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Cellular Targets and Mechanisms in the Cytotoxic Action of Non-biodegradable Engineered Nanoparticles

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Abstract

The use of nanoparticles (NPs) has improved the quality of many industrial, pharmaceutical, and medical products. Increased surface reactivity, a major reason for the positive effects of NPs, may, on the other hand, also cause adverse biological effects. Almost all non-biodegradable NPs cause cytotoxic effects but employ quite different modes of action. The relation of biodegradable or loaded NPs to cytotoxic mechanism is more difficult to identify because effects may by caused by the particles or degradation products thereof. This review introduces problems of NPs in conventional cytotoxicity testing (changes of particle parameters in biological fluids, cellular dose, cell line and assay selection). Generation of reactive oxygen and nitrogen species by NPs and of metal ions due to dissolution of the NPs is discussed as a cause for cytotoxicity. The effects of NPs on plasma membrane, mitochondria, lysosomes, nucleus, and intracellular proteins as cellular targets for cytotoxicity are summarized. The comparison of the numerous studies on the mechanism of cellular effects shows that, although some common targets have been identified, other effects are unique for particular NPs or groups of NPs. While titanium dioxide NPs appear to act mainly by generation of reactive oxygen and nitrogen species, biological effects of silver and iron oxide are caused by both reactive species and free metal ions. NPs lacking heavy metals, such as carbon nanotubes and polystyrene particles, interfere with cell metabolism mainly by binding to macromolecules.

Keywords

Cytotoxicity; genotoxicity; lysosomes; metal nanoparticles; metal oxide nanoparticles; mitochondria; plasma membrane; reactive oxygen species

INTRODUCTION

Nanotechnology is regarded as one of the key technologies of the 21st century. Nanoparticles (NPs) are generally particles smaller than 100 nm in one dimension, but for targeted drug delivery in medicine larger particles are also included. Nano-size materials change their physical and chemical properties and have improved and innovated a variety of industrial, pharmaceutical and medical products. On the other hand, however, materials being innocuous in bulk form often become cytotoxic when they reach nano-size. These adverse cellular effects have been attributed to the high chemical surface reactivity of NPs. While environmental NPs are present in nature and produced unintentionally by humans, engineered NPs are developed for a specific purpose. Natural NPs include organic colloids

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(humic, fulvic acids), viruses, soot (fullerenes, carbon nanotubes), organic acids, magnetite, silver (Ag), gold (Au), Fe-oxides, allophane and sea salts, anthropogenic NPs are combustion by-products, soot (carbon black, fullerenes, functionalized carbon nanotubes (CNTs), platinum group metals, titanium dioxide (TiO_2), silica (SiO_2), Ag, iron, metal-phosphates, zeolites, clays, and ceramics [1]. Similar materials can be found in environmental and engineered NPs but physico-chemical properties of engineered NPs are usually better characterized, allowing a better correlation between particle properties and cellular effects. NPs can be classified into biodegradable and non-biodegradable. The correlation of toxic effect to presence of the NPs is much easier for non-biodegradable than for biodegradable NPs since the former can be detected by more techniques and observed for longer periods. In addition, biodegradable NPs, for instance liposomes, poly(lactic-co-glycolic), and albumin NPs, in general act less cytotoxic and less data are available for the analysis. In the case of cytotoxicity, however, identifying the link of the effect to the localization of biodegradable particles is more difficult because cytotoxicity may also be caused by degradation products and, therefore, independently from the particle structure. Similar reasons apply for loaded NPs and NPs with functional coatings or targeting groups. For these particles, the link between cytotoxicity and particle properties is less clear. If, for instance the particle is coated with a targeting macromolecule, the observed effect could be caused by the particle with targeting molecule or by the particle after removal of the target group. In the case of loaded NPs, the payload has an effect on its own. For the reasons mentioned above, this review will focus on basal cytotoxicity of non-biodegradable NPs, which are neither loaded with active molecules nor functionalized with high molecular targeting groups.

Studies on a panel of NPs revealed that biological effects are influenced by a variety of particle parameters. As a general rule, small size, fibrous shape, positive surface charge, crystalline structure of the surface, and content of heavy metals are linked to greater cytotoxicity. It was also noted that NPs did not share a common mode of action and that both the type of cell damage (e.g. necrosis, apoptosis, inhibition of proliferation) and the underlying mechanism for the same type of cell damage showed particle-related differences (e.g. induction of apoptosis by destabilization of mitochondria or by disruption of lysosomes). Furthermore, in the biological context, the type of cell used for the assessment and the kind of medium or buffer, particularly the presence or absence of proteins, influence the biological effect. Most data were obtained by studies on TiO_2, SiO_2, zinc oxide (ZnO), Au, Ag, CNTs, quantum dots, fullerenes, and iron oxide particles (Fe_2O_3 or Fe_3O_4). Data on these particles are very important since the majority of products used in consumer products, healthcare, and medicine are based on these non-biodegradable NPs.

Evaluation of Biological Effects

Basal cytotoxicity means impairment of cellular functions by disruption of plasma membrane integrity, interference with organelle function, and disruption of the cytoskeleton. In addition to basal, also called general cytotoxicity, two other forms of cytotoxicity can be discerned, selective cytotoxicity and cell-specific function toxicity [2]. Selective cytotoxicity occurs if some cells are more sensitive to the toxicant, for instance as a result of biotransformation, specific receptors, or uptake mechanisms. Cell-specific function toxicity alters the cell in a way that is critical for the organism as a whole. Basal cytotoxicity screening is part of the initial evaluation of a chemical, drug or NPs. The testing procedure and most of the assays have been established for the assessment of conventional compounds in drug development. When these assays were first used on NPs, the striking difference to conventional compounds was recognized.

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EXPOSURE

Conventional chemicals can be applied in any solvent provided this solvent is not toxic. For the cytotoxicity of NPs, however, the suspension medium plays an important role in the observed biological effect. The presence of protein, either as albumin or in complex form as serum, in the incubation medium reduces cytotoxicity for many NPs. Polystyrene particles and iron oxide NPs acted less cytotoxic on non-phagocytic cells in the presence of serum [3]. Coating with protein or other macromolecules occurs within seconds when the NPs come into contact with biological fluids and leads to an irreversibly bound 'hard protein corona' and a weakly bound 'soft protein corona' [4]. The composition of this corona determines the biological effects of the NPs. On the other hand, binding of protein changes the physico-chemical properties of the particles. Protein effects include instability of the suspension with formation of larger NP aggregates in the presence of proteins, masking of the reactive surface of the NPs preventing the interaction of the NPs with the plasma membrane, anti-oxidant effects of the serum, lower cellular uptake, and neutralization of the increased sensitivity of serum-deprived cells.

