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Review

Review

Impact of nanoparticles on DNA repair processes: current knowledge and working hypotheses

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Abstract

The potential health effects of exposure to nanomaterials (NMs) is currently heavily studied. Among the most often reported impact is DNA damage, also termed genotoxicity. While several reviews relate the DNA damage induced by NMs and the techniques that can be used to prove such effects, the question of impact of NMs on DNA repair processes has never been specifically reviewed. The present review article proposes to fill this gap of knowledge by critically describing the DNA repair processes that could be affected by nanoparticle (NP) exposure, then by reporting the current state of the art on effects of NPs on DNA repair, at the level of protein function, gene induction and post-transcriptional modifications, and taking into account the advantages and limitations of the different experimental approaches. Since little is known about this impact, working hypothesis for the future are then proposed.

Introduction

With the extensive development of new nanomaterials (NMs) during the last decades, the determination of their potential health effects has become of the upmost importance. Thus, nanotoxicology has recently emerged, and emphasis has been placed on understanding the consequences of NM exposure for human health and the environment.

With the central role that the DNA molecule plays in the maintenance of genetic information, the possible formation of DNA lesions by NMs, i.e. genotoxicity, has also been raised. Therefore, several experimental approaches have been used to study either the presence of DNA lesions in cells exposed to nanoparticles (NPs) or the consequences of those formed lesions. Since DNA repair processes are responsible for circumventing DNA damage, it thus also appears to be very important to evaluate the effects of NP exposure on these processes. Indeed, several repair mechanisms are effective in cells and continuously manage the diverse types of damage that are produced by a plethora of physical (e.g. ionizing radiation), chemical (chemotherapeutic compounds such as cisplatin) or biochemical [reactive oxygen species (ROS) released by the mitochondria, methyl glyoxal, etc.] genotoxic agents. These repair systems keep the level of DNA damage as low as possible, in order to minimise the probability to induce mutation, cell death or to develop cancer. Regarding the potential genotoxic effect of NMs, the involvement of oxidative stress is frequently reported. The particularity of oxidatively generated DNA lesions is that they are continuously produced in cells by oxidative metabolism. They are also continuously repaired, in order to maintain their background level as low as possible. Elevation of this background level reveals the onset of oxidative stress, which results from imbalance between the production of ROS and the cells' capability to detoxify them. Thus, it should be kept in mind that an alteration of the cell's defenses against ROS would give rise to oxidative stress as would do an increased production of ROS. The same is true for oxidatively generated DNA lesions: increased amounts of these lesions could originate from increased oxidative stress or from decreased DNA repair capacity or from a combination of both. Therefore, any method

that could provide information on the effect of stress towards DNA repair will provide valuable information on the genotoxic potential of a stress-inducing agent.

In the present review article, the objective was to summarise the recent data from the literature that have demonstrated how NP exposure influences DNA repair systems. First, evidence for NP-induced oxidative DNA lesions is reported. Then the different DNA repair pathways present in eukaryotic cells are shortly described, together with their main critical proteins. The possible impact that NPs may cause on these DNA repair systems are considered. Finally, based on the recent literature, the reported effects of NPs on DNA repair, at the level of protein function, gene induction and post-transcriptional modifications are discussed, taking into account the advantages and limitations of the different experimental approaches. To conclude, possible orientations of future research in the field are discussed.

Nanoparticle-induced DNA damage and its management by cell repair systems

NPs generate oxidative DNA lesions

Two accepted paradigms for NP toxicology are their induction of oxidative stress and their pro-inflammatory potential. These phenomena are closely interconnected and may be the source of other cellular damage if not properly handled. The only hypotheses formulated so far to explain NP-induced oxidatively generated DNA damage rely on the implication of ROS. ROS may form at the surface of NPs and be released in cells. Moreover, NPs may alter the cell's capacity to eliminate endogenous ROS. As a consequence, NPs have been shown to induce oxidative damage to DNA, both on DNA bases and on the 2-deoxyribose moiety of the DNA backbone, thereby leading to the formation of oxidized DNA bases, single-strand breaks (SSBs) and abasic (AP) sites. The induction of double-strand breaks (DSBs) by NPs is possible as well, if DNA replicates in proximity to a non-repaired SSB or due to the collapse of stalled replication forks. Oxidized DNA bases and SSBs are efficiently and continuously repaired in healthy cells, provided that cell repair systems are not overwhelmed. If overwhelmed, then lesions will subsist in the genome, potentially leading to cell mortality or mutagenesis. On the other hand, DSBs, either directly generated or resulting from replication of SSBs, as well as blocking base lesions, are considered among the most dangerous forms of DNA damage, because they may lead to chromosomal aberrations if left unrepaired.

More than 80 different types of oxidatively generated DNA base lesions have been identified. Their relative yield of formation greatly depends on the ROS involved. Due to its low redox potential, guanine (G) is a target of all DNA oxidative processes, with 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo) being its main degradation product (1). 8-oxo-dGuo is mutagenic because it can pair with adenine (A) or cytosine (C) with equal efficiency, thus leading to G:C to thymine (T):A transversion. Induction of 8-oxo-dGuo in cells exposed to NPs has been largely documented (2,3), including recommendations on their proper quantification (3). To date, the possibility that NPs might oxidize DNA via products of lipid peroxidation has never been explored. These reactive compounds, i.e. ketonic and aldehydic derivatives [malondialdehyde, acrolein, 4-hydroxy-2-nonenal (4-HNE) or its epoxidized derivatives] (1), may play a role, particularly in the context of glutathione (GSH) depletion via NPs.

Because DNA is constantly susceptible to be attacked, as a result of environmental conditions or normal metabolism, all living cells have evolved a variety of DNA repair pathways, including nucleotide excision repair (NER), base excision repair (BER), non-homologous end joining (NHEJ), homologous recombination (HR) and mismatch repair (MMR), which are the most studied and understood. Detailed reviews of the different DNA repair pathways have already been published; for this reason, the objective is not to extensively describe them herein. Our aim is to focus on the main pathways that can be impacted by NPs, as demonstrated through the published literature.

