Exposure of the murine RAW 264.7 macrophage cell line to hydroxyapatite dispersions of various composition and morphology: Assessment of cytotoxicity, activation and stress response

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

Cellular stress responses leading to the release of cytotoxic mediators are discussed as indicators of the hazard presented by particles, and in particular ultrafine particles or nanomaterials. The present study was designed to investigate effects of the following materials on RAW 264.7 macrophages: three hydroxyapatite materials of various morphologies, i.e., nano-sized with rod-like (HA-NR), plate-like (HA-PP) or needle-shaped (HA-NN) morphology, and an irregularly shaped composite of hydroxyapatite and protein (HPC) in the low micrometer range. Concentrations of 50, 100, 500, 1000 and 5000 \( \mu \text{g/ml} \) were applied and cells were analyzed for viability (XTT-test), cytokine production (TNF-\( \alpha \)), and induction of nitric oxide (NO) after 18 and 42 h. DQ12 quartz and lipopolysaccharide (LPS) served as positive controls. Up to concentrations of 500 \( \mu \text{g/ml} \), cell viability was not considerably impaired by the test samples at both timepoints. Overall, viability was about one order of magnitude higher than with comparable concentrations of quartz. TNF-\( \alpha \) release was induced in all samples after 18 h, with HA-NR showing the most pronounced induction at 100 \( \mu \text{g/ml} \), still clearly below the LPS signal. No or little induction was observed after 42 h. NO production was low after 18 and 42 h. The results support the conclusion that the tested materials exhibit good biocompatibility and are safe to use.

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1. Introduction

Particulate materials, especially in the small size range, are suspected of exerting enhanced inflammatory and cytotoxic effects in biological systems, which can lead to chronic inflammation and related diseases (Borm et al., 2006). Macrophages in different organ systems are a highly efficient defense system that filters and clears small particles. Due to their crucial role in the fate of particulate matter after uptake into the human body, macrophages are considered suitable sensors for analyzing such potential adverse effects. As central effectors and regulators of the inflammatory response, they are involved at all stages of the immune response, which has been reviewed elsewhere (Fujiwara and Kobayashi, 2005; Zhang and Mosser, 2008). Other well-studied functions of macrophages are the phagocytosis of microorganisms as part of the host defense, the killing of ingested pathogens, and assistance in the initiation of T-cell activation by processing and presenting antigens. To fulfill this plethora of functions, macrophages in their activated state are able to produce more than a hundred different substances, including tumor necrosis factor alpha (TNF-\( \alpha \)), reactive oxygen species and nitrogen compounds like nitric oxide (NO). Prolonged, enhanced production of these quite aggressive effector molecules may contribute to the development of diseases such as pulmonary disorders (Dorger and Krombach, 2000; Fubini and Hubbard, 2003).

TNF-\( \alpha \) is an extremely potent peptide cytokine which serves as an endogenous mediator of inflammatory, immune and host defense functions, being capable of acting independently and in conjunction with a variety of other factors to affect the phenotype and metabolism of cells in every tissue of the body (Dinarello, 1997). Very effective elicitors of TNF-\( \alpha \) include bacterial lipopolysaccharides (LPS), which are components of the outer cell wall of Gram-negative bacteria. TNF-\( \alpha \) can be determined in supernatants of macrophages and serves as an important parameter for examining...
the anti-inflammatory, inflammatory and immune stimulating properties of test substances.

NO is a key biological messenger and inflammatory mediator, playing a role in a variety of physiological and pathophysiological processes, including macrophage-mediated cytotoxicity, blood vessel dilatation, smooth muscle relaxation and neurotransmission (Mariotto et al., 2004; Korhonen et al., 2005). Peroxynitrite, a reaction product of NO and superoxide, is presumed to be an extremely potent mediator of cellular and tissue injury in various pathological situations (Forman and Torres, 2001; Pacher et al., 2007). Its levels are inversely related to the viability of RAW 264.7 macrophages (Hirvonen et al., 1996). Its biological effects are related to its reactivity towards a large range of molecules including amino acids, nucleic bases and antioxidants. NO is synthesized from l-arginine using NADPH and molecular oxygen by NO synthase (NOS). There are three forms of NOS, two constitutive and one inducible (iNOS) (Alderton et al., 2001), the latter being stimulated by the actions of cytokines like TNF-α and bacterial endotoxins like LPS (Forsterrmann and Kleintert, 1995; Aktan, 2004).

It has been reported that the size and morphology of materials including hydroxyapatites can influence their interaction with biological systems (Evans, 1991; Laquerriere et al., 2003; Grandjean-Laquerriere et al., 2004, 2005; Ramesh et al., 2007). The present study was designed to investigate the effects of various hydroxyapatite materials on the murine RAW 264.7 macrophage cell line, which is considered as a representative cell line for various types of macrophages routinely used in many studies. RAW 264.7 have been reported to retain many of the characteristics of macrophages in vivo (Raschke et al., 1976; Chapeker et al., 1996). Hydroxyapatite and its composites – including nanoscalar specifications – are well-known materials intended for a variety of biomedical applications, in particular intraosseous implantation, as a bone substitute or implant coating material, and are widely used in practical dentistry (Arts et al., 2006; Huber et al., 2007). The samples in this study were selected for their specific physicochemical properties, including different morphologies and sizes. Due to the high diversity of particulate materials and their applications, a hazard evaluation need to be performed on a case-by-case basis. The presented results are meant to serve as building blocks in such an evaluation.