When conventional compounds are dissolved in the exposure solution, they reach the cells by diffusion. NPs, by contrast, can get into contact with cells by sedimentation (aggregated NPs) and by diffusion (single particles). The relation of these processes is predominantly size-dependent and difficult to determine [5]. A variety of indications are used to indicate NP exposure doses. Mass, number or surface area of particles per volume or mass, number or surface area of particles per exposed cell area are most often employed. These measures, however, give no indication on the amount of NPs that are actually in contact with the cells. Several more recent studies calculated the deposited rate of spherical NPs using *In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) models [6]. The ISDD model by Hinderliter *et al.* used temperature, media height, particle size in solution, agglomeration state and particle density to calculate the deposition of fluorescent carboxylated polystyrene particles, iron oxide, TiO_2, SiO_2, and Au particles in a size range of 30-1000 nm. Particle-dependent minimal deposition was seen between 50-200 nm for all particles, while larger and smaller particles deposited at higher rates. In physiological medium, NPs tend to form agglomerates, which have a marked influence on the deposition rate. Measured deposition of 50-1000 nm plain polystyrene particles on macrophages increased over time and showed a minimum for 100 nm particles [7]. The location of the cells in the well has also an effect on particle deposition; Cho *et al* showed that cells cultured upside-down ingested much less NPs than cells cultured in the standard orientation [8]. It is, therefore, unlikely that cells in suspension take up the same amount of NPs as adherent cells. In order to solve these problems, it is recommended, whenever possible, to measure cellular particle content experimentally and to use this value as base for cytotoxic evaluation.

The selection of the relevant exposure dose poses an additional problem for testing. Conventional cytotoxicity testing employs high doses (about 10 times the expected dose) for maximally 72h in order to identify the therapeutic window of a drug. For NPs, concentrations as high as 100 μg/ml can be reached in drug applications, for environmental exposure the expected doses are much lower. On the other hand, repeated or prolonged exposure or cellular accumulation, one of the typical characteristics of NPs, may take place. While conventional compounds are metabolized and excreted from the cells quite fast, non-biodegradable NPs may persist in animals for prolonged periods. After one single injection of quantum dots, the particles could be detected in mice over a period of 2 years [9]. The use of two-photon spectral microscopy revealed that the particles were metabolized but not excreted. Other NPs, such as iron oxide NPs and CNTs, can slowly be degraded in lysosomes [10] or by myeloperoxidase present in neutrophilic granulocytes [11], respectively. Dissolution of metallic NPs, for instance in lysosomes, can reduce the

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concentration of intracellular NPs but release toxic ions. NPs lacking heavy metal content, positive charge, or reactive surface characteristics, e.g. carboxylated polystyrene particles, can persist in lysosomes. After 3 days they only slightly decreased the activity of lysosomal enzymes and increased lysosomal pH [12]. When exposed repeatedly to low concentrations of small polystyrene particles, cell viability was significantly decreased after 4 weeks, suggesting that accumulation may induce cell damage independently from lysosome damage [13]. In addition to degradation, cells have the possibility to excrete NPs by exocytosis. Exocytosis has been demonstrated for quantum dots, Au NPs, and 30 nm polystyrene NPs [14]. The rate of Au NP exocytosis by HeLa cells was size-dependent; while 35% of the smallest (14 nm) NPs were excreted after 1h, 95% of the 74 nm ones persisted in the cells [15]. It appears that only NPs not ingested in lysosomes undergo exocytosis, while NPs in lysosomes don't [14c]. Since integration of NPs in lysosomes increases over time [12, 16], reaching about 50% for polystyrene particles and SiO_2 NPs after 24h, removal of NPs from cells by exocytosis should be effective only after very short exposure times. Even when excreted from the cells, the fate of the released NPs is unclear and re-uptake appears likely. Due to metabolic and physical changes of NPs in the culture, it is not clear if higher concentrations of NPs for short duration can mimic the effects of lower concentrations for prolonged periods.

CELLULAR MODELS

Cell lines are most often used for cytotoxicity screening since they produce more reproducible results than primary (freshly isolated) cells, differing in quality between preparations. Most standard cell lines are derived from tumors and have escaped cellular senescence. Cell lines can be generated either by selection of normal cells undergoing spontaneous random mutagenesis, by introduction of a viral gene that de-regulates the normal cell division cycle (e.g. SV40 T), or by artificial expression of key proteins required for immortality (e.g. telomerase). Independent from cell line generation, the immortalization per se alters the cellular metabolism by circumventing cellular senescence. A few reports noticed differences in cytotoxicity between cell lines from normal and transformed cells for NPs. Iron oxide NPs, for instance, reacted cytotoxic in the lung cancer cell lines A549 and not in the fibroblast line IMR-90 [17]. Given the fact that pronounced differences in NP cytotoxicity between cell lines are common [3a], and that also cell lines derived from normal cells are altered by the immortalization process, it is questionable if the observed differences are exclusively due to the fact that the A549 cells are derived from the tumor cells and the IMR-90 from normal cells. Different cytotoxicity in cell lines and primary cells are not unusual in cytotoxicity testing of conventional compounds, but differences are usually not very pronounced [18]. In contrast to conventional compounds, cell type differences (phagocytes vs. non-phagocytic cells) have been identified for particles; larger polystyrene particles and thicker CNTs were not cytotoxic to non-phagocytic cells, while they still induced cell damage in phagocytes [19]. It is hypothesized that the generation of reactive oxygen and nitrogen species, which accompanies phagocytosis of large particles causes the observed cell damage.

Conventional cytotoxicity testing uses cells grown as monolayers on plastic surfaces (2D culture). Although this form of culture is relatively easy to perform, suitable for high-throughput screening, allows a good control of the exposure conditions, and is relatively inexpensive, growth on impermeable plastic surface creates artificial changes in the cells. Production of extracellular matrix and intercellular contact is low and proteins associated with tight junctions, such as E-cadherin, occludin, β-catenin, etc., are down-regulated in these 2D cultures. Tight junction proteins, together with collagen type IV and mucins, in contrast, are highly expressed and correctly localized in spheroid cultures [20]. The observed differences may explain why several drugs, such as cisplatin, 5-azacytidine,

emodin, mitoxantrone, which were highly active in 2D culture, lacked efficacy in 3D culture. Better differentiation and tighter intercellular junctions may also explain why small polystyrene particles acted much less cytotoxic when added to cells cultured on microbeads compared to cells cultured on plates [13]. Spheroid cultures can mimic the passage of compounds through the cell layer to reach cells in organs. While 5-fluoruracil killed cells in the outer layer of spheroids, the hypoxia-activated cytotoxin tirapazamine was more active against cells inside the spheroid [21]. According to preliminary observations, passage through cell layers dramatically abolished cytotoxicity of small polystyrene particles, indicating that conventional cytotoxicity screening overestimates the cytotoxic potential of NPs. The lower sensitivity in 3D culture and the lack of mucus or other protective layers suggest that cytotoxicity in-vivo is lower than determined by conventional cytotoxicity testing. The importance of cellular uptake in cytotoxicity was demonstrated by a comparison of phagocytic and non-phagocytic cells. Non-phagocytic cells, showing reduced cytotoxicity, ingested a lower amount of particles, while phagocytes with a generally higher uptake of serum-coated particle uptake showed increased cytotoxicity in the presence of serum [22].