DNA repair pathways

The oxidized DNA bases, such as 8-oxo-dGuo, which are relatively small and do not distort DNA helix, are mostly repaired via the BER pathway. The core reaction of BER is also involved in the repair of SSB and AP sites. BER consists in the recognition of the DNA lesion, followed by excision of the oxidized base by a glycosylase, either monofunctional (UNG, MPG, SMUG etc.) or bifunctional (OGG1, NEIL1, NTH1 etc.), which generates an AP site. Then, the AP site is incised by APE1. Each glycosylase has a limited, specific set of substrates (4,5). For example, 8-oxo-dGuo is recognised by several glycosylases: OGG1, the most studied glycosylase in the context of NP toxicity, preferentially recognises and processes 8-oxo-dGuo when paired with C, T and G. OGG2 recognises 8-oxo-dGuo paired with G and A. In addition hMYH (MUTYH) specifically recognises and excises the A opposite to 8-oxo-dGuo (6). Depending on which glycosylase participates as well as the current state of the cell (i.e. phase of the cell cycle and adenosine triphosphate content) BER can proceed via a short-patch or a long-patch pathway, which both replace missing nucleotides, thanks to polymerases, and seal the DNA end, thanks to ligases. If NPs interfere with the activity of some of these proteins, NP-generated DNA damage will not be appropriately repaired. Inactivation of glycosylases would leave lesions unrepaired. If the proteins operating downstream of glycosylases (e.g. APE1, polymerases, ligases etc.) are inactivated by NPs, then an SSB will remain at the initial site of the oxidized DNA base.

NER repairs a variety of DNA lesions via two sub-pathways: global genome NER (GG-NER), which repairs voluminous, helix-distorting lesions on the entire genome and transcription-coupled NER, which specifically repairs transcription-blocking lesions (7). Some radical-induced base modifications, therefore possibly those induced by NPs, are bulky enough to distort DNA and block replication. It is the case for instance of cyclopurines or adducts like those involving G and 4-HNE. In GG-NER, the damage is recognised by Xeroderma Pigmentosum Group C-complementing protein (XPC), together with RAD23B and CETN2. Alternatively, if the damage does not cause large helix distortions, the lesions are marked by DDB1 and DDB2 and other auxiliary factors that facilitate lesion recognition by XPC. Binding of XPC to the lesion stimulates the binding of the transcription initiation IIH complex [transcription factor II Human (TFIIH)], composed of Xeroderma Pigmentosum Group B-complementing protein (XPB) and Xeroderma Pigmentosum Group D-complementing protein (XPD) and stimulated by Xeroderma Pigmentosum Group A-complementing protein (XPA), which verifies the presence of lesions (8). If a lesion is detected, it is excised by XPF-ERCC1 and XPG, coordinated with the binding of replication protein A (RPA), which stabilizes the opposite strand and XPA. Finally, the gap is filled by polymerases (POLδ, POLε and POLκ), together with auxiliary proteins (PCNA and RFC) and ligated by ligases (LIG1 or the XRCC1-LIG3 complex). XPA is a central protein in NER: acting as a stimulating factor for the TFIIH complex, binding to altered nucleotides in single-stranded DNA and interacting with almost all the proteins of NER. Moreover, post-translational modifications, in particular ubiquitinations, are central

processes that regulate NER initiation and progression (7). Other post-translational modification of some DNA repair proteins are also essential for NER to efficiently process, particularly for preparing the chromatin so that DNA repair proteins can gain access to the damaged areas. Poly(ADP-ribose) polymerase 1 (PARP) plays a central role in this preparation (9). Both XPA and PARP1 are zinc (Zn) finger (ZF) proteins (10,11), and as discussed subsequently, their function may be altered by NPs or metal ions released from NPs upon degradation.

MMR has been reported to recognise and repair 8-oxo-dGuo paired with A, if not recognized by BER. It also repairs 8-oxo-dGuo resulting from oxidation of G in the available nucleotide pool and that would be incorporated into DNA during replication. MMR thus plays a role in the elimination of mutations that may be induced by NPs via unrepaired, bypassed 8-oxo-dGuo. In humans, mispairs are recognised by MutS homolog (MSH) heterodimers to which the MutL homolog 1 (MLH1)/postmeiotic segregation increased 1 homolog 2 (PMS2) complex binds (12). The exonuclease EXO1 is then recruited to the mispair and excises it, with translesional and/or replicative DNA polymerases that serve to fill in the gap (12).

When a DSB arises in a cell, a very early response is phosphorylation of the minor variant of H2A histone, H2AX, leading to the formation of the so-called γ-H2AX. γ-H2AX serves as a platform for the recruitment of other DNA repair proteins but also increases DNA accessibility, recruits cohesins that maintain the proximity between DNA strands during the repair process and modulates the checkpoint response (13). Depending on cell cycle phase, DSBs are repaired either via HR or via NHEJ, thanks to the involvement of the p53 binding protein 1 (14). NHEJ repairs DSB regardless of what phase of the cell cycle the cell is in. It involves the KU70/KU80 complex, DNA-PKcs, ARTEMIS, PNPK and other cofactors that prepare DSB for their repair by polymerases (POLμ and POLλ). This repair mechanism introduces new DNA sequences and could thus be mutagenic. The strands are then ligated by the XRCC4-XLF-LIG4 complex (15). If a second copy of DNA is available and can serve as a template for DNA repair, DSBs are repaired with high fidelity via HR. HR is therefore limited to the S and G2 phases of the cell cycle. It is initiated by binding of a series of RPA proteins by the MRN complex (MRE11, RAD50 and NBS1) together with cofactors including BLM, CtIP, EXO1 and DNA2 (16). RPA proteins are then displaced by RAD51, which finds a homologous sequence in the genome and creates a D-loop. Finally, the D-loop is resolved by synthesis-dependent strand annealing or by migrating the double Holliday junctions that are subsequently cleaved by resolvases.

Current knowledge on NP impact on DNA repair processes

We present here a literature survey focused specifically on the impact of NPs on DNA repair systems and activities. This survey is classified by considering the technique that was employed to probe an impact of NPs, which more or less directly reflects their effects.

