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EVALUATION OF BIOLOGICAL EFFECTS OF NANOMATERIALS. PART I. CYTO- AND GENOTOXICITY OF NANOSILVER COMPOSITES APPLIED IN TEXTILE TECHNOLOGIES

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Abstract
Objectives: The aim of this study was to investigate the cyto- and genotoxicity of nanocomposites (NCs) and generation of reactive oxygen species (ROS) as a result of particle-cell interactions. **Materials and Methods:** Titanium dioxide (TiO_2-Ag) and ion-exchange resin (Res-Ag), both coated with silver (Ag), were examined. The murine macrophage J774A.1 cells were incubated *in vitro* with NC at different concentrations for 24 h. Cytotoxicity was analyzed by the methylthiazolyldiphenyl-tetrazolium bromide reduction test (MTT reduction test). ROS generation was assessed by incubation of cells with dichlorodihydrofluorescein diacetate (DCF) and flow cytometry. DNA damage was detected by comet assay and included single-strand breaks (SSB), alkali-labile sites (ALS) and oxidative DNA damage after formamidopyrimidine glycosylase (FPG) treatment. The tail moment was used as an indicator of DNA damage. **Results:** TiO_2-Ag was not cytotoxic up to 200 μg/ml, whereas IC_{50} for Res-Ag was found to be 23 μg/ml. Intracellular ROS levels were elevated after 4 h of exposure to Res-Ag at the concentration of 50 μg/ml. Both types of NC induced fragmentation of DNA strands, but only one of the composites caused damage to purine bases. TiO_2-Ag induced SSB of DNA at concentrations of 10 and 5 μg/ml. For Res-Ag, a concentration-dependent increase in tail moments was observed. **Conclusions:** Silver-coated nanocomposites (both TiO_2-Ag and Res-Ag) may cause genotoxic effects in murine macrophages J774A.1. Res-Ag increased generation of ROS which suggested that toxicity of Res-Ag in murine macrophages is likely to be mediated through oxidative stress. This paper will support industry and regulators alike in the assessment of hazards and risks and methods for their mitigation at the earliest possible stage in material and product development.

Key words:
Nanosilver composites, Cytogenotoxicity, Genotoxicity, DNA damage, Textile technology

INTRODUCTION

A rapid increase in global production and application of nanomaterials has resulted in the development of nanotechnologies and, consequently, in potential human exposure. Based on the data published by the Institute of Occupational Medicine, UK, it can be asserted that nanoparticles may be a source of exposure in the following industrial sectors: manufacture and storage of dyes, pigments and cement; production of pharmaceuticals and chemicals (including cosmetics), as well as in research centres where nanoparticles

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are produced and investigated [1]. According to the cited authors, over 100 thousand people employed in the UK pharmaceutical industry are exposed to this kind of particles, and the estimated data provide evidence that the number of persons potentially exposed to nanoparticles in the "nano" production plants will double in five coming years [1].

In the United States, the number of persons exposed to nanoparticles is estimated at about 2 million workers [2]. It should be emphasized that in many branches of industry, substances harmful in terms of the size of particles are not investigated. Therefore, the number of persons exposed to nanoparticles seems to be underestimated [2].

Characteristics of nanoparticles facilitate their use in numerous areas, but at the same time they make them more harmful as compared to large particles. Potential exposure to nanoparticles (especially to engineering nanoparticles released from nanocomposites, NCs) becomes more and more realistic. Therefore, the assessment of health effects (including late ones) of NC commonly used in many branches of industry is now an issue of crucial importance. According to Scientific Committee on Emerging and Newly Identified Health Risk (SCENIHR) report [3], certain nanomaterials and composite materials may contain internal or external structures at the nanoscale that were incorporated to confer nanospecific characteristic. As the external dimensions of NC would be typically larger than 100 nm then, based solely on external size, most NC would not be considered to be nanomaterials. If the internal structure with a size at the nanoscale would be an element to be included in the definition, then NC will be included in the definition of nanomaterial. There are also nanocomposites where one phase is the solid bulk [3].

