inflammation, fibrosis, neoplastic lesions and lung tumours in rats has been well established (reviews 37, 38). Lung tumors associated with experimental exposure to such particles are generally of two types, i.e., those originating from alveolar type II cells, called bronchoalveolar tumours, and squamous or epidermoid tumors which are associated to bronchiolarisation and are considered to arise from areas of squamous metaplasia.[39] The tumours include adenomas (BA), adenocarcinomas (BAC), squamous cell carcinomas (SCC), adenosquamous carcinomas and squamous keratinizing cysts (SKC). From the studies described above, it has become clear that NP (TiO_2, carbon black) induces lung tumors in rats at considerably lower gravimetric lung burdens than their larger sized analogues and the retained particle surface metric has been used to describe the lung tumor rate in chronic inhalation studies.[40] It is now generally accepted that the continued presence of high levels of non-toxic particle surface leads to the impairment of alveolar macrophage clearance, culminating in rapid buildup of particles, chronic inflammatory response, fibrosis and tumorigenesis, known as the so-called rat lung overload. The overall pattern is one of chronic inflammation that occurs upon saturation of lung clearance by overloading of macrophages[41] at which point particle accumulation starts and inflammatory cell influx increases sharply.[42] The inflammatory cell influx is held responsible for the lung tumors after chronic particle exposure to PSP due to their mutagenic activitiy and actions on cell proliferation.[43, 38] This point is illustrated by our findings in a chronic animal study where we compared tumor rates induced by different NP after treatment with the same gravimetric dose (Table III). The amount of lung tumors induced by three different insoluble NP is proportional to their surface area. Diesel particles with the lowest surface area (34 m^2/g) induced 22 tumours in 46 animals, while the carbon NP (300 m^2/g) induced 40 tumors in 45 animals. Important to note is the fact that the high-surface amorphous silica NP induced few lung neoplasms due to its high solubility *in vivo*.

Still, the surface dose concept is probably an oversimplification for several reasons. First, ultrafine particles at similar surface area appear to exhibit significant differences in inflammatory activity.[26] Secondly, it is unclear whether ultrafine particles have a different lung distribution between alveolar spaces, macrophages and interstitium,[25] and how relevant this is for tumor formation. In addition, ultrafine TiO_2 induced a higher tumor rate compared to fine TiO_2 after intratracheal instillation of relatively high doses, whereas the chronic inflammatory response at time of sacrifice (129 weeks) was similar to that observed after instillation of fine TiO_2.[44] Thirdly, at high local concentrations of ultrafine particles, these particles should be considered to penetrate target cells and exert direct genotoxic effects. Carbon black particles, but not their

Table III. Lung tumor incidence in rats 129 weeks after intratracheal administration of a 30 mg dose of three different NP with low-toxicity, untreated control rats and diesel exhaust particles as a positive control. The soluble NP aerosil caused three adenoma, while the other insoluble NP cause lung tumours dependent on the administered surface area.

Particle	Size (nm)	Surface area (m^2/g)	Tumors			
			Benign	Maligne	BA[1]	SCT[2]
Control	NA[3]	NA	0/91	0/91	0	0
TiO_2	30	50	16/45	24/45	22	18
Carbon Black	14	300	32/48	16/48	21	27
Diesel	ND[4]	34	21/46	1/46	8	14
Aerosil	14	200	3/39	0/39	3	0

Female Wistar rats (190 gr) were treated with multiple intratracheal injections (6 mg/injection) at weekly intervals to reach the final dose (30 mg). Control animals (Female Wistar, eight weeks) were untreated. Between 125 and 129 weeks of age, rats were sacrificed, lungs embedded and three lung lobes were cut in sections for histopathology. Tumors were evaluated by histopathological evaluation of two HE-stained sections per lobe. Abbreviations: [1] Sum of bronchial adenomas and adenocarcinomas,[2] Sum of squamous cell tumors, including squamous carcinomas and squamous keratizing cysts (SCT),[3] NA (not applicable), and[4] ND (not determined).