Probing the impact of NPs by measuring the activity of DNA repair systems

The best probe for DNA repair impairment is the observation of reduced activity of DNA repair systems. Only a few studies report such result. First, direct impact of NPs on DNA repair processes has been reported by studying the repair kinetics of DNA lesions induced by NPs. Zijno *et al.* demonstrated that zinc oxide (ZnO)-NPs induced chromosomal damage in Caco-2 cells, via the cytokinesis-blocked micronucleus assay (17). In this cell line, both ZnO and titanium dioxide (TiO_2)-NPs induced DNA strand breaks and formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites in the comet assay. Both NPs also increased the basal level of 8-oxo-dGuo, as measured by high-performance liquid chromatography/electrochemical detection. When the cells were then allowed to recover in cell culture medium that did not contain NPs, these lesions disappeared, suggesting efficient DNA repair (17). However, the lesions were more rapidly repaired in potassium bromate ($KBrO_3$)-exposed cells, where $KBrO_3$ served as a positive control, compared with cells exposed to TiO_2-NPs. Repair of 8-oxo-dGuo lesions was totally abolished in ZnO-exposed cells, whereas repair was only slightly inhibited in cells exposed to TiO_2-NPs (17). OGG1 content did not change in ZnO-NP–exposed cells and slightly increased in TiO_2-NP–exposed cells, as compared to control cells (17). The authors thus suggest that BER DNA repair blockade occurs after the damage recognition and excision by DNA glycosylases. It is also possible that OGG1 was still present, but in an inactive form because this enzyme is susceptible to oxidation. NPs would thus moderately affect, via oxidation, the activity of other DNA repair proteins from this pathway (17). The formation of micronuclei in ZnO-NP–treated cells would be a consequence of the division of a cell containing unrepaired DNA lesions (18). The same approach of DNA repair kinetics study was used by Demir *et al.* to probe ZnO-NP interaction with DNA repair systems. Altered DNA repair was shown in ZnO-NP–exposed cells, but not in cells exposed to ZnO microparticles (MPs) (19). On the contrary, cerium oxide (CeO_2)-NPs were shown to accelerate the repair of ultraviolet (UV) radiation B-induced DNA damage (20). A hypothesis to explain this impact is that NER and BER rely on the activity of enzymes that contain redox-sensitive [4Fe-4S] clusters, which are cysteine–metal coordination groups and allow rapid detection of DNA damage through electrochemical gradients (20). Due to the antioxidant activity of CeO_2-NPs, which depends on the Ce^{3+}/Ce^{4+} redox switch, these enzymes were activated in UV-exposed cells (20). Direct impairment of DNA repair processes was also proven in cells treated with silver (Ag)-NPs. Indeed pretreatment of HepG2 hepatocytes with Ag-NPs or TiO_2-NPs, then treatment with X-rays, led to DNA strand breaks, as assessed by the comet assay. A decreased rate of strand break rejoining was observed in HepG2 cells pretreated with Ag-NPs, but not TiO_2-NPs (21). Finally, direct impairment of DNA repair processes was shown in A549 cells chronically exposed to TiO_2-NPs for 2 months, i.e. subcultured for 2 months in culture medium containing TiO_2-NPs. In these cells, post-exposure to a sublethal dose of methyl methanesulphonate (MMS) led to significant cytotoxicity (22). It also caused significant increase of DNA strand breaks induced by MMS (22). Proteomics analysis showed increased levels of serine/threonine protein phosphatase 5 and serine/threonine kinase receptor-associated protein in these chronically exposed cells, both of them being involved in the DNA damage response (23). Increased acetylation of p53 was demonstrated, together with cell cycle slow down (23). Together, these results suggest that chronic exposure to TiO_2-NPs sensitise cells towards genotoxic agents, possibly via impairment of DNA repair processes.

Direct impact of NPs on DNA repair activities was also demonstrated in acellular assays, using a multiplexed array probing both BER and NER activities (Figure 1) on A549 cells exposed to various types of TiO_2-NPs (24). This assay relies on the ability of protein pools extracted from NP-exposed cells to repair plasmids carrying specific types of DNA lesions (Les.A to Les.C in Figure 1). These plasmids are spotted on a microarray, together with an undamaged plasmid (Ctl in Figure 1). The microarray is incubated with the cellular machinery that would enable DNA repair, in which fluorescently labelled nucleotides are added. The repair of the lesions

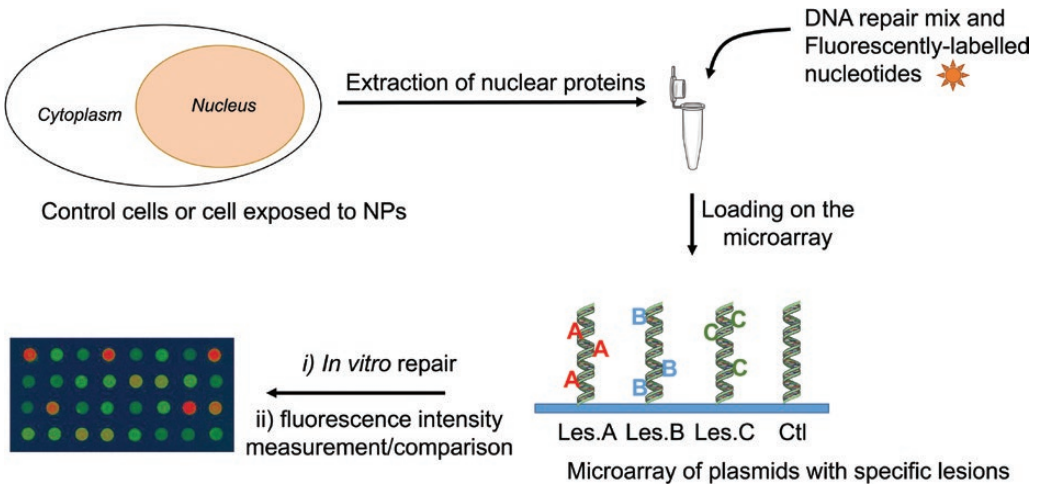

Figure 1. Microarray used for the evaluation of DNA repair activity in cells exposed to NPs. Nuclear proteins from cells exposed to NPs or from control (unexposed) cells are extracted and mixed with an *in vitro* repair mix containing fluorescently labelled nucleotides (star). This mixture is loaded on the microarray, consisting in a slide on which are spotted plasmids carrying a specific DNA lesion ('Les.A', 'Les.B' or 'Les.C' in this figure), known to be repaired via the BER or the NER. The repair reaction is allowed to proceed on this microarray *in vitro*. Fluorescence intensity is then measured on each spot of the microarray (plasmids with lesions or undamaged plasmid, i.e. 'Ctl' in this figure), because if repair is effective then fluorescent nucleotides are incorporated in the plasmids. The fluorescent intensity thus reflects the ability of NP-exposed cells to repair each specific lesion (25). Figure available in colour online.

carried by these plasmids is followed by fluorescence measurement (25). Dramatic reduction of DNA repair activities, via both the NER and the BER pathways, was observed after 24 h or 48 h of TiO_2-NPs exposure in A549 cells, concomitantly with the presence of DNA strand breaks and oxidatively damaged DNA (24). Both anatase and rutile TiO_2-NPs, either spherical or elongated-shaped, with diameters ranging from 12 to 140 nm caused this impairment. The cellular response differed only for a mixed anatase/rutile, 21 nm diameter TiO_2-NP, which caused a slight increase of DNA repair activity in cells exposed for 24 h, then the dramatic decrease of DNA repair activity was also observed with this NP after 48 h of exposure (24). Since TiO_2-NPs also led to an increase of intracellular ROS formation in exposed cells, a hypothesis was that TiO_2-NPs could oxidatively inactivate some DNA repair proteins (24). The same impact was recently observed in BEAS-2B cells, and a deeper interpretation allowed us to reconsider these results. Dramatic reduction of DNA repair activities were effectively observed; however, the relative importance of the different DNA repair pathways were different in TiO_2-NP–exposed cells, as compared to control cells. The repair via NER was highly decreased, while repair via BER was less drastically affected (26). This is consistent with a preservation of BER activities that would be necessary for the repair of oxidatively generated DNA damage that were observed in both A549 and BEAS-2B cells (26).