In the textile industry, nanocomposites are most frequently added to fibre-forming polymers during production processes of synthetic and man-made fibres [4] or incorporated into textile materials as mono- or multifunctional modifiers of polymers applied using the coating technique. Functional nanocomposites-modified textile materials characterized by such properties as non-flammability, electro-conductivity, photocatalytic, self-cleaning or antibacterial activity are already known [5–8].

Nanofibres, fibrous membranes, sensors, composites, nanofibre-based three-dimensional structures, nanolayers or functional coatings are used worldwide [9].

Biological nanofilters, barrier materials, active dressings, systems of drug release, tissue-engineering materials assimilable artificial skin or tissue implants form an important category of applications [10].

Inclusion of nanocomposites into polymer materials is linked not only with difficulties of technical nature, e.g. optimal monoparticle dispersion in the polymer matrix, but also with proper control of dosage to ensure that it is harmless to the environment. The durability of their binding within the material is extremely important in the case of material surface modification [11]. Progress made in the development of nanotechnology has rendered it possible to intensify effectiveness of nanocomposites, by e.g. increasing their photocatalytic properties through surface modification with noble metals [12].

Human exposure to NC may potentially be on the increase. Exposure to various nanoparticles may be associated with a number of adverse health effects [13–15]. Several studies demonstrated an association between exposure to particulate matter (PM) and adverse health effects, not only respiratory but cardiovascular diseases as well [16,17]. Exposure to various NP may be associated with several disturbances at molecular levels [13,15,18]. Cytotoxic effects of NP on macrophages include inflammation and irritation [19]. During inflammatory reactions, reactive oxygen and reactive nitrogen species (ROS/RNS) are produced, which play a major role in lipid peroxidation and loss of membrane integrity [20].

The mechanisms underlying particle-induced cellular changes, which ultimately may result in carcinogenesis are not fully understood. Therefore, the objective of this study was to investigate the cyto- and genotoxicity of nanocomposites and the generation of ROS resulting from particle-cell interactions.

MATERIALS AND METHODS

Materials

Titanium dioxide coated with silver ions (TiO_2-Ag), produced by Research Institute for Man-Made Fibres (VÚCHV), Slovakia, and zirconium phosphate-based ceramic ion-exchange resin containing silver (Res-Ag), produced by Milliken & Company, USA were tested. TiO_2-Ag is added to synthetic fibres and polymers during manufacture processes as well as to polymer coating of textile materials to provide antibacterial and photocatalytic performance for end-use articles (Photo 1). Res-Ag also possesses antimicrobial properties. Scanning electron microscope JEOL JSM-35C was used to analyze the nanocomposites. The mean diameter of tested nanocomposites was 320±80 nm for TiO_2-Ag, while for Res-Ag it was 460±82 nm (Photo 2). The weight percentage of ionic silver on TiO_2-Ag surface was 1.5%, and for Res-Ag surface was indicated as 10% (according to manufacturers specifications).

The mass specific surface area of those nanocomposites was measured by physical adsorption of gas