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extracts (median diameter: 100 nm), caused strand breaks in A549 (human alveolar epithelial cell line) and macrophage-like THP-1 cells at concentrations of 160 ng/ml,[45] while ultrafine TiO_2 (20 nm) induced micronuclei in Syrian Hamster embryo (SHE) cells at 1 $\mu g/cm^2$ during 12 hr incubation, with concomitant signs of apoptosis as well.[46]

5. SYSTEMIC EFFECTS OF NANOPARTICLES

Epidemiological studies have shown that patients with existing cardiovascular disease have an increased risk of death at increased levels of ambient PM.[5, 47] Theories that try to explain these effects can be discriminated into direct effects and indirect effects of PM, as illustrated in Fig. 4. The translocation of NP in the lung toward effector organs via the circulation is considered a important direct pathway for systemic effects of NP after inhalation.

5.1. Particle Translocation

Conflicting studies have been reported regarding particle translocation after inhalation or instillation of NP in the lung. Oberdörster and co-workers[48] observed rapid translocation toward the liver of more than 50% of ^{13}C NP (26 nm size) within 24 hours in a rat model. Kreyling et al.,[49] however, observed only minute (<1%) translocation of iridium NP (15–20 nm size) into the blood of rats, reaching not only the liver, but also the spleen, kidneys, brain and heart. Conflicting results in human studies are also reported. Nemmar et al.[50] demonstrated a rapid 3–5% uptake of radio-labeled carbonaceous NP into the bloodstream within minutes of exposure and subsequent uptake in the liver. In contrast, Brown et al.[51] could not find any detectable particulates (<1% of inhaled NP, limit of detection) beyond the lungs, and cleared fractions via airways and gastro-intestinal tract. However, Nemmar and co-workers[50, 52] emphasized, in their hamster model, the importance of surface properties such as charge, since polar surface components yielded different translocation rates across the respiratory epithelium into circulation. Apart from that study, little attention has been paid to surface characteristics and charges that could influence this process as is well known in drug delivery.[53] However, other studies of drug delivery across the blood-brain barrier further confirmed the importance of surface properties, showing that particle surface components may bind to the ApoEreceptor, which mediates crossing of this otherwise very tight barrier.[15] In addition, current discussion focuses on another transport function of the vesicular caveolae that transport from the luminal to the mucosal side of epithelial and endothelial cells. Transport within caveolae for macromolecules with molecular radii of several nanometers seems to exist across the alveolar–capillary barrier as a path-

