Research Paper

Crucial role of chelatable iron in silver nanoparticles induced DNA damage and cytotoxicity

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\textbf{A R T I C L E  I N F O}

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\textbf{A B S T R A C T}

Damage to mitochondria and subsequent ROS leakage is a commonly accepted mechanism of nanoparticle toxicity. However, malfunction of mitochondria results in generation of superoxide anion radical (O$_2^-$), which due to the relatively low chemical reactivity is rather unlikely to cause harmful effects triggered by nanoparticles. We show that treatment of HepG2 cells with silver nanoparticles (AgNPs) resulted in generation of H$_2$O$_2$ instead of O$_2^-$. As measured by ROS specific mitochondrial probes. Moreover, addition of a selective iron chelator diminished AgNPs toxicity. Altogether these results suggest that O$_2^-$-generated during NPs induced mitochondrial collapse is rapidly dismutated to H$_2$O$_2$, which in the presence of iron ions undergoes a Fenton reaction to produce an extremely reactive hydroxyl radical (OH). Clarification of the mechanism of NPs-dependent generation of OH and demonstration of the crucial role of iron ions in NPs toxicity will facilitate our understanding of NPs toxicity and the design of safe nanomaterials.

1. Introduction

Iron is an essential component of many enzymes involved in a variety of biological processes, including electron transfer, oxygen transport, DNA synthesis and repair [1]. Despite its necessity for almost all living organisms, iron in excess is dangerous. In the presence of ferrous ions, hydrogen peroxide undergoes the Fenton reaction to produce an extremely reactive hydroxyl radical (OH). Radical reactions initiated by OH may result in damage to the macromolecules, such as DNA, lipids and proteins [2,3]. Iron overload has been linked to increased risk of coronary heart disease, inflammation, neurodegenerative disease and cancer [4,5]. Iron content was also reported to correlate with the amount of oxidative damage to DNA [6,7] and with urinary excretion of 8-hydroxy-2-deoxyguanosine [8].

Iron uptake and storage are carried out by different proteins, thus there is a pool of chelatable iron ions (chelatable iron pool, CIP) that reflects a junction of metabolic pathways of iron-containing molecules. Although the CIP represents only a minor fraction of total cellular iron (3–5%), it is easily accessible and engaged in formation of reactive oxygen species (ROS) [9]. Increased ROS production leads to an imbalance between generation of free radicals and their neutralisation by cellular antioxidative defence mechanisms and causes disturbance of the redox equilibrium, known as oxidative stress. Being highly reactive, ROS are able to modify cellular components, causing cyto- and genotoxic effects.

An increase in ROS due to nanoparticles (NPs) treatment is well documented. It has been shown to be a key factor in the biological effects of NPs, both in vivo and in vitro [10–13]. Although low concentrations of ROS are generated during cell respiration under normal physiological conditions, the presence of NPs markedly increases ROS formation, likely due to the interference with mitochondrial or non-mitochondrial ROS producing enzymes. Indeed, an NPs-dependent increase in production of superoxide anion radical (O$_2^-$) by NADPH oxidase, accompanied by intracellular production of the other ROS was recently reported for AgNPs [14] and ultrafine particles [15]. It was also shown that different non-metal NPs co-localize with mitochondrial markers [16]. Also AgNPs of varying size and shape accumulate in the mitochondria [17]. Thus, it seems plausible to assume that AgNPs accumulation could be a direct cause of mitochondrial damage and malfunction of the respiratory chain resulting in ROS generation. For

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example, in BRL 3A rat liver cells exposed to AgNPs (15 and 100 nm) the cellular level of ROS increased in a AgNPs concentration-dependent manner and reached a maximum after 6 h [18]. Moreover, in three human cell lines treated with AgNPs, the extent of ROS production correlated with intracellular nanoparticle accumulation and genotoxicity, and negatively with long term cell survival [19]. The recent critical review on AgNPs toxicity leaves no doubts that ROS induction due to the malfunction of mitochondria might be a major cause of detrimental effects exerted by AgNPs on living cells [20].

Here we investigate the mechanism of AgNPs toxicity with special attention paid to generation of ROS and the role of iron in the formation of NPs-induced oxidative damage to DNA and toxicity.

2. Materials and methods

2.1. Chemicals and cell culture

All chemicals, cell culture media ingredients, etc. were purchased from Sigma-Aldrich (Poland) unless otherwise indicated. HepG2 cells were obtained from ATCC. Cells were cultured in cell culture medium with 10% Fetal Bovine Serum at 37 °C in a 95% moist atmosphere with 5% carbon dioxide. Serum at 37 °C.