Not all cytotoxicity screening assays available on the market are suitable for the evaluation of NPs. Conversion of assay compounds by NPs, interference with photometrical, chemoluminescent or fluorescent detection methods, and binding or inactivation of metabolites are some of the most important examples for the interference of NPs with screening assays [5, 23]. For a relevant assessment of cytotoxicity, appropriate controls to identify potential interaction with NP are required. Most often, the use of more than one cytotoxicity assay is recommended. If interference is identified in the evaluation, an alternative screening assay, modification of the assay procedure, or inclusion of NP-specific controls, such as color control, may be appropriate measures. If, for instance, interference with the commonly used formazan bioreduction assay MTT by chemical reactivity of the NPs is detected, the measurement of alternative assays, such as ATP-content, quantification of protein or of DNA can be tried. Alternatively, if the presence of colored NPs associated with the cells is the source of interference, the cells can be washed prior to incubation with tetrazolium salt. When the soluble formazan salt (reaction product) is formed, the supernatant is transferred to another plate for evaluation.

Despite specific limitations for the assessment of NPs, conventional cytotoxicity assays will remain a state-of-the-art procedure to assess cellular effects of NPs since these in-vitro assays have a relatively good predictive value for toxic effects in-vivo [24].

After identification of adverse cellular effects by cytotoxicity screening assays, the type of cell damage is determined with assays for apoptosis, necrosis, and inhibition of proliferation to identify the mode of action of the compounds or particles. It turned out that, even when NPs induced a similar type of cell death, the underlying mode of action could be different. TiO_2 and carbon black NPs induce apoptosis by activation of caspase within 2h; TiO_2 by destabilization of mitochondria and carbon black by rupture of lysosomes [25].

Mechanism of Cytotoxicity

The influence of multiple parameters on the cytotoxic action prompts the testing of each particle type. The identification of common principles of toxic action could be helpful in the identification of toxic NPs and prediction of toxicity. Generation of reactive oxygen and nitrogen species (ROS) and action by dissolved ions were identified as common mechanisms for metal and metal oxide NPs and will be discussed in more detail. Interactions with proteins are involved in the cytotoxicity of NPs, both by changing their physico-chemical parameters and as targets for cytotoxicity. These effects are described in

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subsections of ‘Exposure’ (see above) and ‘Cellular targets, intracellular proteins’ (see below).

REACTIVE OXYGEN (ROS)

Reactive oxygen species are defined as molecules containing one or more oxygen atoms, which are more reactive than molecular oxygen. The most common ROS are superoxide radical, hydroperoxyl radical, hydroxyl radical, nitric oxide, and hydrogen peroxide. Reactive nitrogen species, mainly peroxynitrite, nitrogen dioxide, derive from reactions between superoxide and nitric oxide radicals. The average life-lives of these species is inversely related to their reactivity; the most reactive hydroxyl radical has a half-life of 10^{-9} seconds, while the less reactive superoxide radical has a half-life of 10^{-6} seconds.

ROS originate in the cell mainly from enzymatic activity of myeloperoxidase and cytochrome P450 enzymes, oxidases and flavoproteins in the peroxisomes, of NADPH oxidase at the plasma membrane, of cytoplasmic xanthine oxidase, from autooxidation of hemoglobin (Fenton reaction), riboflavin and catecholamines, transient metals, and from the electron transport chain of the mitochondria (Fig. **1a**). NO is synthetized by NO synthase expressed in endothelial cells, macrophages and neuronal cells. In the following the term ROS will be used for all reactive (oxygen and nitrogen) species. External factors for ROS generation include photoactivation and particle dissolution. In physiological concentrations, ROS are important regulators of cellular processes, such as growth, survival, proliferation, cell cycle arrest, apoptosis, differentiation, migration of cells, inflammation, and changes of extracellular matrix. Pathologically increased ROS cause oxidative damage of lipid (lipid peroxidation), protein (modification of amino acids, fragmentation and aggregation of proteins) and DNA (mutations and altered gene transcription) (Fig. **1b**). When intracellular levels of ROS rise to pathological concentrations antioxidant enzymes such as heme oxygenase 1, superoxide dismutase, catalase, and glutathione peroxidase are induced as the first line of defense. At higher ROS levels activation of pro-inflammatory signaling pathways, such as the JNK and NF-κB cascades, occurs. Higher and more prolonged oxidative stress levels induce cellular perturbation and result in a decrease in mitochondrial membrane potential, leading to cell death [26].

NPs may increase ROS levels by depletion of ROS scavengers (for instance by binding to these scavenger molecules), by reactivity at the NPs surface, by transition metals (as material or as contaminant), and by influencing intracellular production, e.g. by interaction of NPs with lysosomes and mitochondria. Cu, TiO_2, and iron oxide NPs produce ROS in acellular systems [27]. The reductive environment of the cytoplasm inhibits this reaction so that lower concentrations were measured in the presence of cells than in acellular systems [27a]. Highly reactive hydroxyl radicals may arise at the surface of NPs. Silanol groups at the surface of iron oxide NPs are able to lyse plasma membranes [28]. ROS production by transition metal oxides is presumably caused by oxygen vacancies on their surface. Charge-accepting NO_2 or O_2 molecules in vicinity to these vacancy sites may lead to de-coupling of the molecules and initiate oxidative stress. Huang *et al.* hypothesized that metallic and semiconducting properties of NPs were involved in this effect [29]. Metallic NPs may generate ROS by two types of reactions inside the cells. The Fenton-type reaction describes the reaction of metal ions with hydrogen peroxide to produce an oxidized metal ion and a hydroxide radical. The Haber-Weiss-type reaction involves an oxidized metal ion, reduced by superoxide, and hydrogen peroxide to generate oxidized metal ions and hydroxyl radical (Fig. **2**). The Fenton-type reaction is particularly seen for iron and copper NPs, while chromium, cobalt, and vanadium NPs can be involved in both types of reactions [30].

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FREE METAL IONS

For several metal and metal oxide NPs, cytotoxicity cannot be attributed solely to ROS generation.