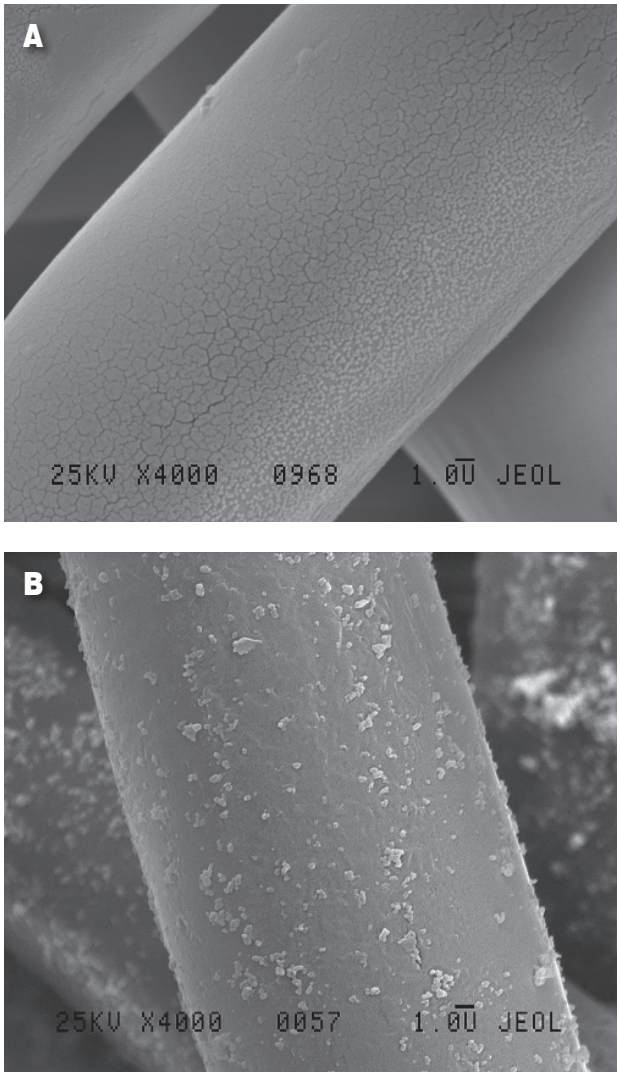


Photo 1. Surface of not modified (A) and TiO_2-Ag modified (B) fibres.

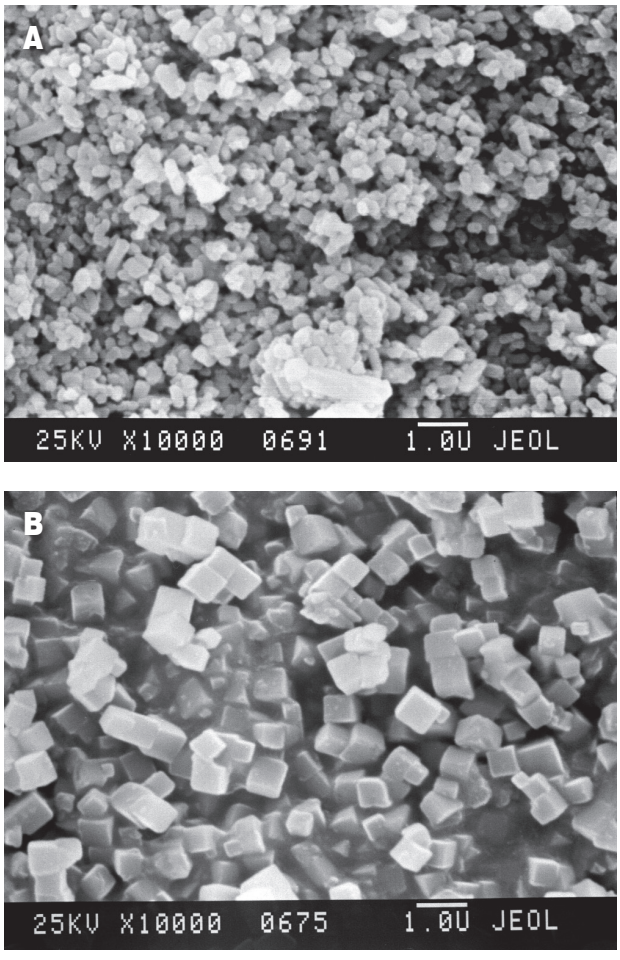


Photo 2. Microscopic images of TiO_2-Ag (A), and Res-Ag (B), both coated with silver ions.

molecules on a solid surface (Brunauer, Emmet, Teller; BET method) [21].