2.2. Nanoparticle preparation and characterization

AgNPs of nominal size 20 nm were purchased from Plasmachem GmbH, Germany. The stock solution (2 mg/mL) was prepared by suspending of 2 mg AgNPs in 800 µL of distilled water, followed by sonification (4.2 kJ/cm², Bronson, USA). Immediately after sonification 100 µL of 15% BSA and 100 µL of a 10× concentrated phosphate buffered saline [22]. Size and ζ-potential of AgNPs aggregates in suspension were determined by the dynamic light scattering (DLS) method (Zetasizer S, Malvern Instruments, Malvern, United Kingdom).

2.3. Neutral Red assay

The Neutral Red (NR) assay was used to assess proliferation of HepG2 cells after treatment with AgNPs, DFO or their combination. The assay was performed as described in [22]. In brief, HepG2 cells were seeded in 96-well microplates (TPP, Switzerland) at a density of 1 × 10⁴ cells/well in 100 µL of culture medium. Twenty four hours after seeding, cells were treated as described above. After treatment cell culture medium was removed, the cells were washed with 150 µL PBS and incubated for 3 h at 37 °C with 100 µL of neutral red solution at a final concentration of 50 µg/mL. Next the NR solution was aspirated, cells were washed with 150 µL of PBS and 200 µL of an acetic acid-ethanol solution (49% water, 50% ethanol and 1% acetic acid) was added to each well. After 15 min of gentle shaking, optical density was read at 540 nm in plate reader spectrophotometer Infinite M200 (Tecan, Austria). At least three independent experiments in six replicate wells were conducted per experimental point.

2.4. Alkaline comet assay

The comet assay (single cell gel electrophoresis) was performed as described in [23]. Briefly, an aliquot of cell suspension was mixed with an equal volume of 2% low melting point agarose (Type VII, Sigma), put on a microscope slide pre-coated with 0.5% normal agarose (Type I-A, Sigma) and left on ice. After agarose solidification, the slides were immersed in ice-cold-lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris and 1% Triton X-100, pH 10). After 1 h lysis, the slides were placed on a horizontal gel electrophoresis unit filled with a fresh electrophoretic buffer (1 mM Na₂EDTA (sodium ethylenediamine tetra-acetate) and 300 mM NaOH) and allowed to stay in the buffer for 40 min for DNA unwinding. Next, electrophoresis was performed (1.2 V/cm, 30 min, 10 °C). After electrophoresis, the slides were washed with 0.4 M Tris, pH 7.5 (3 × 5 min) and stained with DAPI (4’,6-diamidino-2-phenylindole), 50 µL per slide (1 µg/mL).

Basically the same procedure was applied for the measurement of DNA base damage. The treated cells were incubated on slides with the formamido-pyrimidine DNA glycosylase (FPG, New England Biolabs, UK), as described in [24]. Briefly, after lysis, the slides were washed 3 × 5 min with the FPG buffer (40 mM Heps (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid), 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8) at 4 °C. Further, 50 µL of FPG solution (4.8 × 10⁻² U) in the buffer was placed on each slide, covered with cover glass and incubated for 30 min in a light-protected box at 37 °C. The slides were stained with DAPI (1 µg/mL) and analysed as described above. Image analysis of the data was performed with the Comet Assay IV Image Analysis System (Perceptive Instruments, UK). Fifty randomly selected comets per slide were analysed, two slides per experimental point. The percentage of DNA in the comet tail was used in this study as a measure of DNA damage.

The induction of DNA damage by a combined treatment with AgNPs and DFO was compared to the “expected value”. The “expected value” concept is based on the assumption that action of both factors (NPs and DFO) is independent, and their combined toxicity is a sum of toxicities of each factor alone (neutral effect). If the combined toxicity is lower than the “expected value”, the sparing effect is observed. If the combined toxicity is higher than the “expected value”, the synergistic (potentiating) effect is observed.