In addition to these approaches that probe global DNA repair activities, some studies reported impact of NPs on the activity of isolated DNA repair proteins, especially OGG1. Direct impact of Ag-NPs on this enzyme was demonstrated in human cell models and *Caenorhabditis elegans*. DNA base oxidation was shown in human Chang liver cells exposed to 5–10 nm-sized Ag-NPs, where 8-oxo-dGuo content was higher in treated cells than in control cells (27). This was inversely correlated with decreased OGG1 gene expression, protein content and activity. This impact was also correlated to decreased expression, translocation to the nucleus and activity of nuclear factor erythroid 2 (NRF-2), a transcription factor that controls the expression of redox genes (27). Impact of Ag-NPs on 8-oxo-dGuo content, OGG1 expression and activity was also reported in Jurkat T cells and *C. elegans* by Chatterjee *et al.* (28). Higher impact was observed on *p38 MAPK* knock-down vs. wild-type Jurkat T cells and on *pmk-1* mutant compared with wild-type *C. elegans* (PMK-1 is the ortholog of the human p38 MAPK in *C. elegans*). The authors also observed higher expression and activity in Ag-NP–exposed cells of human MutT homolog 1 (hMTH1) and *C. elegans* NDX-4, which are phosphatases (8-oxo-dGTPases) that are responsible for the removal of 8-OH-dGTP from nucleotide pools (28). This underlines the oxidative impact of Ag-NPs on DNA, but also their impact on the activity of glycosylases, which are responsible for the first step of BER. At the molecular level, copper(II) oxide (CuO)-NPs were shown to affect Poly (ADP-ribose) polymerase 1 (PARP-1) activity in A549 cells (29). In these cells, exposed to CuO-NPs (20 nm) and -MPs (200 nm), decreased hydrogen peroxide-mediated poly(ADP-ribosyl)ation was observed. It coincided with increased direct and indirect genotoxicity of NPs (but only indirect genotoxicity of MPs) as well as both apoptotic and necrotic cell death. These particles substantially dissolved in cell culture medium and in reconstituted lysosomal fluid. Interestingly, the impact of CuO-NPs correlated with copper (Cu) content in the nucleus, which reached 1 mM in cells exposed to 10–20 µg/mL CuO-NPs while its basal level in A549 cells was 15 µM (29). Inactivation of PARP-1 was attributed to Cu overload in these cells, which would react with redox-sensitive sites in PARP-1, leading to local unfolding (29). Lastly, Ag-NPs, CeO_2-NPs and cobalt oxide (Co_3O_4)-NPs were shown to affect Fpg activity *in vitro* and would therefore interfere with the comet assay in its Fpg-modified version (30).

Impact of NPs on the expression of genes encoding DNA repair proteins and on the intracellular content of DNA repair proteins

Besides measuring the activity of DNA repair processes, other approaches, which are more indirect, consist of measuring the intracellular content of DNA repair proteins or of the genes that encode DNA repair proteins (thereafter designed as 'DNA repair gene expression'). These approaches do not directly inform on DNA repair activities, because DNA repair proteins can be present but in an inactive form. *A fortiori* expression of DNA repair proteins can be modulated,

while DNA repair activity remains stable. The advantage of these approaches is that they are rapid, cost-effective and allow the screening of a large panel of proteins involved in DNA repair processes.

Several targeted studies showed impact of NPs on the content of some proteins involved in the DNA damage response. Among them, those by Piao *et al.* and Chatterjee *et al.* cited above described altered content of OGG1 protein in Ag-NP–exposed cells (27,28). In addition, increased content of p53, RAD51 and MSH2 was demonstrated in A549 cells exposed to CuO-NPs (31). However, the techniques used in these former studies do not allow high or medium-scale throughput screening of the impact of NPs or other toxicants on DNA repair protein contents. Dizdaroglu *et al.* recently developed a method based on liquid chromatography–mass spectrometry (MS)/MS that allows higher throughput screening quantification of DNA repair proteins (32). It would now be interesting to apply this technique to samples derived from experimental animals or in cells exposed to NPs. Moreover, proteomics would give an overview of the proteins impacted by NP exposure, including DNA repair proteins. The advantage of proteomics approaches is that they analyse the cellular proteome without *a priori*, rather than targeted studies in which the measured proteins are chosen to prove a hypothesis. Classical proteomics studies using 2D gels often miss DNA repair proteins, because these studies analyse total protein extracts from exposed cells. The content of DNA repair proteins in these extracts is very low as compared to other proteins. Using a 2D-gel proteomics approach, we recently demonstrated the impact of ZnO-NP on the repair of methylglyoxal adducts in DNA in J774 macrophages (33). Methylglyoxal is a metabolite produced in cells during the oxidative stress response, which reaches the nucleus, thus causing DNA damage. In J774 cells, we showed that the intracellular content of three proteins involved in the metabolism of methylglyoxal was increased: two aldose and one hydroxyacylglutathione hydrolase. We also identified increased contents of two proteins involved in the degradation of altered deoxyribonucleotides, namely deoxyribonucleotidase and deoxyribonucleotide *N*-hydrolase. These observations were concomitant with increased contents of methylglyoxal DNA adducts in ZnO-NP–exposed cells (33). We thus identified a novel type of DNA lesion caused by NP-induced oxidative stress, together with increased contents of proteins involved in its elimination.

A better way to analyse DNA repair proteins would be to analyse the proteome of nuclear extracts, but to our present knowledge, it has not been done in cells exposed to NPs. We only identified one proteomic study showing altered DNA repair protein contents in NP-exposed cells. This study was conducted on Caco-2/HT29MTX intestinal cells, small airway epithelial cells and THP1 macrophages that were exposed to either multiwalled carbon nanotubes (MWCNTs) or TiO_2 nanobelts (TiO_2-NBs), i.e. high aspect ratio NPs. This study compared the proteomic impact of such exposure with its transcriptomic impact. Apoptosis was one of the top enriched biological processes in TiO_2-NB–exposed cells in both the proteomic and transcriptomic experiments. A metacore network created around this pathway, using direct interaction between the nodes of these two data sets, was shown to include several DNA repair proteins, among them KU70/80, RAD51, PARP1, BRCA1, GADD45 alpha and -beta and APE1 (34).