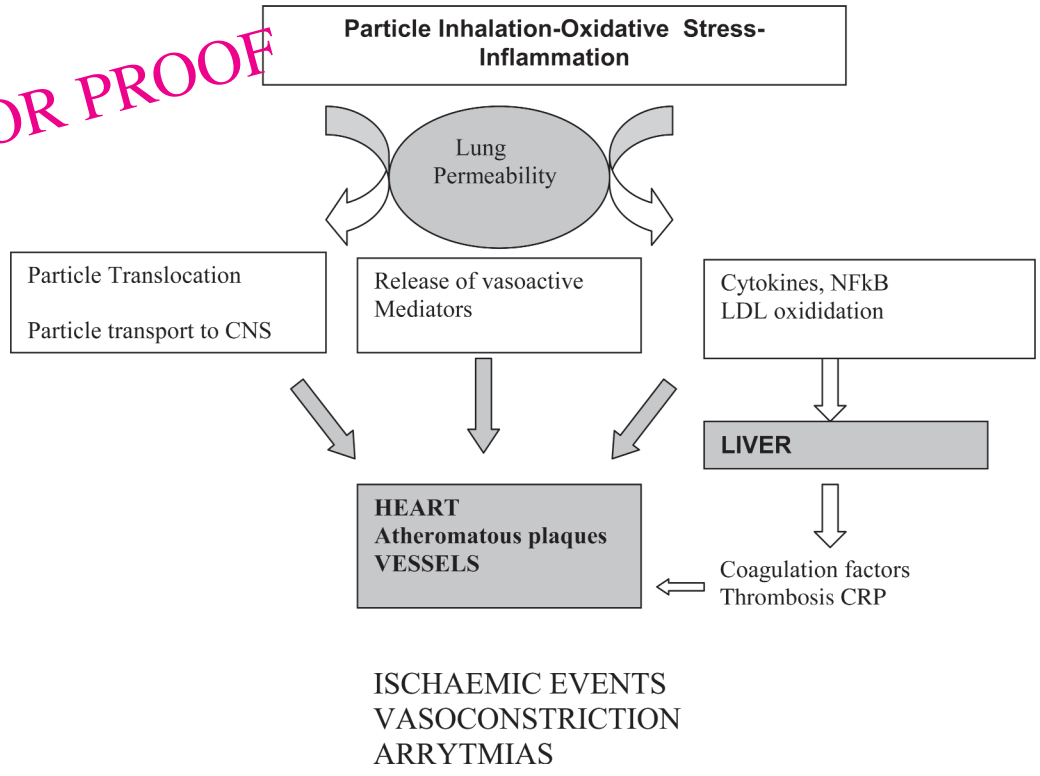

Fig. 4. Schematical presentation of mechanisms and their sequence, suggested to play a role in particle induced systemic effects, centrally mediated through lung permeability.

way for protein delivery from lung to blood. This might be another mechanism for solid UFP transport, given that the openings of the caveolae range between 0.04 and 0.1 μm.[54]

Another direct pathway of NP effects may be transportation toward extra-pulmonary organs via neurons, including trans-synaptic transport, as pointed out in a recent editorial.[55] Such a mechanism was first reported by Howe and Bodian[56] for 0.03-μm polio virus in monkeys and was later described for nasally deposited colloidal 0.05-μm gold particles moving into the olfactory bulb of squirrel monkeys.[57] Carbonaceous NP may translocate along the same pathway to the central nervous system (CNS) based on their presence in the olfactory bulb of rats after inhalation.[55]

5.2. Indirect Effects of NP Exposure

Among indirect effects, secondary to NP inhalation in the lung, inflammation has been considered to affect target organs by mediators that become systemically available (Fig. 4). However, inhalation studies with NP at particle numbers found in the general environment did not demonstrate pulmonary inflammation as described at higher doses. As discussed above, not all NP have similar particle surfaces, some NP maybe inflammogenic at low exposures. A number of mechanisms have been supposed that could be considered as indirect mechanisms:

- Seaton et al.[58] suggested that in susceptible individuals, exposure to NP will invoke alveolar inflammation, and that the release of inflammatory mediators can trigger systemic hypercoagulability of the blood, thereby increasing the risk for cardiovascular events.
- A second mechanism is the disturbance of autonomic imbalance by NP,[59] having direct effects on the heart and vascular function. This hypothesis has recently been reinforced by the finding that NP are transported along axial nerve endings to the brain after which they can affect CNS function.[55] Effects have also been found on large and small arteries after *in vivo* exposure to PM[60, 61]
- A third mechanism is the progression and destabilisation of atheromatous plaques by inhalation of PM.[22] Although this mechanism remains to be investigated using NP, NP properties should be able to invoke the same destabilisation mechanisms (inflammation, LDL oxidation, lipid peroxidation) as the PM used in earlier studies.

A large series of molecular epidemiological studies have variously supported aspects of the plausibility of the above mechanisms. One of the first studies[62] re-analysed blood parameters from a large multinational trial on cardiovascular risks (MONICA) performed between 1984 and 1988, and reported a higher blood viscosity during an air pollution episode that coincided with the survey in 1985. Initially, they reported a higher plasma viscosity,[62] and later, an increase in C-reactive protein,[63] an acute phase protein from the liver and a well-known risk factor for sudden cardiac death. Recent studies from the same research group in Erfurt (Germany) have identified NP as an important variable explaining cardiac deaths due to increased ambient particle exposure.[12] In fact, the association increased as the particle size decreased, and individuals with cardio-vascular diseases were more likely to die than others.