For better characterization of nanocomposites under study, the volume specific surface area (VSSA) was calculated using the density and mass specific area [3]. VSSA characterises the entire particulate surface area per volume of a solid and/or powder material. A practical advantage of the VSSA parameter is it simple calculation from two parameters usually available for each commercial nano- or micro-structured powder material; density (g/cm^3), and the mass specific surface area (m^2/g) of nanostructured material [3].

Cell culture

Murine macrophages J774A.1 (American Type Culture Collection; ATCC#TIB-67) were maintained in cDMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% heat-inactivated foetal bovine serum, 25 Mm HEPES, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin; Sigma–Aldrich Chemical Company; St. Louis, MO, USA). All tissue culture reagents contained < 10 pg/ml endotoxin contamination as certified by the manufacturer. The cells were screened for *Mycoplasma* sp. infection using indicator cell line 3T6 cells (ATCC#CCL-96) and MycoTech Kit (Gibco BRL).

Cytotoxicity assay

The cell viability was assessed using the methylthiazolyldiphenyl-tetrazolium bromide reduction test (MTT reduction test). In this assay, yellow tetrazolium salt MTT is reduced metabolically to purple formazan product, which is quantified colorimetrically [22]. In brief, J774A.1 macrophages were plated onto 96-well microplates (3×10^4 cells/well seeded on a prior day) and incubated in the absence or presence of test compounds for 24 h. At the end of the experiment, the supernatants were removed and the cells were incubated with 100 μl MTT (0.5 mg/ml) for 3 h. After discarding MTT solution, DMSO (50 μl) was added to each well. The optical density of solubilized formazan product was determined using a spectrophotometer (with a 550 nm and 620 nm filter as a reference). Results were expressed as the percent of cell survival (OD of exposed vs. OD of control non-exposed cells). The IC_{50} value (concentration evoking 50% inhibition of growth/viability of cells) for each compound was calculated.

Measurement of reactive oxygen species (ROS) generation

ROS generation was visualized by incubation of cells with dichloro-dihydrofluorescein diacetate (DCF) at a final concentration of 10 μM [23]. The fluorescent dye was added to NC for the last 30 min of the exposure period. After incubation, cells were washed twice with PBS, removed from culture dishes following a short trypsin treatment, resuspended in PBS containing 2 μg/ml propidium iodide (PI) and examined by flow cytometry (Cytomics FC 500 MPL, Beckman-Coulter, USA). Only PI-negative viable cells were included in the analysis.

Comet assay

The assay was performed according to the method of Singh et. al. [24], as modified by Mc Kelvey-Martin et al. [25]. Cells were embedded in agarose gels on microscope slides and lysed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base pH = 10,1% Triton X-100) at 4°C for at least 1 h. Then, DNA was denaturated in an electrophoretic buffer (1 mM Na_2EDTA, 300 mM NaOH, pH > 13) to allow DNA unwinding and producing single-stranded DNA and expressing alkali labile sites for 20 min at 4°C and electrophorezed in the same alkaline conditions (30 min, 0.9 V/cm, 25 V and 300 mA). Finally, the slides were neutralized by rinsing three times with 0.4 M Tris buffer, pH = 7.5, and dried for staining and analysis. To examine the oxidative damage, the modified

comet assay was used as described by Collins et al. [26]. After lysis, slides were washed three times for 5 min with enzyme buffer (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES-KOH, 0.2 mg/ml bovine serum albumin, pH = 8) and incubated at 37°C for 30 min with FPG (formamidopyrimidine glycosylase) at 1 μg/ml in the enzyme buffer. The slides were then processed as described earlier. Slides were stained with 5 μg/ml DAPI (4',6-diamidine-2-phenylindole dihydrochloride) and 50 cells in total were analyzed using a fluorescence microscope (Olympus BX40, Tokyo, Japan) equipped with an image analysis system (Lucia Comet Assay; Precoptic C., Prague, Czech Rep.).