The literature reporting the impact of NPs on the expression of genes encoding DNA repair proteins is more important. Modulation of DNA repair gene expression may be a sign of induction of repair activity by NPs, via increased activity of transcription factors that regulate the expression of DNA repair proteins. Altered expression of these transcription factors may simply be a response of the cell to DNA damage. Alternatively, it may be a consequence of direct interaction of NPs with the transcription factor, which may either activate or inactivate it. In that sense, NP impact on the expression of DNA repair genes may be indirect (as a consequence of DNA damage) or direct (if NPs interact with transcription factors). Upregulation of DNA repair genes may possibly lead to increased DNA repair activity but not necessarily. Downregulation of DNA repair gene expression would lead to less DNA repair proteins in the cells and therefore impair DNA repair processes. Indeed, it would alter the renewal of DNA repair proteins, if they are inactivated or if their intracellular content decreases because they adsorb on the surface of NPs.

From toxicogenomics studies, very low modulation of gene expression was observed in Jurkat T cells exposed to Ag-NPs (35), while more intense transcriptional regulation was observed in A549 or Hep-G2 cells also exposed to Ag-NPs (36,37). Among the pathways impacted by Ag-NPs detected in these studies, altered expression of genes involved in the DNA damage response or DNA repair were identified (36,37). More precisely, in HepG2 cells, modulation of the p53 signalling pathway and NRF-2–mediated oxidative stress response pathway were observed after exposure to 20 and 50 nm Ag-NPs (37). The authors observed upregulation of gene expression in Ag-NP–exposed cells. Among the upregulated genes, they identified the gene encoding GADD45, which is implicated in the DNA damage response and cell cycle control and which plays a central role in cellular transformation (37). Upregulation of GADD45 was also observed in A549 cells exposed to 16 nm Ag-NPs at 24 h post-exposure. In these cells, the authors also observed downregulation of a series of genes encoding histones (36). Upregulation of proliferating cell nuclear antigen (PCNA) was observed at 48 h, while p53 was downregulated at 24 h (36). Together, these data suggest an impact of Ag-NPs on cell cycle progression, probably linked to a response to DNA damage.

Downregulation of DNA repair gene expression was reported in targeted studies and often coupled with the evaluation of NP genotoxicity. Implication of GADD45 in the toxicological response to Ag-NPs was also reported in a study by Asharani *et al.* (38) in which U251 human glioblastoma cells and IRM-90 normal human lung fibroblasts were exposed to 6–20 nm Ag-NPs. These NPs were previously reported to induce DNA damage and cell cycle arrest (39). The expression of *GADD45A* was downregulated in IRM-90, while the expression of *GADD45G* was upregulated. In this cancer cell line, upregulation of *GADD45G* was correlated to deregulated expression of a series of genes involved in DNA repair pathways, especially via BER (*MBD4*, *APEX1*, *OGG1*, *MUTYH*) and MMR (*MUTYH*, *ABL1*, *PMS1*, *MSH2*). PCNA and RPA1, which also play central roles in several DNA repair pathways, were downregulated in both cell lines. At the protein level, p53, p21, PCNA and cyclin B contents were decreased, all of which are involved in cell cycle control and indirectly involved in DNA repair processes. In addition, increased cleavage of caspase 3 and PARP, as well as increased phosphorylation of p53, demonstrated onset of apoptosis in some conditions (38). Finally, *in vivo*, a recent study showed that oral exposure of mice to polyvinylpyrrolidone-coated Ag-NPs also led to the induction of genotoxic damage and mutagenic lesions. These NPs modulated the expression of 36 DNA repair genes (of 84 tested) in the liver of exposed animals. Most of these genes were downregulated, with BER being the most affected, compared with other DNA repair pathways (40). *In vivo*, exposure of rats by oral gavage to MWCNT or C60 fullerenes led to increased 8-oxo-dGuo contents in the liver and lung of treated animals and increased expression of OGG1 in their liver (41). OGG1 activity appeared to be unchanged (41). The molecular mechanisms behind this DNA repair gene downregulation is currently unknown.

Epigenetic regulation of DNA repair proteins

Epigenetic regulation has been explored as a cause of transcriptional modulation of DNA repair activity by NPs. Two targets were studied, the first one being modulation of the translation of proteins involved in the DNA damage response, via deregulation of microRNA (miRNA). The second target was modulation of the transcription of genes involved in the DNA damage response via methylation or demethylation of their promoters. Again, either large-scale or targeted approaches were used. Eom *et al.* used an miRNA microarray to probe the impact of NPs on epigenetic regulation of DNA repair processes. miRNAs are short non-coding RNAs that bind to target mRNAs when incorporated in an RNA-induced silencing complex (RISC). This binding leads to inhibition of the translation or favours degradation of the mRNA. In this microarray, three miRNAs had modulated expression, miR-504, miR-33 and miR-302, all three being regulators of the translation of mRNA involved in DNA damage response and apoptosis (35). miRNA-504 is a negative regulator of the expression of p53 (42), while miR-33 regulates cell cycle progression via inhibition of the expression of cyclin-dependent kinase 6 (CDK6) and cyclin D1 (CCND1) and its overexpression causes cell cycle arrest in the G1 phase (43). Overexpression of miR-302 in ovarian cancer cells inhibits cell proliferation and colony formation, arrests cell cycle in the G0/G1 phase and induces apoptosis (44). Modulation of DNA repair promoter gene methylation was reported for PARP-1 in cells exposed to TiO_2-NPs (45) and SiO_2-NPs (46). First, Gong *et al.* showed that SiO_2-NPs dramatically decreased the expression of PARP-1 in HaCaT cells, concomitant with decreased PARP-1 protein content. This was inversely correlated to increased methylation of the PARP-1 promoter. Knock-down of the DNA methyltransferase (DNMT1) gene in HaCaT cells, or chemical inhibition of DNMT1, restored the normal phenotype, proving that PARP1 promoter hypermethylation was correlated with the activity of DNMT1 (46). Bai *et al.* showed that exposure of A549 cells for 24 h to 22 nm TiO_2-NPs caused hypermethylation of PARP1 promoter. Pretreatment of cells with an ROS scavenger or with a chemical inhibitor of DNMT1 restored normal methylation levels, suggesting that hypermethylation resulted from oxidative stress and was mediated via increased activity of DNMT1 (45). The mode of action of TiO_2-NPs on methyltransferase is currently not documented. In a large-scale approach, we investigated the methylation profile of 20 DNA repair gene promoters in A549 cells exposed to 21 nm, mixed anatase/rutile TiO_2-NPs. The rationale for performing this study was that if DNMT1 was involved in DNA repair gene promoter hypermethylation, then it might not specifically impact PARP-1, and other promoters might be hypermethylated as well. We showed that 4 h exposure to these NPs increased the methylation of APE1 and POLD3 promoter, while 24 h of exposure increased the methylation of APE1, MRE11 and PMS2 (26). Hypermethylation was thus a more overall mode of action of TiO_2-NPs; still, it could not explain the overall repression of DNA repair gene expression that we observed (26).