Quantum dots, Ag, ZnO, and iron oxide NPs, presumably act predominantly by free ions. When NPs are dissolved, intracellular concentrations can rise higher than the respective IC_{50} values for the free ions. Cytosolic and mitochondrial Zn^{2+} concentrations, for instance, increase significantly at NP sizes of <50 nm [31]. Increased Zn^{2+} concentrations inhibit cellular respiration [32], and Ag^{+} ions act on the same sites as Ca^{2+} resulting in an increase of Ca^{2+} from sarcoplasmic reticulum [33]. To identify the role of free metal ions in the toxicity of NPs, assessment of pH-dependent leaching of metal ions and inclusion of the respective salt concentrations in the experiments is recommended.

Dissolved Fe^{2+} from iron oxide NPs acting by generation of highly reactive hydroxyl radicals through the Fenton reaction appears to be less important for the cytotoxic effect than hydroxyl radicals generated at the NP surface [28b]. Release of metal ions can also influence gene transcription. Iron response elements are present on transferrin receptor mRNA and on ferritin mRNA and allow binding of iron response element binding proteins. Iron blocks the binding in these proteins and leads to degradation of mRNA for the receptor [34]. Iron ions released from iron oxide particles by this mechanism can reduce translation of transferrin receptor mRNA.

The effect of metal ions must also be taken into account when working with non metal NPs, where contamination with heavy metals from the preparation may persist, for instance for CNTs. Contamination with nickel, cobalt and gold was significantly correlated to the ability of multi-walled CNTs to induce apoptosis in lymphocytes [35].

Cellular Targets

Cellular plasma membrane, lysosomes, mitochondria, nucleus, and intracellular structural (cytoskeleton) and globular proteins were identified as important targets for NP-based cytotoxicity (Fig. **3**, Table **1**).

PLASMA MEMBRANE

As a physical barrier between cytoplasm and extracellular space, the plasma membrane serves as a portal of entry to NPs. This entry can take place via active mechanisms (endocytosis), by passive diffusion without persistent membrane damage, or by disruption of plasma membrane integrity. Holes in the plasma membrane smaller than 1 μm in diameter can be sealed, and, therefore, NPs can cross the plasma membrane passively without causing manifest cell damage. Membrane disruption with subsequent re-sealing has, for instance, been reported for cellular entry of 14 nm SiO_2 particles [36]. Carboxylated polystyrene particles appear to generate plasma membrane discontinuities in a similar order of magnitude as 0.1% Triton ×100 [37]. Disruption of membrane integrity may occur either by direct mechanical action on membrane components, mostly to membrane lipids, as for Au NPs [38], or by generation of ROS with subsequent oxidation of membrane lipids, for instance, by iron oxide NPs and SiO_2 NPs [28]. Mechanisms for non-plasma membrane-disruptive entry of NPs into cells are changes in membrane fluidity [39]. Although zwitterionic quantum dots increase membrane fluidity, decrease of membrane fluidity as a result of lipid peroxidation accompanies the entry of TiO_2 NPs. Au NPs are transported encapsulated in lipid vesicles through plasma membranes [40]. SiO_2 NPs disrupt the plasma membrane when the lipid membrane wrapping capacity is exceeded. Alternatively, the strain caused by coverage of the NPs by lipid membrane is released by transient pore formation

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[41]. Physical interaction with membrane compounds plays an important role in NP penetration of cells. Cationic gold NPs create defective areas across the entire surface of the outer leaflet of the bilayer and a hydrophilic pore with highly disordered lipids at the edge is formed [38].

Interaction of NPs with plasma membrane proteins may influence membrane receptors, NADPH oxidase, and ion channels. Activation of the epidermal growth factor (EGF) signaling cascade has been demonstrated for carbon black and carbon NPs [42], but other NPs also act via receptor tyrosine kinase cascades (RTKs). RTK signaling cascades are relatively well studied since constitutional activation of these pathways is involved in a variety of diseases, particularly in cancer. Upon binding of the ligand, usually a growth hormone or a cytokine, these receptors dimerize and regulate cell cycle progression, proliferation, survival, differentiation, inflammation, migration, and apoptosis through a series of kinases and phosphatases (Fig. **4**). Small negatively charged superparamagnetic iron oxide NPs activate the same signaling pathways as EGF [43]. This activation was shown to occur in the absence of serum and, therefore, appears to be a direct particle effect. Multi-walled CNTs, carbon black and SiO_2 particles can activate platelets through activation of PLC [27b], carbon NPs act via Ras/Raf/ERK signaling [42a], and Ag NP hydrogels induce genotoxicity by JAK/STAT signaling [44], while activation of PI3K/PDK1 pathway and of Src appears to be caused by reactive oxygen species, not by receptor binding of the NPs [45].

NADPH oxidase is a membrane protein that transports electrons from the cytosolic site across the membrane to molecular oxygen. Upon activation by a variety of stimuli, such as cytokines, mechanical stress, hyperlipidemia, thrombin, growth factors, etc., the polypeptides p47phox, p67phox, and p40phox translocate from the cytosol to the inner membrane layer to form the active enzyme and generate ROS [46]. Binding of NPs to NADPH oxidase appears, for instance, to be involved in ROS generation of cerium oxide (CO_2) in fibroblasts [47].

Voltage-gated Ca^{2+} channels allow Ca^{2+} ions to enter cells producing transient intracellular Ca^{2+} signals, essential for the transduction of cellular signals in electrically excitable cells. Voltage-gated Ca^{2+} channels are classified as T, L, N, P, Q and R, and are distinguished by their sensitivity to pharmacological blocks, single-channel conductance kinetics, and voltage-dependence. On the basis of their voltage activation properties, the voltage-gated calcium classes can be further subdivided. Activation of these channels with subsequent increases in intracellular Ca^{2+} concentrations was first demonstrated for environmental carbon NPs [48]. Later, it was realized that also engineered NPs, TiO_2 and polystyrene NPs, increase intracellular Ca^{2+} concentrations by binding to Ca^{2+} channels, while ZnO increased intracellular Ca^{2+} concentrations by a combination on membrane damage, channel interaction, and Ca^{2+} release from intracellular stores [49]. Activation of these channels appears, for instance, to be involved in the exocytosis of pro-inflammatory secretory granules from rat mastocytes by polystyrene particles [50]. In addition to interference with Ca^{2+} signaling, 20 nm polystyrene NPs can activate K^+ channels and cystic fibrosis transmembrane conductance regulator Cl^- channels [49c] and quantum dots impair functional properties of sodium channels in hippocampal neurons [51].

Rhodamin 123 transport by P-glycoprotein 1, also termed multidrug resistance protein 1, was decreased in the presence of carboxylated and sulfatated polystyrene particles [52]. Since this protein acts as efflux pump for xenobiotics and endogenous substrates, its inhibition by NPs could be particularly relevant in pharmacology.