Three experiments were performed for each type of nanocomposite. This method detects single-strand breaks (SSB), alkali-labile sites (ALS), and oxidative DNA damage after FPG treatment. The image analysis provided a variety of parameters for each comet, including tail length and % DNA in the tail. Tail moment was used as an indicator of DNA damage. This parameter expresses both the migration of DNA fragments forming the tail and the relative amount of DNA in the tail.

Statistical analysis

The results represent mean from three experiments with standard deviation (SD). The differences between the mean values of tail moment in NC treated cells and vehicle control groups were evaluated with ANOVA followed by Student's t-test. A value of $p < 0.05$ was considered to indicate statistical significant differences.

RESULTS

The specific surface area and VSSA value of nanosilver composites

The mass specific surface area (S_{BET}) of those NCs measured by BET method was found to be 8.95 m^2/g for TiO_2-Ag, and 2.61 m^2/g for Res-Ag. VSSB highly differs between those nanosilver composites and was 35.8 m^2/cm^3 and 0.78 m^2/cm^3 for TiO_2-Ag, and Res-Ag, respectively.

Influence of NCs on cell viability

The mouse macrophages J774A.1 were exposed to increasing concentrations of two NCs: TiO_2-Ag or Res-Ag, both coated with Ag for 24 h to assess cytotoxicity (Figures 1 and 2). TiO_2-Ag was not cytotoxic (Figure 1) up to 200 μg/ml, whereas Res-Ag was found to produce cytotoxicity (calculated IC_{50} was 23±3 μg/ml (Figure 2).

Intracellular levels of ROS

To determine intracellular ROS levels, cells were labelled with dichloro-dihydrofluorescein-diacetate (DCF) and fluorescence was measured by flow cytometry. Changes in the mean fluorescence intensity (MFI) relative to untreated control cultures were interpreted as increase or decrease in the amount of internal ROS. Incubations were

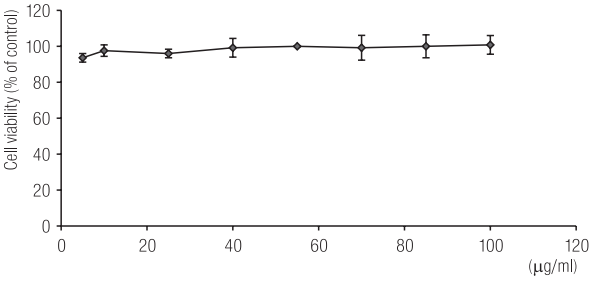


Fig. 1. Influence of TiO_2-Ag (24 hrs exposure) on mouse macrophages J774A.1 viability — MTT reduction assay.

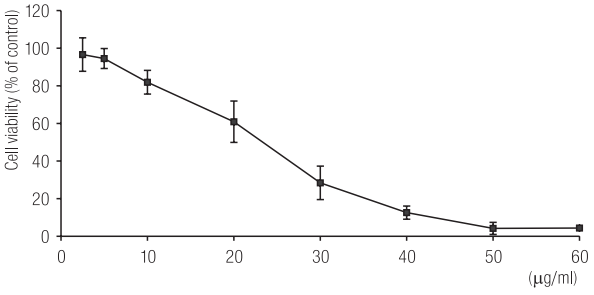
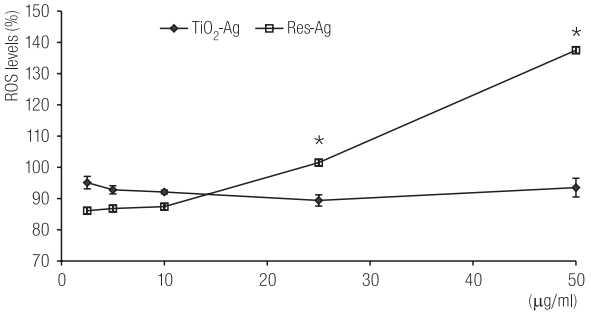


Fig. 2. Influence of Res-Ag (24 hrs exposure) on mouse macrophages J774A.1 viability — MTT reduction assay.