Working hypotheses

NPs have been shown to accumulate in almost all cell types, mainly via endocytosis (47). Direct diffusion through the plasma membrane has also been hypothesized, although it is certainly a less likely process as compared to the high amount of NPs reaching the cytoplasmic compartment by endocytosis. Being trapped in cytoplasmic compartments, NPs may directly or indirectly alter the function of DNA repair proteins. NPs may prevent DNA repair protein synthesis and post-translational modifications or avoid their recruitment to the nucleus when necessary (Figure 2). The nucleotide pool that is necessary for correct re-synthesis of DNA during the repair process can also be impacted by NPs.

NPs with very small diameter [<5 nm (48)] may gain access to the nucleoplasm by transiting through nuclear pores and thereby come into contact with the DNA repair machinery. Moreover, proteins bearing a nuclear localisation sequence and decorating NP surface may serve as a cargo and transport NPs to the nucleoplasm by borrowing the machinery that is normally dedicated to the nuclear transport of essential biomolecules (48). Alternatively, NPs may gain access to the nucleoplasm and DNA repair machinery by taking advantage of nuclear envelope breakdown during the M phase of

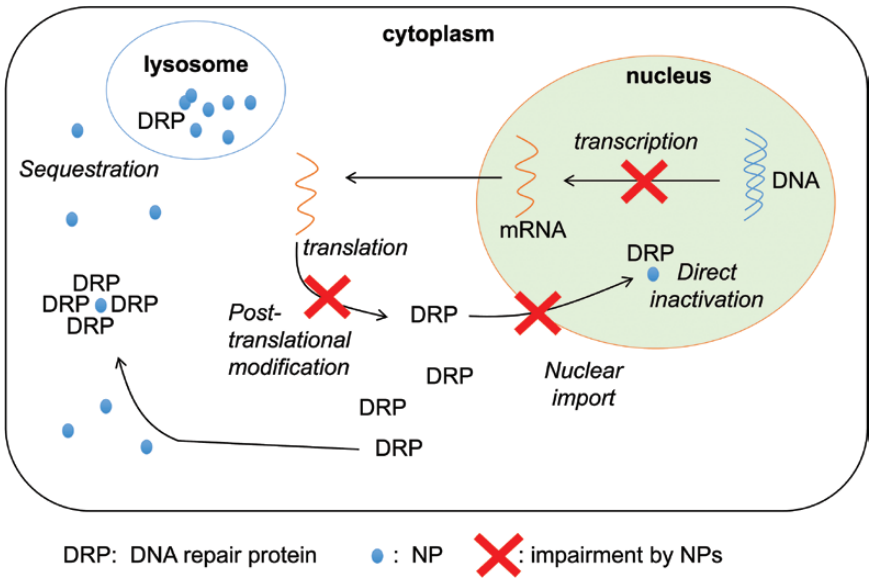

Figure 2. Impairment of DNA repair processes by NPs. NPs might directly or indirectly impair the activity of DNA repair proteins by altering their gene expression, i.e. transcription, mRNA translation, post-translational modification(s) or nuclear import for proper localisation to their site of action. In addition, NPs may sequester DNA repair proteins in the cytoplasm, either in the cytosol or in cytoplasmic compartments, such as lysosomes. Figure available in colour online.

the cell cycle. This may result in direct impairment of DNA repair processes, i.e. alteration of DNA repair protein action or of their proper location on damaged DNA.

Several strategies for NM grouping that can be used for risk assessment have been proposed recently. Among them, the one that was developed in the context of the EU-funded FP7 project MARINA (49,50) has identified four main groups of NMs: (i) main group 1: soluble NMs, which would release metal ions in their surrounding environment; (ii) main group 2: bio-persistent, high aspect ratio NMs such as carbon nanotubes that may be of concern because of their bio-persistence and fibre-like effect; (iii) main group 3: passive NMs, which would carry a passive surface i.e. without surface reactivity; and (iv) main group 4: active NMs. This grouping strategy can be used to forecast the impact that NPs may have on DNA repair processes.

Impact of metal ions released from NPs on DNA repair protein functions

NPs from main group 1 may dissolve and release toxic metal ions; for instance, in the case of Ag-NPs, CuO-NPs, ZnO-NPs, cadmium selenide (CdSe), cadmium telluride and possibly other quantum dots such as InP or $CuInS_2$. From the current state of knowledge, the toxicological impact of soluble metal-containing NPs has primarily been shown to rely on the impact of toxic metal ions released from the NP. Their impact on DNA repair processes is thus highly linked to that of the metal they are composed of (51). The mutagenicity of carcinogenic metal compounds is often low. Their carcinogenicity is considered to be a consequence of their impact on DNA repair processes (51). Arsenic, cobalt, cadmium and nickel are among these metals, as they are considered as co-mutagenic by impairing DNA repair by excision/synthesis pathways, i.e. BER and NER (51).

Being internalised in cells primarily via endocytosis (47), NPs reach the lysosomal compartment where the pH drops to around 5. These pH-sensitive NPs would be subject to acidic destruction and would release metal ions. NPs would also degrade due to complexation of their surface atoms with some highly affine ligands. For example, CuO-NPs have been shown to dissolve to a great extent in lysosomal fluids *in vitro* (29). Furthermore, Ag-NPs sulphidise in environmental or biological fluids, leading to the transformation to silver sulphide (52). In mammalian cells, Ag-NPs have been shown to accumulate, dissolve and reprecipitate as Ag/S NPs or deposit Ag ions on S-containing sheet-shaped cellular structures (Figure 3) (53–55). Intracellularly, the fate of Ag-NPs thus depends on the concentrations of –SH-containing ligands such as GSH and metallothioneins, which are known to be highly affine for Ag (56).