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LYSOSOMES

Lysosomes are obvious targets for NPs since many types of NPs (iron oxide, polystyrene, Au, quantum dots) are taken up by endocytosis and stored in lysosomes [53]. In addition to accumulation, NPs have been reported to cause destabilization of lysosomes and permeabilization of lysosomal membranes [54]. Partial lysosomal membrane permeabilization results in ROS generation and apoptotic cell death, while massive permeabilization induces cytosolic acidification and necrosis. Interference of NPs with lysosomes can result in oxidative cell damage by ROS, mitochondrial damage, and decreased elimination of macromolecules (lysosomal dysfunction) and of damaged organelles (reduced autophagy) from the cells. The effect of NPs on lysosomes was mainly studied in non-phagocytic cells and the following results obtained. Storage of quantum dots in lysosomes was accompanied by morphological damage, such as swelling of lysosomes [55]. The cytotoxic effect of anionic CeO_2 NPs [56] was correlated to localization in lysosomes while cationic CeO_2 NPs and polystyrene particles caused disruption of lysosomes [57]. Multi-walled CNTs interact with lysosomal membranes and increase lysosomal permeability [19c]. ZnO NPs, by contrast, destabilize lysosomes by release of Zn^{2+} ions [58]. Cationic NPs induce swelling of lysosomes by buffering of H^+ and increase of the lysosomal pH [59]. Lysosomal changes in pH and enzyme activities were observed upon exposure to 15 – 200 nm TiO_2 NPs and 10 nm ZnO NPs [25, 54a, 60]. NPs, such as 110 nm polystyrene NPs and TiO_2 NPs, however, also increased intralysosomal pH in macrophages [60-61]. It can be presumed that lysosomal permeabilization and release of lysosomal enzymes by NPs is similar to the action of micro SiO_2 NPs in phagocytic cells [62]. The degree of interference with lysosomal metabolism decreases with persistence of NPs in lysosomes: after 3d exposure to non-cytotoxic concentrations of polystyrene particles, the particles persisted in the lysosomes but interference with lysosomal pH and function of lysosomal enzyme was lower than after exposures for 24h [12].

Lysosomes may also act as a toxicant for NPs since a linear correlation of lysosome content and toxicity was identified in superparamagnetic iron oxide particles [63]. It is hypothesized that lysosomes generate Fe^{2+} ions, which together with O_2 produce Fe^{3+} ions and O^{2-} radical (Fenton reaction). Free iron ions can pass mitochondrial membranes and further increase the concentration of radicals. In addition, release of leachable metal ions from metal oxide NPs increases the cytotoxic effect of NPs. Solubility of iron, zinc and copper oxide is higher at low pH and particles may dissolve in the acid environment of the lysosomes. It is suggested that the toxic effect of iron oxide, ZnO, and CuO NPs, at least in part, is caused by leachable metal ions. Toxicity may be caused either by the high cytosolic concentration of these metals or by generation of oxidative stress [31, 64].

In addition to inhibition of lysosome function, NPs can affect autophagy [65]. This process requires normal function of lysosomes and is the main cellular process for removal of large compounds, for instance organelles, from the cell. Autophagy dysfunction is defined as either excessive induction of autophagy or blockade of autophagy flux. Several diseases are accompanied by defective autophagy but the precise role of this defect in these diseases is currently unclear. In cancer, for instance, defective autophagy promotes cell transformation and development of cancer, while in the later stages autophagy allows the cancer cells to survive regardless of deprivation of nutrients. Disruption of autophagy in neurodegenerative diseases appears to have a promoting role. The role of autophagy in the toxic action of NPs has been recently reviewed by Stern *et al* [66]. Increase in autophagosomes, determined as increase of Microtubule-associated protein 1A/1B-light chain 3 (LC3) levels, has been observed for a variety of NPs, such as fullerenes, gold NPs, iron oxide NPs, rare-earth oxide NPs, quantum dots, CNTs, TiO_2 NPs, and SiO_2 NPs [17, 67]. LC3 is a cytosolic protein, which during formation of autophagosomes, is conjugated to phosphatidylethanolamine. The

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conjugate is first recruited to autophagosomal membranes and, after fusion of autophagosomes with lysosomes, degraded by lysosomal proteases [68]. Detection of LC3 either by immunoblotting or immunocytochemistry has become a common marker for autophagy. Both mechanisms, increased production of autophagosomes and inhibition of autophagosome flux, appear to occur during NP exposure. Quantum dots, Au NPs, fullerenes, and rare earth oxide NPs induce autophagosome formation as important mode of action [67b, 67d, 69]. Other studies report blockage of autophagy flux as the main mode of action for Au NPs and fullerenes [67c, 70]. Since NPs with different composition and surface functionalities induce autophagy, it is suspected that increase in autophagy was inherently linked to the nanosize of these particles. The observation that NPs, but not larger particles of neodymium oxide, induced this response [71] corroborates this hypothesis. Oxidative stress with subsequent accumulation of damaged proteins and organelles is suspected as one inductor of autophagy by NPs [72]. This hypothesis was supported by the prevention of autophagy formation induced by fullerenes by antioxidants [67f] and the link between exposure to superparamagnetic iron NPs, TiO_2 NPs, and SiO_2 NPs, generation of ROS and autophagosome formation [67a]. Damage of lysosomes by osmotic swelling and membrane rupture by increased intralysosomal pH, membrane damage by oxidative stress, and disruption of lysosomal trafficking by disruption of the actin cytoskeleton are suggested as potential mechanisms for the increase of autophagosomes [66]. The question remains open whether induction of autophagy is a consequence of oxidative cell damage or a specific reaction of the cells to NPs with the aim to remove them from the cell. This hypothesis is corroborated by the finding that autophagosomes commonly contain NPs and that ubiquitinated proteins co-localize with NPs (e.g. [73]).