* Statistically significant as compared with TiO_2-Ag ($p < 0.05$).

Fig. 3. ROS production (%) measured by flow cytometry.

conducted for 4 h at increasing concentrations of NC. A rise in intracellular ROS levels was observed for Res-Ag at 50 μg/ml concentration after 4 h while TiO_2-Ag did not markedly changed MFI at the tested concentrations (Figure 3).

Induction of DNA damage

The levels of DNA damage induced by NC are presented in Table 1. Titanium dioxide-Ag induced concentration-dependent DNA SSB and oxidative DNA damage after exposure for 24 h. The tail moments in cells treated with TiO_2-Ag at 0.25, 2.5, 5 or 10 μg/ml were significantly higher compared to respective controls. Moreover, TiO_2-Ag-induced tail moments were further significantly raised when oxidative DNA damage was compared to the respective DNA SSB (Table 1).

Similarly, Res-Ag induced only DNA SSB in concentration-dependent manner (Table 2). DNA damage significantly higher than in control cells was observed at concentration of 2.5 μg/ml, while 0.25 μg/ml exerted no effect. The tail moments after treatment with FPG in cells treated with Res-Ag were at the same level as the respective DNA SSB (Table 2).

DISCUSSION

Numerous studies concerned with toxicity of nanoparticles were performed, mainly on cultured cell lines [18,27–29], but also on animals [30,31]. According to IUPAC report [32], a nanocomposite is a composite in which at least one of the phase domains has at least one dimension of the order of nanometers. Ajayan et al. [33] claim that nanocomposites are the combination of a bulk matrix and nanophase(s). Therefore, as nanocomposites have an integral structure on the nanoscale, they would be considered to be nanomaterials [3].

Very little is known on nanocomposites toxicity in general, and on cyto-and genotoxity in the cell lines in particular. Our study demonstrated that nanocomposites differed

Table 1. DNA damage in cultured mouse macrophages J774A.1 induced by nanosilver composites of titanium dioxide (TiO_2-Ag)

NCs (μg/ml)	DNA damage					
	DNA fragmentation (SSB) (mean ±SD)			oxidative DNA damage (FPG) (mean ±SD)		
	tail DNA %	tail length	tail moment	tail DNA %	tail length	tail moment
Control	2.52±0.35	3.04±0.24	0.32±0.04	2.75±0.46	4.12±1.63	0.35±0.04
10	12.77±0.81	12.01±2.94	4.85±0.50[a]	12.91±1.23	17.16±0.88	6.35±0.85[a,b]
5	8.53±1.40	9.51±0.80	3.14±0.47[a]	10.61±2.09	14.01±3.03	4.88±1.64[a,b]
2.5	5.99±1.04	6.57±1.27	1.54±0.33[a]	6.80±0.81	8.64±1.58	2.13±0.39[a,b]
0.25	4.88±0.93	5.28±0.67	1.07±0.13[a]	5.88±2.08	6.84±2.81	1.27±0.18[a]

[a] Significantly different vs. control ($p < 0.05$) (ANOVA).
[b] Significantly different vs. SSB ($p < 0.05$) (Student's t-test).

Table 2. DNA damage in cultured mouse macrophages J774A.1 induced by nanosilver composites of resin coated by silver (Res-Ag)

NCs (μg/ml)	DNA damage					
	DNA fragmentation (SSB) (mean ±SD)			oxidative DNA damage (FPG) (mean ±SD)		
	tail DNA %	tail length	tail moment	tail DNA %	tail length	tail moment
Control	1.73±0.41	2.73±0.78	0.11±0.03	1.66±0.25	2.16±0.43	0.13±0.04
10	4.56±0.98	5.33±0.91	0.97±0.05[a]	5.26±0.31	5.37±1.08	0.98±0.03[a]
5	3.22±0.73	4.85±2.20	0.50±0.12[a]	4.11±0.86	4.88±1.95	0.58±0.17[a]
2.5	2.41±0.31	4.00±1.46	0.32±0.04[a]	2.97±0.40	3.72±0.98	0.30±0.06[a]
0.25	1.60±0.10	2.19±0.30	0.14±0.05	1.84±0.17	2.28±0.17	0.13±0.04