Other epidemiological studies showed that elevated exposure to ambient pollution is associated with the triggering of myocardial infarction,[64] changes in cardiac rhythm and autonomic function,[65] or endothelial dysfunction.[60] Furthermore, in a hyperlipidaemic rabbit model, exposure to inhaled particles induced a morphology of atherosclerotic plaque that is predisposed to rupture.[22] Clearly, further research is needed, but the research reported to date has direct relevance to public-health policy, since both coal-burning and traffic emissions continue to be major sources of particulate exposure worldwide. Hoek and colleagues[6] followed 5,000 adults aged 55–69 years over 8 years and determined the relative risk for cardiopulmonary mortality associated with ambient particulate exposure dominated by carbonaceous NP originating from traffic emission. Living within 100 m of a highway or within 50 m of a major road was associated with a significantly increased relative risk of 1.95 (95% CI 1.09–3.51). Furthermore, a ban on coal sales in Dublin resulted in a 35·6 $\mu g/m^3$ (70%) reduction in particulate air pollution as measured by black smoke. Clancy and coworkers[66] took advantage of this opportunity with their straightforward analysis. Adjusted death rates for respiratory and cardiovascular deaths declined by 15·5% and 10·3%, respectively, as a result of decreasing pollution. Clancy and colleagues reasonably discuss alternative explanations for the decline in mortality. Yet, the results are extremely suggestive that NP of carbonaceous soot, sulphur oxides, or a combination of both, have a deleterious effect on the cardiopulmonary system.

6. SCREENING HAZARDS OF NP IN THERAPEUTIC APPLICATIONS

Since one of the anticipated applications of NP lies in novel drug delivery techniques, NP will be used to target drugs to specific tissues and to increase their biological half life. Also, a number of particles are used for imaging purposes to visualise extravasation or tumor vascularisation. A successful NP for drug conjugation and delivery must be able to have its pharmacokinetics modified at the organ and cellular level to promote increased delivery or efficiency, while minimising exposure of sensitive normal tissues to the drug and avoiding toxic effects of the NP carrier.

The discussion among toxicologists as to the fraction of NP that translocates from the lung into the blood is,

therefore, less relevant. In fact, the lung as a barrier is bypassed in most applications and these NP should be considered to reach all target cells of the cardiovascular system, hepatic system and kidney, as well as interact with the pool of immune competent cells. Several cells and/or receptors on these organs have been found sensitive to NP or NP-constituents, and NP can disturb normal cell function and its autocrine or paracrine signalling in various ways. This is schematically illustrated in Fig. 5, which shows a cell with its receptors as contacts to the outer world and its intracellular machinery. NP can be internalised in cells (see Fig. 3) and, therefore, should be able to interact with signalling processes. The selection of an NP for drug delivery purposes follows guidelines as defined for other bio-materials, which are based on their biocompatibility as measured by platelet adhesion and activation, neutrophil attachment, angiogenesis and cell spreading.[67] Based on the observations of NP after inhalation, we suggest exploration of the concept described previously for inhaled particles and the special case of NP. A summary of proposed, partly alternative methods can be found in Table IV. Special attention is given to two types of testing that are not standard in any guideline for NP, but may be highly relevant. The first is the potential interaction of NP with endogenous proteins, and the second is testing for particle durability.