MITOCHONDRIA

Mitochondria as targets for NPs can aggravate cytotoxicity by increasing intracellular ROS-levels. Morphological (ultrastructural) and biochemical studies show that several types of NPs (quantum dots, Ag, TiO_2 and alumina NPs, and CNTs) can damage mitochondria by causing morphological alterations (swelling of mitochondria) or decrease of the mitochondrial membrane potential [74]. Direct injury of mitochondrial membranes is assumed for SiO_2 NPs, which were identified inside the organelles [75]. Increased mitochondrial membrane permeability caused by mechanical disruption, through disturbance of the respiratory chain, and through changes in Bax and Bcl-2 expression, is involved in the toxic effect of Ag NPs on mitochondria [76]. As a consequence of the greater mitochondrial membrane permeability cytochrome C as initiator of the intrinsic (mitochondrial) way of apoptosis is released. Generation of ROS caused by highly reactive silanol groups at the particle surface is an important mechanism in the mitochondrial effects of SiO_2 particles [75]. ZnO NPs may cause mitochondrial damage by toxic concentrations of Zn^{2+} ions [77]. Consequently, overexpression of microsomal glutathione transferase 1 protected MCF-7 cells against cytotoxicity by SiO_2, not by ZnO NPs. Impairment of mitochondrial function can also be caused by decreased production or down-regulation of mitochondrial DNA-encoded proteins. SiO_2 NPs, for instance, decrease levels of cytochrome C oxidase subunit II and NADH dehydrogenase subunit 6 [78]. In exposures, where the decrease of mitochondria potential by ZnO NPs was caused through ROS generation, a relation to abnormal autophagic vacuoles has been identified [79]. It was reported that mitochondrial depolarization preceded autophagy but the connection of mitochondrial dysfunction and autophagy blockade is currently not well understood. Exposure to NPs and to smoke, induce a combination of autophagy blockade, mitochondrial dysfunction and increase in ubiquinated proteins in alveolar macrophages [67e, 80]. This shared cellular reaction pattern suggests a common mechanism for decreased lung clearance.

Mitochondria cross talk with the endoplasmic reticulum by Bcl-2 proteins and Ca^{2+}-signalling. Endoplasmic reticulum stress has been shown to be involved in cytotoxicity caused by Ag NPs and SiO_2 NPs [81]. Damage of Golgi membranes by TiO_2 and iron oxide NPs currently has not been definitively proven [82].

NUCLEUS

Action of toxicants in the nucleus is usually linked to genotoxicity. Direct action of NPs on the sugar-phosphate backbone, for instance depurination, oxidation, methylation, formation of adducts of bases that can subsequently cause single strand breaks, can either be repaired or lead to apoptosis. Nearby single strand breaks (SSBs), SSB duplication, in-strand crosslink can lead to double strand breaks (DSBs) and chromosome breaks, which cannot be repaired. In addition, inhibition of repair enzymes may also cause an increased rate of genotoxic events, while protein lesions and abnormalities of the mitotic spindle are causes for chromosome loss.

Many NPs (<50 nm) can get into the nucleus [41, 76a], but localization in the nucleus is not a prerequisite for action on the DNA; intracellular NPs can also gain access to the genetic material during mitosis when the nuclear membrane breaks down and genotoxic changes may also be caused by interference with the mitotic spindle.

Ag, ZnO, TiO_2, and Cu NP cause genotoxicity mainly by generation of ROS and oxidative DNA-damage [83]. Effects of ROS in the nucleus are point mutations in the DNA and DSBs. TiO_2 NPs can insert themselves between base pairs or form covalent Ti-O or Ti-N bonds, while Ag NPs directly interact with DNA by other, not further characterized, mechanisms [76a, 84]. Quantum dots, containing cadmium and lead, cause genotoxic damage by release of these metals, which subsequently may inhibit DNA repair and induce methylation of nucleotides [85]. In addition to that, direct binding of CdSe quantum dots to adenine and thymidine-rich regions of the DNA was attributed to the genotoxic effect of these NPs [86]. Aberrant clusters of topoisomerase I induced by SiO_2 NPs can cause alterations in DNA transcription [87].

Up-regulation of p53 and DNA repair enzymes, for instance Rab51 and phosphorylated H2AX, is interpreted as indication of genotoxicity by Ag NPs, although the mechanism of DNA damage by these particles was not identified [88]. TiO_2 NPs act by a combination of mechanisms; they induce SSBs and oxidative DNA lesions and impair the ability for DNA repair by interaction with nucleotide excision repair (NER) and base excision repair (BER) pathways [89]. Sterical blockade of cytokinesis is a presumed mechanism of genotoxic damage by CNT [90] and interaction of single walled CNTs with the mitotic spindle, microtubule, centrosomes and condensed chromatin cause genotoxicity [91].

The least well understood mechanism of genotoxicity has been observed upon exposure to CoCrNPs. These particles induced genotoxicity in fibroblasts cultured below a bi-layered sheet of choriocarcinoma cells. The mechanism includes connexin 43, pannexin channels, ATP transmission, and Ca^{2+} wave propagation and mitochondria-derived ROS generation, but no changes in protein transcription [92].

Epigenetic changes include modifications of DNA and histones and influence the transcription rate of genes. Histone acetylation and DNA methylation are key processes in cell differentiation and transformation. NPs can cause epigenetic changes by modulation of DNA methyltransferase activity (Cd NPs), decreased mRNA expression of methyl-CpG-binding domain protein expression (SiO_2 NPs), decrease of histone deacetylase activity (Au NPs), and histone 3 hyperacetylation (quantum dots) [93]. ROS are involved in DNA hypomethylation and expression of methylation DNA-regulated genes. Epigenetic changes

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by direct effects of NPs include formation of histone aggregates through interaction with quantum dots and binding of Au NPs to SH-groups in histone deacetylase [94]. Although not a direct consequence of DNA damage, NPs may inhibit translation, either by hindering the export of mRNA through enlarged NP-loaded lysosomes, or by reduced expression of ribosome subunit S2 [76a].

INTRACELLULAR PROTEINS

Almost all types of NPs intensely interact with protein and form a protein coat, which is believed to determine their biological trafficking and toxicity [95]. Interactions of NPs and proteins, in principle, may i) have no effect on protein function, ii) have a stabilizing effect, iii) inhibit the normal function of the protein, iv) induce or inhibit the formation of protein aggregates, and v) present hidden epitopes to immune cells (Fig. **5**). Several types of sensors take advantage of the retention of catalytic activity upon binding to NPs. Immobilization of acetyl cholinesterase on multi-walled CNTs, Au nanoparticles, zirconia nanoparticles, cadmium sulfide NPs or quantum dots are used as electrochemical sensors for organophosphorous pesticides [96].

Amphiphilic NPs can bind to heat-damaged proteins and act as chaperones [97]. The stabilizing effects have been observed for TiO_2 NPs, which were able to assist in the refolding of thermally denaturated proteins, namely α-chymotrypsin, RNase A and papain [98].

The same type of particles, on the other hand, induced conformational changes in tubulin and inhibited tubulin polymerization [99], impairing cell division, cellular transport, and cell migration. When cellular extracts were evaluated for binding to TiO_2 NPs, SiO_2 NPs, and polystyrene NPs, the structural protein actin was identified as the most commonly bound protein [100]. This binding suggests that NPs could inhibit actin function, such as vesicle and organelle transport, cell signaling, maintenance of cell junctions, cell motility, etc. Interference of single-walled CNTs with cytoskeletal proteins (e.g. actin) may cause cell division defects [101]. Binding to SiO_2 particles destroyed the catalytic activity of carbonic anhydrase II [102]. Other intracellular enzymes that were altered by the contact with NPs are the microsomal CYP450 oxidases, which metabolize chemicals and drug compounds. Small (<50 nm) polystyrene, Au and Ag particles inhibited the activity of isolated CYP450 enzymes, particularly CYP3A4 [103]. CYP450 enzymes are located in the outer leaflet of the endoplasmic reticulum membrane and could get in contact with NPs in the cytoplasm. Exposure to NPs could, therefore, lead to delayed oxidation and excretion of drugs.