2.5. Detection of H₂O₂ in mitochondria

2.5.1. Cell transfection with Hyper Mito plasmid

pHyPer-dMito plasmid was purchased from Evrogen (Russia) To ensure that maximum transfection rate is achieved, the electroporation parameters have been optimized for HepG2 cells. Cells were harvested in the exponential growth phase, diluted in culture medium DMEM and the number of cells was determined using a Countess Automated Cell Counter (Invitrogen). One million HepG2 cells were spun down and resuspended in 800 µL of Eppendorf Hypoosmolar Electroporation Buffer. Ten micrograms of plasmid DNA were added and mixed well. Afterwards the cell suspension was transferred to 4 mm gap width electroporation cuvettes and electroporation was carried out using the Eppendorf Multiporator set to following parameters: 500 V, 3 pulses and 100 µs time constant at room temperature. After pulsing the cells were allowed to remain in the cuvettes for 5 min and then carefully transferred into 3 mL of fresh culture medium DMEM with 10% FBS and cultivated in 6-well culture plates at least 24 h.
2.6. Detection of H$_2$O$_2$ in cytosol

Cells were incubated for 2 h with 50 µg/mL AgNPs and dihydroethidine 123 was added to the final concentration 5 µM for 40 min. Then, cells were harvested, resuspended in DMEM with 10% FBS and change of fluorescence intensity was measured with a LSRII flow cytometer (BD) on PE-channel (FL-2).

2.7. Detection of O$_2^-$ in mitochondria

Cells were incubated for 2 h with 50 µg/mL AgNPs and MitoSox Red probe was added to the final concentration 5 µM for 40 min. Then, cells were harvested, resuspended in DMEM with 10% FBS and change of fluorescence intensity was measured with a LSRII flow cytometer (BD) on FITC-channel (FL-1).

2.8. Detection of O$_2^-$ in cytosol

Cells were incubated for 2 h with 50 µg/mL AgNPs and dihydroethidine were added to the final concentration 5 µM for 1 h. Then, cells were harvested, resuspended in DMEM with 10% FBS and change of fluorescence intensity was measured with a LSRII flow cytometer (BD) on PE-channel (FL-2).

2.9. Statistics

If not otherwise indicated, significance of the difference of means was evaluated by Student t-test for independent samples. The time and AgNPs concentration dependence of generation of H$_2$O$_2$ in mitochondria was analysed by two-way ANOVA. Interaction of cytotoxic effects of DFO and AgNPs was evaluated by combination index method [21].

3. Results and discussion

Many TEM microphotographs clearly show that AgNPs of various sizes and shapes accumulate in the mitochondria. Thus, it is plausible to assume that NPs could be the direct cause of mitochondrial damage and malfunction of respiratory chain resulting in the ROS generation and oxidative stress. However, the primary product of failing electron transport chain is O$_2^-$. [25]. Due to its low chemical reactivity, O$_2^-$ is rather unlikely to cause harmful effects triggered by the presence of NPs [26]. In addition, being negatively charged O$_2^-$ does not pass freely through cell membranes and must be either protonated or converted to another uncharged compounds, to leave the mitochondria and exert detrimental effects in cytoplasm or nucleus. This discrepancy hampers our understanding of the basis of NPs induced toxicity. We anticipated that O$_2^-$ generated by mitochondria is dismutated to H$_2$O$_2$, and then 'OH is produced as a result of an iron-driven Fenton reaction. To solve this enigma we designed a set of experiments focused on the effects of iron chelation on AgNPs toxicity. In this study we used spherical AgNPs particles of nominal size 20 nm. The hydrodynamic diameter of AgNPs in Williams medium supplemented with 10% FCS was 78.22 ± 2.3 nm, polydispersity index 0.286, zeta potential – 33.6 mV. A detailed characteristics of the AgNPs used in this study, including its aggregation in time in different culture media was already published [22]. However, ions released from NPs to culture medium are a confounding factor, when NPs toxicity is measured. Understanding the importance of this issue, we have previously measured AgNPs dissolution in experimental conditions similar to those used in this study. The results suggested only a negligible role of silver ions in our experimental design, even during long incubation times [19].