[a] Significantly different vs. control ($p < 0.05$).

in the cytotoxicity produced. TiO_2-Ag was not cytotoxic, whereas Res-Ag was cytotoxic. Hussain et al. [18] studying different sizes of nanoparticles including Ag, molybdenum, TiO_2 and iron oxide found that Ag reduced cell viability in a concentration-dependent manner during a 24-hr incubation. In our study, NCs coated with Ag nanolayer differed in the effects on cell viability. It is likely that the NCs differed in surface physicochemical properties such that different recognition by the mouse macrophages J774A.1 may had occurred. The studies by Kagan et al. [19] indicated important role of surface characteristics of nanoparticles in their recognition by macrophages. Barnes et al. [34] indicated that the surface of nanoparticles should be taken into account not only in view of the stability of particle structure, but mostly due to possible electrostatic interactions between surface of nanoparticles and cellular proteins. All these observations on differences in recognition of nanoparticles by cells may have important implications for the relationship between the potentially toxic health effects of nanomaterials and their surface modification.

As evidenced in our study, TiO_2-Ag did not markedly affect ROS generation, whereas Res-Ag increased it moderately (40% as compared to TiO_2-Ag) after 4 h at the concentration of 50 μg/ml. Oberdorster [35] showed that fullerens induced oxidative stress in a fish model, which resulted in significantly enhanced lipid peroxidation. It is worth noting that for a number of nanoparticles oxidative stress-related inflammatory reactions were reported [36]. It is also postulated that nanoparticles may exert adverse effects via either direct interaction with DNA, release of ions with toxic properties from soluble nanoparticles, or by generation of ROS [34]. Investigations of the genotoxic activity (comet assay) of crystalline silica and high concentrations of amorphous silica nanoparticles demonstrated an increased ROS generation.

However, Lin et al. [37] failed to show an association between the genotoxic effect and size of amorphous silica particles because of particle aggregation. Studies carried out by Barnes et al. [34] also failed to confirm the genotoxic activity of amorphous silica samples within a wide range of concentrations (from 4 to 40 μg/l). Our results suggest that TiO_2-Ag increase DNA damage (both SSB and oxidative DNA damage) in J77A1.1 macrophages despite the lack of significantly changed level of intracellular ROS (Table 1, Figure 3). Res-Ag produced SSB in the absence of oxidative DNA damage, but caused a significant increase of ROS levels (Table 2, Figure 3). The mechanisms underlying particles-induced cell changes remain unknown. Hussain et al. [18] suggest that silver-coated nanoparticles

toxicity is related to oxidative stress due to significant depletion of glutathione level, reduced mitochondrial membrane potential and increase in ROS levels. Our data show that Res-Ag NC may cause increased generation of ROS (Figure 3) that may ultimately lead to the observed cytotoxicity (Figure 2) which is in good accordance with the data published by Hussein et al. [18]. Silver-coated nanocomposites, both TiO_2-Ag and Res-Ag, may cause genotoxic effects in murine macrophages J774A.1, and Res-Ag in particular cause increased generation of ROS, which suggests that toxicity of Res-Ag in murine macrophages is likely to be mediated through oxidative stress.