Proteins that necessitate metallic cofactors to be active are probably affected by metal ions released from NPs. Generally, by releasing metal ions, NPs would affect metal homeostasis in the cells, therefore altering all the cellular processes that necessitate this homeostasis. For example, ZF proteins are a heterogeneous family of proteins that share the property of carrying one or several ZF domains. These domains typically contain 30–40 amino acids and form a 3D structure resembling a finger, maintained by the coordination of a Zn(II) ion. In the finger, Zn(II) ions interact with chain donors of histidines or cysteines. The classical ZF motif, abbreviated CCHH, contains two Zn-binding domains, the first one composed of two cysteines and the second one composed of two histidines. In both of them, the –SH-containing amino acid is separated by two or more non-bonding amino acids, forming a first loop where one Zn(II) ion binds. Other ZF motifs either contain four cysteines or three cysteines and one histidine, all of them adopting the same structure upon Zn(II) binding. Depending on their sequence, these motifs adopt different secondary structures that modulate their properties. The function of ZF motifs is primarily to interact with sequences of nucleic acids (DNA, RNA) and proteins. The presence of Zn(II) in the ZF domain stabilises its 3D structure, which is necessary for this interaction, but it does not directly participate in this interaction. Metal ions released from NPs may substitute to Zn(II) in the ZFs, leading to the distortion of the protein active site and finally to their partial or total inactivation. They may also, via their pro-oxidant potential, induce production of some ROS that oxidise cysteines in the ZF domains and consequently impair proper Zn(II) binding. Indeed *in vitro* experiments have shown that singlet oxygen is able to release Zn from ZF analogs (57). These modes of action have already been reported and reviewed for metal ions (58) but not so much for NPs. Most ZF proteins are transcription factors, but several DNA repair proteins containing ZF motifs have also been identified. The most studied ZF DNA repair protein is the bacterial Fpg, which is classically used in the comet assay to incise the so-called Fpg-sensitive sites. These sites include 8-oxo-dGuo, the imidazole ring-opened purines 2,6-diamino- 4-hydroxy-5-formamidopyrimidine (FapyGua) and

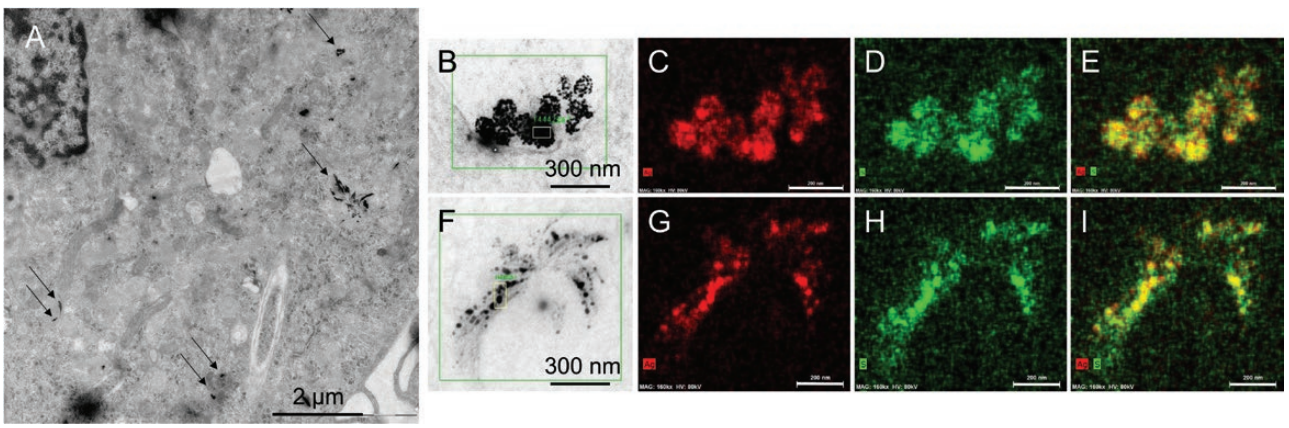

Figure 3. Intracellular dissolution of Ag-NPs. Intracellular dissolution was probed in murine primary macrophages exposed to Ag-NPs coated with polyvinylpyrrolidone. Electron microscopic observation (**A**, **B**, **F**) and energy-dispersive spectroscopy (EDS) analysis (**C**–**E**, **G**–**I**) of macrophages exposed for 24 h to NPs. B and F are closer views of typical Ag-containing deposits observed in macrophages (identified by arrows in A); their EDS analysis shows that they contain both Ag (C, G) and S (D, H). Merge of Ag and S distribution patterns in this area (E, I). More information can be found in literature (53,54). Figure available in colour online. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

4,6-diamino-5-formamidopyrimidine (FapyAde) and 8-hydroxyadenine (59). Other examples of ZF domain-containing DNA repair protein are XPA, which is involved in the recognition of helix-distorting DNA lesions that are repaired via BER and PARP1, which is involved in the recognition of DNA strand breaks which lead to the synthesis of poly(ADP-ribose). p53, largely involved in the DNA damage response, also contains ZF domains. Fpg has been described as being inhibited by a variety of metal ions, such as Cd(II), Cu(II) and Hg(II), while XPA is inhibited by Cd(II), Cu(II), Ni(II) and Co(II) (51). As previously stated, its activity is also decreased by treatment with Ag-NPs, due to the release of Ag ions, and by CeO_2 and Co_3O_4-NPs (30). Moreover, since CdSe quantum dots are known to dissolve intracellularly, leading to the release of Cd(II), these NPs may inactivate both Fpg and XPA. PARP-1 has also been shown to be inactivated by metal complexes made of platinum, ruthenium and gold (Au) (60) and would thus probably be inactivated by metal ions released from NPs. As stated previously, it is also inactivated in cells treated with Cu ions (61) and CuO-NPs and MPs (29).

Active NPs and their impact on DNA repair proteins

Among poorly soluble NPs, those from Group 4 as defined by Arts *et al.* (49) are active. This is due to their surface properties, as they may activate or inversely inactivate biological molecules and reactions. For instance, CeO_2 and TiO_2 belong to this main group. The Ce atoms at the surface of CeO_2-NPs have been shown to be subject to surface oxidation–reduction, cycling between their +III and +IV oxidation states and consequently leading to redox modification of surrounding biomolecules. Therefore, CeO_2 particles have been shown to be potent biological antioxidants (20). Conversely, TiO_2-NPs are well-known photocatalytic agents, as they become highly active following sunlight exposure and may cause the release of reactive species, i.e. ROS or reactive nitrogen species (RNS). Release of reactive species has also been reported when TiO_2-NPs are manipulated in the dark, i.e. their surface is intrinsically reactive, even if not exposed to light (62). These redox properties might lead to the inactivation of DNA repair proteins, as previously shown for the OGG1 protein. This glycosylase is inactivated by reversible oxidation of critical residues (63) which was demonstrated to occur in cells exposed to Cd(II) (64). This inactivation only relied on oxidation of hOGG1 and led to sequential relocalisation of the protein to nuclear granules and then to cytoplasmic stress granules (64).