CeO_2, quantum dots, and CNTs induced protein aggregation and formation of fibrils [104]. Such effects could be particularly dangerous because the formation of aggregates has been linked to a variety of neurodegenerative diseases. The binding of proteins, however, may also display protective effects in these diseases since fullerenes, polymeric NPs and quantum dots have also been shown to prevent the formation of protein aggregates [105].

CONCLUSIONS

Better characterization of NPs, estimations of cellular dose, and identification of limitations in conventional cytotoxicity screening considerably improved the knowledge in biological effects of NPs. Some metal and metal oxide NPs appear to act mainly by one mechanism: Ag, ZnO and iron oxide NPs, for instance, appear to act predominantly through free ions, while surface related effects, generation of ROS, play an important role in the cytotoxicity of SiO_2 and TiO_2 NPs. NPs without metal content, such as polystyrene particles or CNTs preferentially act through binding and interaction with proteins. Knowledge of the broad

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panel of potential cellular targets might be helpful in the identification of effects of new NPs.

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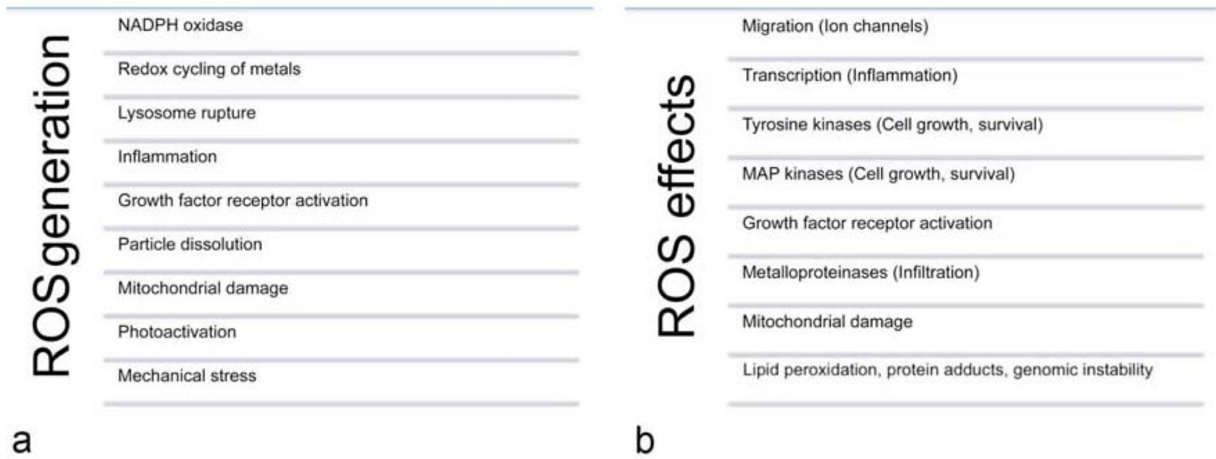

Fig. (1). Sources (a) and cellular effects (b) of reactive oxygen species (ROS).

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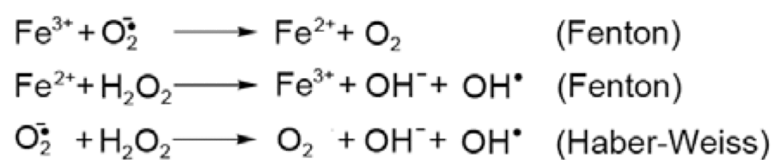

Fig. (2). Illustration of Fenton reaction, generating either superoxide or hydroxyl radicals in the presence of iron ions, and Haber-Weiss cycle, generating hydroxyl radicals from hydrogen peroxide and superoxide.

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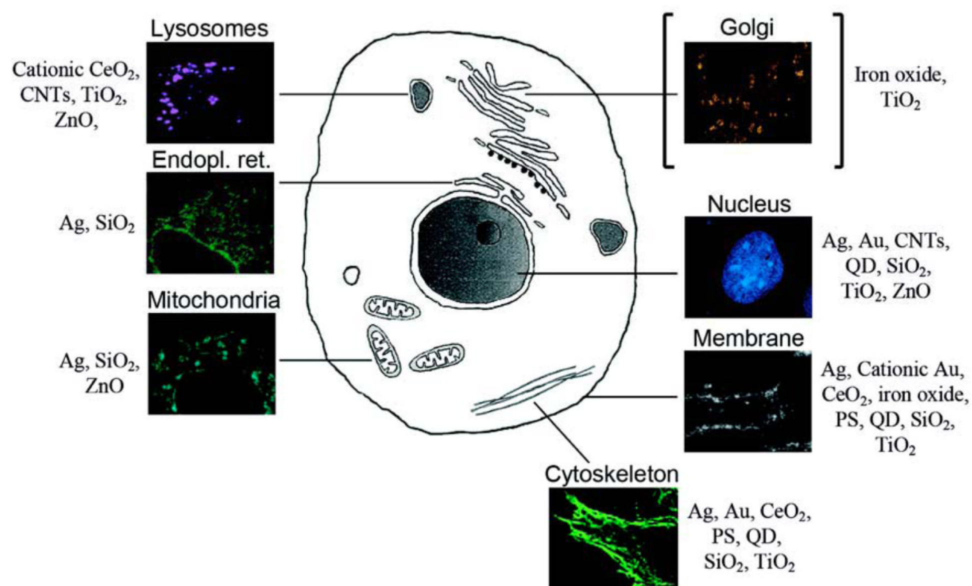

Fig. (3). Targets for cytotoxicity of nanoparticles with examples.