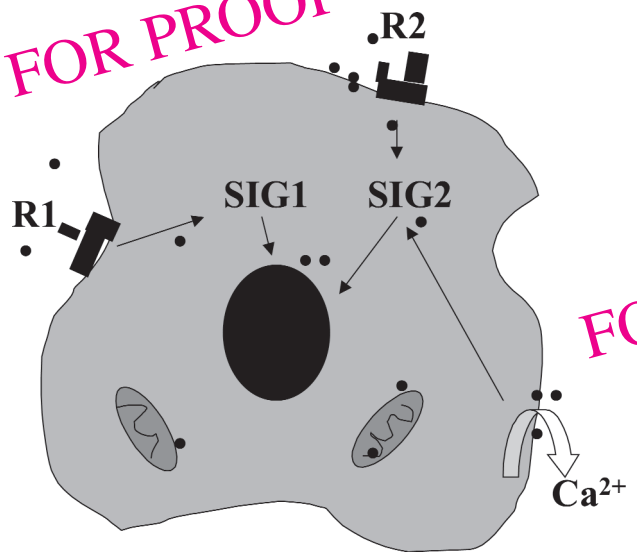

Fig. 5. Schematic illustration as to how NP can theoretically interfere with normal processes in the cell. On the cell membrane, NP can bind or interfere with normal agonist binding to receptors (R1, R2). Consequently, they can block/activate downstream signalling pathways (SIG1, SIG2) that, in turn, switch on other intracellular processes. Alternatively, NP can directly affect SIG1 or SIG2 by absorption or radical generation after endocytosis and release into the cytoplasm. These events, along with ionic homeostasis (e.g., Ca^{++}), regulate normal cell function. NP have also shown to cause mitochondrial damage and nuclear changes, although it remains unsure whether NP can translocate into these subcellular compartments.

6.1. Protein Binding and Complexation

Apart from being used as protein carriers in drug therapy, one should consider that after release of the carrier protein, NP can interact with endogenous proteins at the site of deposition. NP below 40 nm have a size similar to large proteins and, therefore, we hypothesize that:

- NP may form complexes with endogenous proteins
- depending on NP surface properties, NP may complicate different endogenous proteins
- different NP-protein complexes may have different biokinetics, including translocation across membranes
- endogenous proteins of these complexes may have a different activity or even a different function.

Preliminary studies using different ultrafine particles indicate different binding to a number of proteins in native rat broncho-alveolar lavage fluid (Semmler & Kreyling, unpublished observations). If these preliminary studies are confirmed, the biokinetics of different NP-protein complexes could provide the basic explanation for the different observed translocation patterns described by the various studies as reviewed in chapter 5. At the same time, functional changes of proteins of such complexes may be another mechanism by which particularly small NP, with their large surface area as a binding interface, may induce protein mal-functioning, which may lead to the pathogenesis and adverse health effects. Figure 6 illustrates some of the possible scenarios.

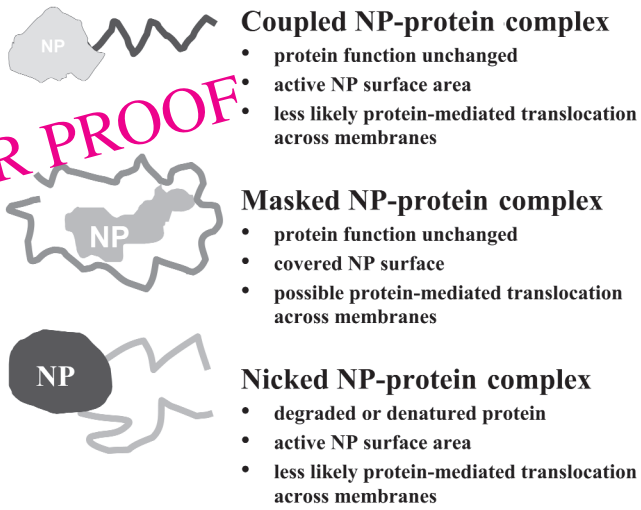

Fig. 6. Schematic presentation of potential interactions between Nanoparticles and proteins. The first example shows the intended (covalent) binding of a protein to an NP as a drug-delivery tool. The second example shows how proteins may absorb on the NP surface, thereby masking the particle properties and loosing functional protein. The third example shows how NP can bind and breakdown proteins through their active surface area.

Table IV. Overview of test methods that can be used to explore potential hazards of NP particles before their use as drug delivery tools.

Hazard	Test system	Biomarker
Acute damage and responses after iv administration	Red blood cells	Hb in supernatant
	Whole blood system	Release of inflammatory markers (IL-8, TNF, IL-6)
		Release of blood coagulation factors
Acute phase response (APR)	Hepatocytes, lung cells, liver perfusion	Fibrinogen
		C-reactive protein factor VII
		Heat shock proteins
Increased permeability	Endothelial or epithelial cell layers	Permeability of H_2O or DTPA
Destablisation of atheromatous plaques	Watanabe-rabbit, Apo-E-/- mice	Plaque morphology, CRP serum
Effects of autonomic nervous system	Langendorff heart vascular strips	Altered response to endogenous mediators
Adjuvant activity	Rat Ovalbumin sensitisation	IgE formation
Immune effects	PLN assay	T-cells and cytokine profiles
Surface activity	EPR, DCFH	Radical formation (OH, O_2, H_2O_2)
Oxidative stress	Alveolar macrophage, epithelial cell	Isoprostane, radical formation (O_2, H_2O_2)
Absorption of endogenous proteins	2D-gel imaging	
Toxicity	Various cell types, primary and lines	LDH release
		MTT conversion dye-exclusion
Biopersistence	Durability/Dissolution in flow-through cells	