Sequestration of DNA repair proteins in the NP protein corona

For all groups of NPs described by Arts *et al.* (49), a common mechanism that may impair DNA repair protein function is their sequestration on the surface of NPs. Indeed, NPs are known for their high affinity for proteins, thereby forming the so-called protein corona, which confers to the NP its biological identity. The concept of protein corona emerged in the late 2000s (65–68) and is now recognised as a major parameter governing bio-nano interactions and NP impact on living organisms (69). This notion was then refined by the introduction of concepts of 'soft' and 'hard' protein corona. Indeed, the adsorption of molecules on the surface of NPs is a dynamic process. For example, depending on the composition of the surrounding medium, the adsorbed molecules desorb and are replaced by molecules with higher affinities. The hard corona is defined as the first layer of biomolecules that firmly adsorb on NP surface and may not be easily replaced by other ones. This is considered as a signature of the passage of NPs in a particular environment. On the other hand, the soft corona is composed of molecules that are adsorbed on the surface of the hard corona with rapid exchange rates. These proteins exchange as soon as the surrounding environment of the NP changes (68).

DNA repair proteins may be captured in the corona and hence inactivated simply by sequestration, because they are no longer available for DNA repair. This sequestration is of particular concern for DNA repair proteins, which are poorly abundant in the cell and necessitate nuclear localisation to be active. Moreover, due to their large specific surface area, NPs would store large amounts of these proteins on their surface, eventually leading to their deprivation. DNA repair proteins can also be inactivated, because adsorption on NP surface would affect their conformation (Figure 4). To our knowledge, conformational change of DNA repair proteins

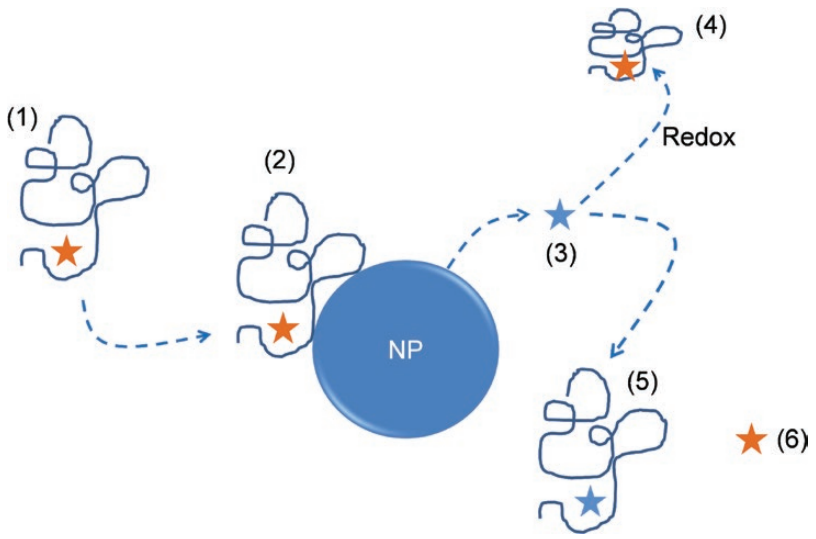

Figure 4. Mechanisms of DNA repair protein inactivation by NPs. Active DNA repair proteins (1) containing a metallic cofactor (orange star) that might adsorb on the surface of the NP (2) and thus get sequestered in an improper location. NPs might release metal ions [(3) blue star] that might induce oxidative stress (4), which in turn inactivates the DNA repair protein by oxidising its active site. The released ion might may replace the metallic cofactor in the active site of the protein (5) leading to a conformational change and to the release of the cofactor (6). Figure available in colour online. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

upon adsorption on NP surface has never been investigated, but it is well described for other types of proteins. For instance, lactate dehydrogenase (LDH) is inactivated by silicon dioxide (SiO_2), Au and CdSe NPs, and this causes interference with the classical LDH method for cytotoxicity assessment (70). Furthermore, transferrin is inactivated when adsorbed on superparamagnetic iron oxide NPs because of conformational modification due to the physical contact (71) between the protein and NPs.

Although it is not completely understood at present, the physical interaction between NPs and proteins is driven by the physico-chemical characteristics of the NP, i.e. its size which defines its surface curvature, (72) its surface state and reactivity. The physico-chemical properties of the protein is a critical parameter as well. For instance, SiO_2-NPs were shown to preferentially bind proteins with low aromatic residue contents, i.e. phenylalanine, tryptophan, tyrosine and histidine. They rather bind proteins with low structural rigidity and low amounts of π–π interactions (73). Moreover, unfolded green fluorescent protein (GFP) was shown to have higher affinity for these SiO_2-NPs than its folded form (74). In addition, these SiO_2-NPs also adsorb HSP60, the chaperonin that is responsible for GFP folding. As a consequence, SiO_2-NPs both adsorb GFP and interfere with its proper folding, which are the two mechanisms leading to its inactivation (74). Another example is that of TiO_2-NPs, which are known for their high affinity for phosphate groups. Due to this property, they have been used for decades for affinity purification of phosphoproteins in proteomics analyses (75). Consequently, TiO_2-NPs would rather bind the phosphorylated forms of proteins, leading to interference with cellular processes involving phosphorylation–dephosphorylation reactions, such as kinase cascades. These cascades play central roles in intracellular signalling, especially in DNA repair processes (76); consequently, this could be a mechanism of DNA repair impairment by TiO_2-NPs. Moreover, DNA is a poly-anion, carrying phosphate residues, for which TiO_2-NPs might also have high affinity. These NPs, if they are able to reach the nucleoplasm might therefore bind to DNA and avoid proper bonding between damaged sequences and DNA repair proteins.

Concluding remarks

The recent literature clearly indicates that the toxicity of NMs could have different origins. Regarding genotoxicity, the induction of oxidative damage to DNA due to oxidative stress is well-documented. In addition, the possibility for NMs to impact the repair machinery of the contaminated cells as reported recently can be a double-edge sword, by not only increasing the level of DNA lesions but also reducing their efficacy of repair. As a long-term consequence, this may increase the induction of mutation. Moreover, humans are never exposed to a single genotoxic agent, but most of the time to low doses of a mixture of different compounds and also possibly to physical agents. Therefore, if a contamination with NPs impairs the DNA repair machinery, simultaneous exposure with other DNA damaging agents could have more severe consequences. For instance, a co-contamination with a DNA-damaging drug in the presence of NPs may reduce the kinetic and efficacy of repair of the induced DNA lesions, thus potentially increasing the biological consequences (mutations, chromosomal aberrations etc.) of the drug. This could be of particular importance for exposure of humans by inhalation of polluted atmospheres potentially containing NPs and also well-known genotoxic compounds such as polycyclic aromatic hydrocarbons.

Consequently, it seems particularly important to study the effects of NPs on the DNA repair machinery. For such a purpose, omics approaches may not be sufficient and measuring the efficacy of repair directly into cells or cells extracts should be preferred. Further work is thus required to provide new information and to determine the possible origin of such an inhibitory effect. In addition, appropriated models (3D models, co-culture etc.) and exposure conditions (chronic *vs*. acute with biologically relevant doses) should be preferred in order to better estimate the effect on living organisms.

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