According to our knowledge, no studies investigating the potential toxic effect of nanocomposites are accessible and no clear guidelines are presently available to quantify these effects. Our paper gives important information about silver coated nanocomposites cytotoxicity and genotoxicity relevant for textile application, but several question arise as to what is the mechanism(s) responsible for the observed cyto- and genotoxicity. It is known that there are some critical points in toxicity evaluation of nanomaterials: particle size, surface area, and chemicals adsorbed or bound on the surface (e.g., silver-coated nanocomposites). Two nanocomposites under investigation in this study moderately differ in particle size (320±80 nm and 460±82 nm for TiO_2-Ag, and Res-Ag respectively) and surface area (8.95 m^2/g and 2.61 m^2/g, for TiO_2-Ag, and Res-Ag respectively), while the percentages of ionic silver on the surface highly differ between the tested nanocomposites (1.5% and 10% for TiO_2-Ag, and Res-Ag respectively). Higher difference in VSSA was observed (35.8 m^2/cm^3 for TiO_2-Ag and 0.78 m^2/cm^3 for Res-Ag). It seems that differences in cytotoxity observed in our experiment are caused by different quantity of ionic silver on the surface of nanocomposites, and that is probably why Res-Ag induced a higher cytotoxic effect than TiO_2-Ag which is non-cytotoxic (trace amount of ionic silver). Cytotoxic effect is probably closely related to the induced oxidative stress. It is most probable that ionic silver was released from the surface to the cell medium and could induce ROS production.

This observation is in contrast to Bosetti et al. [28], who have tested in vitro biocompatibility, cytogenetic, cytotoxic and cell physiological aspect of silver-coated stainless steel external fixation pins. It was shown that silver-coated stainless steel evidenced a very high release of particulate silver coating by the material, but cells in contact apparently were not influenced by this release [28]. The study by Bosetti's team have shown that silver is neither genotoxic nor cytotoxic as compared to stainless steel, a material widely used as a metal implant [28]. We have proved in this study that both TiO2-Ag and Res-Ag are genotoxic, but in a different mechanism(s). Both nanocomposites induced concentration-dependent DNA SSB, but only TiO_2-Ag demonstrated oxidative DNA damage after FPG treatment (Table 1, Table 2). To date we are not able to offer a viable explanation of this phenomenon. We speculate that surface area (and consequently VSSB), as well as crystal structure (which is extremely important for TiO_2 toxicity testing) may by one of important factors in toxicity studies of nanocomposites. It is well known that nanoparticles have larger surface area per unit mass than microparticles.

As for TiO_2 there are many studies published on toxicity evaluation, and precise data were recently reviewed in very elegant paper by Morimoto et al. [38]. Papers from other authors cited by Morimoto et al. [38] about toxicity testing of nanomaterials are not conclusive. Intratracheal instillation of TiO_2 particles in different sizes, and in inhalation studies in rats proved that particles with a smaller diameter caused a greater pulmonary inflammatory response at the same mass burden (cf. Morimoto). As it has been documented that the effects were more pronounced when the doses were expressed as surface area rather than mass in the experiment, and that the dose-response relationship was a straight line, there are opinions that larger surface area may be a factor responsible for inflammatory response to TiO_2 [38–40]. Yamamoto et al. [41] described

cytotoxicity testing of ceramic particles of different shape and size, and found that size effects were not the key factors in nanoparticle-induced cytotoxicity. Warheit et al. [42], reported different biological effect dependent upon surface area but not upon particle size.

We fully agree with Morimoto et al. [38] that one of possible reason for inconclusive results is that many studies have been performed using particles with different particle size, surface area, and impurities and even crystal structures. Therefore it is very difficult to discriminate between the sole effect of physical parameters (such as surface area and particle size) and many other factors.

Data on safety and potential hazard of nanoparticles, nanomaterials and nanocomposites are urgently needed. Nanocomposites produced on a growing scale cannot be introduced to a common use without prior assessment of their toxicity in both *in vitro* and *in vivo* studies. Thus, the different cyto- and genotoxic mechanism(s) of silver-coated NCs requires further investigations. Research to more fully characterize the toxicity of nanocomposites with respect to particle number, surface area, crystal structure, and chemical composition is needed and, to ultimately confirm health safety of using such nanocomposites, further *in vivo* testing should be considered.

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