Abbreviations: IL-8, interleukin-8; TNF, tumor necrosis factor; Il-6, Interleukin-6; DTPA, probe to measure permeability; CRP, C-reactive protein; Apo-E, Aplolipoprotein-E; PLN, Poplithal Lymph Node assay; EPR, Electron Paramagnetic Resonance; DCFH, Dichlorofluorescine; 2D, 2-dimensional; LDH, lactate dehydrogenase; MTT, dimethylthiazol-diphenyltetrazolium bromide.

6.2. Durability

Studies on the pathogenicity of fibers have provided information about the physicochemical factors which determine the pathogenesis of synthetic fibers in general. Longer fibres (>20 μm) are difficult to clear by phagocytosing cells and, therefore, clearance of these fibres is mainly determined by their biopersistence or durability. Both the half-life after instillation in rat lungs, as well as the solubility of solid materials in flowthrough or static procedures, have been used to describe successfully biopersistence in the lung.[68] Therefore, the NP, or the skeleton, should be biodegradable or soluble. If the skeleton is not degradable at all, the molecular weight of the co-polymers should be limited to <40,000 Dalton to ensure renal elimination as a back-up for clearance. The solubility of synthetic vitreous fibres has been related to the chemical content and ranked in terms of solubility. For fibres, dissolution is measured in simulated organ fluids in flowthrough dissolution systems, in which physiological fluids are passed through a sample of fibres.[69] Similar principles or test systems could be used or developed for NP to mimic the biopersistence in various target organs such as kidney and liver. Dissolution rates should also be measured using fluid at pH 4.5 which simulates the acidic pH of the phagolysosomes in macrophages. Extensive testing of particle cores and various surface modifications should, in the end, lead to empirical relations between particle composition, *in vitro* dissolution and *in vivo* retention.[68] Criteria for dissolution and/or *in vivo* retention can then be developed based on links between durability and several potential endpoints of chronic NP administration.

7. CONCLUSION

A major gap of communication has been present so far between know-how on Nanoparticles studied for the application of NP as drug carriers and particle toxicology and epidemiology. One discipline is trying to develop NP that deliver drugs to target organs with a high specificity and long-term effect by increasing half lives of the drug. The other is intensely seeking mechanisms of local and systemic toxicity associated with exposure to NP. Whereas toxicology is trying to understand the mechanisms of NP translocation and how these minute amounts of NP might invoke systemic response, pharmacology intends to use NP for systemic delivery of drugs. Therefore, translocation from lung to blood and other organs is a mutual area of research and a valuable interface for exchange of know-how. In this review, we have indicated what effects of NP have been found by toxicologists and epidemiologists, and how this know-how could be used to develop screening for safe NP drug delivery.

Bio-compatibility determines the success of combining both worlds. The success of an NP in drug delivery is dependent on its acute and chronic interaction with biological systems where they deposit or interact. Interactions between cells and NP are mediated by the surface characteristics of both the material and the target cells. On one hand, proteins, extra-cellular matrix and cell recognition play an important role. On the other hand, the physicochemical properties of NP, including their size, play an important role in the pharmaceutical and dynamical phase of the NP-drug conjugate. However, NP might elicit a biological response in one tissue (e.g., bone), but not in

another (e.g., blood). In addition, inhalation toxicology tells us that NP usually invoke responses in those with existing diseases. Since drugs are primarily used in those with diseases, it should be stressed that toxicological testing of NP should be done in various models that reflect human diseases. Therefore, it is recommended that a close interaction between both areas of research be established, and that this exchange will lead to screening methods that can be used to develop both safe NP for drug delivery and a better understanding of NP toxicology after inhalation.

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