3.1. AgNPs-induced mitochondrial stress

Overproduction of ROS is proposed as a crucial mechanism for the toxicity of engineered NP, including AgNPs [10,11,13]. Thus, four different fluorescent probes were used to assess production of H$_2$O$_2$ and O$_2^-$ in mitochondria and cytoplasm of AgNPs-treated cells. HyPer-dMito indicator protein was used to estimate the mitochondrion specific production of H$_2$O$_2$. In cells treated with 50 µg/mL of AgNPs fluorescence from Hyper-Mito protein increased to 140% of the control level (Fig. 1). Further analysis of time- and concentration-dependence of AgNPs effects by two-way ANOVA revealed statistically important effect of AgNPs concentration (P = 0.001) and time (P = 0.002). Post-hoc analysis revealed a significant difference from the control (untreated) in cells after 120 and 150 min of treatment with 25 and 50 µg/mL AgNPs (Fig. 2). In contrast, measurements of mitochondrial O$_2^-$...
formation with MitoSox Red probe revealed only slight, if any, increase in O$_2^•$- formation. The increase in mitochondrial H$_2$O$_2$ production corresponded to the level of H$_2$O$_2$ in cytosol (150% of the control), as measured by dihydrorhodamine 123 assay. Also, in accordance with the mitochondrial O$_2^•$- production, no increase of O$_2^•$- level was observed in cytosol, as measured by dihydroethidine assay (Fig. 1).

While, NP dependent production of ROS is usually evaluated with fluorescent dyes of limited specificity, such as dichlorodihydrofluorescein diacetate, that preclude exact determination of the nature of ROS produced, in this study we used a HyPer-dMito protein, the specificity of which was previously confirmed [27], leaving no doubts that H$_2$O$_2$ is produced by mitochondria of intact cells treated with AgNPs. Moreover, mitochondrial dysfunction and generation of H$_2$O$_2$ by mitochondria isolated from rats exposed to TiO$_2$NP was recently reported using a H$_2$O$_2$ specific dye – Amplex Red [28]. This is in agreement with a number of works on isolated mitochondria, in which production of H$_2$O$_2$ rather than O$_2^•$- was used as an indicator of mitochondria failure. It is thus believed that O$_2^•$- produced by a failing electron transport chain is dismutated by mitochondrial superoxide dismutase to H$_2$O$_2$ that can freely pass mitochondrial membranes. It is also in a good agreement with steady-state concentrations of O$_2^•$- (0.2–0.3 nM) and H$_2$O$_2$ (10–100 nM) in mitochondria [29].

3.2. Effect of DFO on AgNPs cytotoxicity

Nevertheless, production O$_2^•$- and/or H$_2$O$_2$ by mitochondria of NPs treated cells does not explain the observed nanoparticle geno- and cytotoxicity. Both compounds are not very reactive and rather unlikely to damage DNA or cause cell death. However, trace amounts of un-toxicity. Both compounds are not very reactive and rather unlikely to damage DNA or cause cell death. However, trace amounts of un-

3.3. Efect of DFO on DNA damage induction by AgNPs

We have recently shown that formation of ROS and oxidative damage to DNA correlates well with a decrease in cell viability, measured by the ability of cell to form colonies after the AgNPs treatment [19]. Thus, induction of DNA damage by AgNPs in the presence of iron chelator, deferoxamine (DFO), was estimated using the comet assay. DFO alone did not induce DNA damage. In contrast, AgNPs treatment induced a significant increase in the percent of DNA in comet tail indicating induction of DNA damage. Both induction of single stand breaks (SSB), as measured by alkaline comet assay, and induction of oxidatively damaged DNA bases, as measured by FPG-glycosylase modified assay, were observed. Pre-treatment of AgNPs-exposed cells for 2 h with DFO caused a significant decrease in induction of DNA damage. In DFO pre-treated cells the level of AgNPs-induced SSB was significantly lower than expected from the additive action of both compounds (Fig. 5).

Though our results point to the importance of lysosomal iron in NP induced toxicity, mitochondria themselves, as a site of iron-sulphur cluster synthesis, have a high intrinsic concentration of chelatable iron [4,38]. Moreover O$_2^•$- was proven to oxidize the [4Fe–4S] clusters of dehydratases, such as aconitase, realising Fe$^{+2}$ ions and further increasing the availability of iron for the Fenton reaction [44]. Whereas the actual origin of iron ions involved in NP-induced toxicity needs further investigations, our results leave no doubts that its chelation has a sparing effect and diminishes, both NP geno- and cytotoxicity.

Altogether, our results strongly suggest that chelation of un-shielded iron ions, in particular lysosomal iron, abolishes the toxic effect of AgNPs. This points to the crucial role of Fenton chemistry in oxidative-stress dependent NPs toxicity and solves the discrepancy between generation of relatively biologically inert compounds, such as...
and/or H2O2, by NPs-exposed mitochondria and the detrimental effects of NPs predominantly dependent on highly active •OH radical. A putative role of iron in AgNPs toxicity is summarized in Fig. 6. Whether this mechanism is universal for other types of NPs needs to be veriﬁed experimentally. However, having in mind the ubiquitous abundance of iron ions in living cells, the process seems to be prevalent as far as oxidative stress due to mitochondria dysfunction is concerned.