Most NPs act on more than one target. For the Golgi apparatus (in brackets), damage by iron oxide NPs and TiO_2 NPs is suspected but definite proof is lacking. Abbreviations: PS: polystyrene NPs, QD: quantum dots

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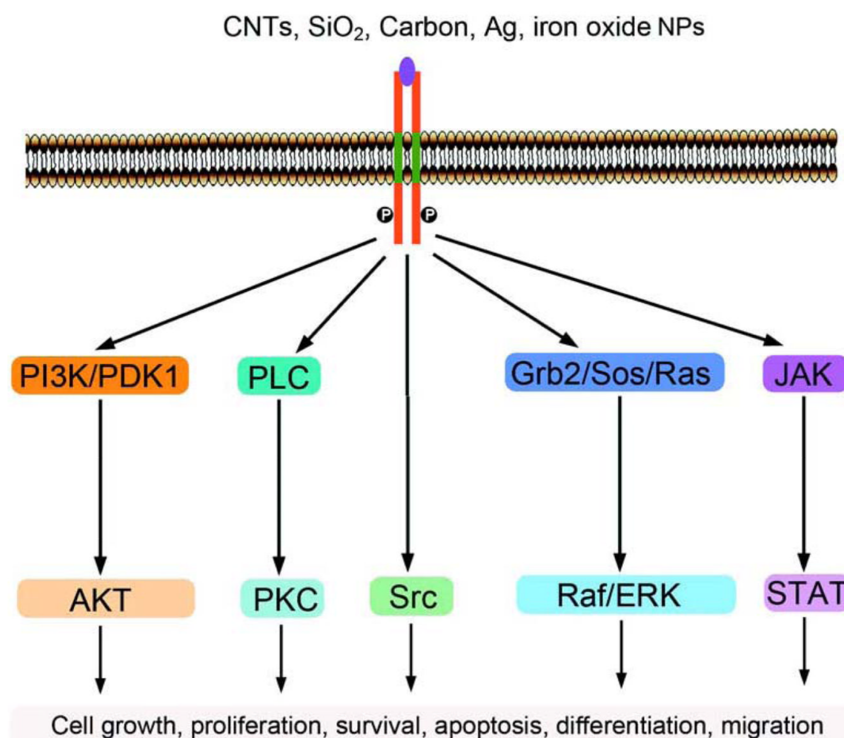

Fig. (4). Involvement of receptor tyrosine kinase (RTKs) in the cellular effects of NPs.

Upon ligand binding and activation, the intracellular catalytic domain of RTKs catalyzes receptor autophosphorylation of cytoplasmic tyrosine residues. Assembly of activated protein complexes triggers signaling cascades that can activate or repress genes involved in cell growth, proliferation, differentiation and survival by PI3K/PDK1 → AKT, PLC → PKC, Src, Grb2/Sos/Ras → Raf/ERK, and JAK → STAT pathways.

Abbreviations: P, phosphorylated tyrosine residue; PI3K, phosphatidylinositol 3-kinase; Pdk1, phosphoinositide-dependent protein kinase-1; Akt, oncogenic kinase initially isolated from a transforming mouse retrovirus; PLC, phospholipase C; PKC, protein kinase C; Src, oncogene of the chicken Rous sarcoma virus, Sos, (son of sevenless); Grb2, (growth factor receptor-bound protein 2) are adaptor proteins; Ras, oncogene first isolated in rat sarcomas; Raf, oncogenic kinase initially isolated from a transforming mouse virus; ERK, extracellular-regulated kinase; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

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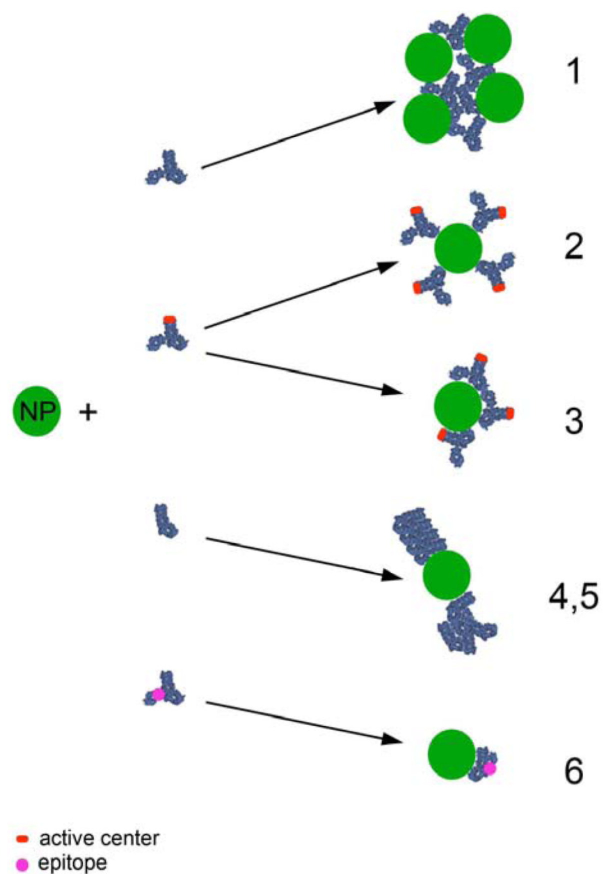

Fig. (5). Potential effects of the interaction of NPs and proteins.

Upon binding of proteins to NPs, the NPs may aggregate (1), in case of an enzyme, the activity may be unchanged if surface-mediated distortion or blockade of the active center is absent (2), while the activity is inhibited if perturbation of the enzyme structure takes place (3). NPs can act as chaperone by facilitating correct folding of a protein (4) or increase aggregation of denaturated protein and formation of fibrils (5). By the binding to NPs hidden epitopes could be exposed (6).

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Table 1

Overview on the kind of adverse cellular action of non-biodegradable NPs

Target	Mechanism	Type of NP
Plasma membrane	Mechanical damage	SiO_2, cationic Au, polystyrene
	Damage by ROS	Iron oxide, TiO_2, SiO_2
	Changes in membrane fluidity	Quantum dots
	Activation of membrane-associated receptors	CNTs, SiO_2, Ag, CeO_2, TiO_2, iron oxide, polystyrene
Lysosomes	Disruption of membrane integrity	Cationic CeO_2, CNTs
	Damage by free metal ions	ZnO
	Intralysosomal pH increase	TiO_2, ZnO
Autophagy	Increase of autosome formation	Fullerenes, Au, iron oxide, rare-earth oxide, quantum dots, TiO_2, SiO_2
	Blockage of autosomal flux	Fullerenes, Au
	Autophagy by ROS	Fullerenes, TiO_2, SiO_2
Mitochondria	Disruption of membrane integrity	SiO_2
	Membrane permeability increase	Ag
	Damage by free ions	ZnO
	Down-regulation of mitochondrial DNA-coded proteins	SiO_2
	Damage by ROS	ZnO
Nucleus	Oxidative DNA damage	Ag, ZnO, Cu, TiO_2
	Point mutations	Ag, TiO_2
	Inhibition of DNA repair by free ions	Quantum dots
	Mechanical binding to DNA	Quantum dots
	Inhibition of transcription	SiO_2
	Inhibition of cytokinesis	CNTs
	Epigenetic changes	SiO_2, Cd, Au, quantum dots
Intracellular proteins	Structural protein actin	TiO_2, SiO_2, polystyrene
	Enzymes (carboanhydrase, CYP450)	SiO_2, Au, Ag, polystyrene
	Formation of fibrils	CeO_2, quantum dots