Fig. 3. Cytotoxicity of AgNPs in HepG2 cells pre-treated with DFO (25 μM). (a) 2 h pre-treatment. (b) 24 h pre-treatment, taking controls as 100%. Expected values reﬂect a sum of toxicity of AgNPs and DFO (additive effect). Whereas DFO induced only slight toxicity, treatment with AgNPs resulted in a marked decrease of cell survival. However, when cells were pre-treated with DFO, toxicity of AgNPs was signiﬁcantly lower. 1 – denotes statistically signiﬁcant difference of means from respective DFO + AgNPs treatment; 2 – denotes statistically signiﬁcant difference of means between experimental and expected values. Mean ± SD, n = 4, Student t-test, P < 0.05, df = 6. Since some expected values were below zero, their signiﬁcance was not evaluated. Statistical evaluation was performed on raw data presented in Supplementary Table 1.

Fig. 4. Analysis of combined effect of AgNPs and DFO on survival of HepG2 cells according to combination index method [21]. Insert presents Combination index (CI) values for Drug Combo: Ag + DFO (Ag + DFO [10:1]), see CompuSync report file AgNPs+DFO d 2.pdf (Supplementary materials) for full analysis. Fa – Fraction affected; CI values above 1 means antagonistic effect. A representative ﬁgure for 3 independent experiments. The analysis by combination index method clearly indicates that combined treatment with DFO and AgNPs in 1:10 ratio has strong sparing effect (antagonism) over a wide range of concentrations, as compared to the toxicity of AgNPs alone.

Fig. 5. Genotoxicity of AgNPs (10 μg/mL) in HepG2 cells pre-treated with DFO (100 μM) for 2 h. Expected values reﬂect a sum of genotoxicity of AgNPs and DFO. 1 – denotes statistically signiﬁcant difference of means from control; 2 – denotes statistically signiﬁcant difference of means from DFO + AgNPs treatment (mean ± SD, n = 4), Student t-test, P < 0.05, df = 6. Pretreatment with DFO has a sparing effect on AgNPs genotoxicity.

Fig. 6. A putative mechanism of induction of oxidative stress in AgNPs treated cells. [1] In mitochondrion. Superoxide anion radical (O2•−) generated by leaking mitochondrial electron transport chain is either dismutated to hydrogen peroxide H2O2 by superoxide dismutase 2 (SOD2, in matrix) or superoxide dismutase 1 (SOD1, in intermembrane space), or protonated to form hydroperoxyl radical (HO2•). Unlike O2•−, HO2• and H2O2 easily cross cell membranes, and so can penetrate between mitochondrial compartments or to cytoplasm. H2O2 formed in the mitochondrion can migrate to cytoplasm or undergo iron-catalysed Fenton reaction to form very reactive hydroperoxyl radical (•OH). [2] In cytoplasm. HO2• can be deprotonated to form O2•−, that is further dismutated to H2O2 by SOD1. O2•− is also generated by NADPH oxidase (NOX). H2O2 formed in cytoplasm can migrate to the mitochondrion or undergo iron-catalysed Fenton reaction to form very reactive •OH. O2•−/HO2• = electron transport chain. mtCIP, cytCIP = mitochondrial or cytosolic chelatable iron pool. DFO = iron chelator, deferoxamine.

O2•− and/or H2O2, by NPs-exposed mitochondria and the detrimental effects of NPs predominantly dependent on highly active •OH radical. A putative role of iron in AgNPs toxicity is summarized in Fig. 6. Whether this mechanism is universal for other types of NPs needs to be veriﬁed experimentally. However, having in mind the ubiquitous abundance of iron ions in living cells, the process seems to be prevalent as far as oxidative stress due to mitochondria dysfunction is concerned.
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Author contributions

A.G. designed part of experiments, contributed in the data analysis and manuscript writing, M.W. made comet assay experiments, S.M-W. was responsible for cytotoxicity tests, M.Z. measured generation of H2O2 in mitochondria including pHyPer-dMito transfection, D.W. measured other ROS, M.K. designed study outline and part of experiments, analysed data and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redxol.2018.01.006.

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