2. Materials and methods

2.1. Particle preparation

Test samples used in this study are listed in Table 1a together with their abbreviations used in this article. All test samples were available as aqueous dispersions. Rod-like nano-hydroxyapatite (HA-NR), plate-like nano-hydroxyapatite (HA-NP) and hydroxyapatite–protein-composite (HPC) were synthesized by precipitation in aqueous solution at constant pH value. Chemical composition and morphology of the particles were controlled by pH during precipitation and the absence or presence of protein. An amount of 44.10 g (0.30 mol) calcium chloride dihydrate (p.a., Fisher Scientific GmbH, Schwerte, Germany) was diluted in 2 l deionized water at room temperature in a precipitation vessel. In case of HPC preparation 35 g denatured collagen (Gelatine, Gelita AG, Eberbach, Germany), was predissolved in 350 ml deionized water at ca 50 °C and added to the calcium solution under stirring. In a second vessel 0.6 mol ammonium phosphate (Sigma–Aldrich, Taufkirchen, Germany) was dissolved in 300 ml deionized water at room temperature. Solutions were adjusted to pH 7 for HPC and HA-NP and pH 9 for HA-NR. The phosphate solution was then pumped into the calcium solution, which was stirred during the whole reaction period. pH was kept constant by adding base with a pH-controlled pump. The precipitate was separated, washed, portioned and gamma-sterilized. HPC, HA-NR and HA-NP were not dried prior to testing.

Needle-shaped nano-hydroxyapatite (HA-NN; trade name Os-tim®); was purchased from Heraeus Kulzer, Hanau. For a detailed description of the material see Huber et al. (2006).

2.2. Particle characterization

2.2.1. Water and apatite content

The water content of the wet sediments of HPC, HA-NP and HA-NR was analyzed using a Sartorius MA 100 Moisture Analyzer. Approximate 5 g of the wet sediment was weighed in a flat aluminum plate, homogeneously distributed and immediately heated up to 125 °C until the balance stops the program by itself (weight loss below approximate 3 mg/30 s). The protein used for HPC is stable at temperatures of 125 °C. The measured weight loss therefore corresponds to water only. In case of the pure hydroxyapatites HA-NP and HA-NR the residue corresponds to the apatite content. Each analysis was done in duplicate.

2.2.2. Apatite and protein content of HPC

The sediment of HPC consists of water, hydroxyapatite and protein. The content of hydroxyapatite was analyzed by calcination. A dry crucible was filled with approximate 2 g of the wet HPC sediment, heated up slowly in 1 h up to 800 °C, kept at high temperature for further 30 min and then cooled down to room temperature. The residue after calcination corresponds to the hydroxyapatite content of HPC. The weight difference prior and after calcination was done in triplicates.

Table 1

This provides an overview of test samples used in this study (a), their chemical composition and specific surface (b), and their particle size and morphology (c).

<table>
<thead>
<tr>
<th>Test sample a</th>
<th>Abbreviation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyapatite–protein-composite</td>
<td>HPC</td>
<td>synthesized at SusTech Darmstadt</td>
</tr>
<tr>
<td>Nano-hydroxyapatite, rod-like</td>
<td>HA-NR</td>
<td>synthesized at SusTech Darmstadt</td>
</tr>
<tr>
<td>Nano-hydroxyapatite, needle-shaped</td>
<td>HA-NN</td>
<td>purchased from Heraeus</td>
</tr>
<tr>
<td>Fine hydroxyapatite, blunt-ended Needles</td>
<td>HA-FN</td>
<td>synthesized at SusTech Darmstadt</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test sample b</th>
<th>Ca/P ratio</th>
<th>Protein content (wt%)</th>
<th>Specific surface (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>1.95 ± 0.28</td>
<td>36.2</td>
<td>67</td>
</tr>
<tr>
<td>HA-NR</td>
<td>2.02 ± 0.20</td>
<td>–</td>
<td>166</td>
</tr>
<tr>
<td>HA-NN</td>
<td>1.67 (Huber et al., 2006)</td>
<td>–</td>
<td>106 (Huber et al., 2006)</td>
</tr>
<tr>
<td>HA-FN</td>
<td>2.12 ± 0.13</td>
<td>–</td>
<td>27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test sample c</th>
<th>Morphology</th>
<th>Average particle size (nm)</th>
<th>Reference for microscopic pictures</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>Irregular shaped</td>
<td>1200 × 2100</td>
<td>Albrecht et al. (TIV-D-08-00441), Fig. 1a</td>
</tr>
<tr>
<td>HA-NR</td>
<td>Mainly nano-sized rods</td>
<td>5 × 90</td>
<td>Albrecht et al. (TIV-D-08-00441), Fig. 1b</td>
</tr>
<tr>
<td>HA-NN</td>
<td>Needles (Huber et al., 2006)</td>
<td>3 × 20 × 100</td>
<td>Huber et al. (2006)</td>
</tr>
<tr>
<td>HA-FN</td>
<td>Intermediate morphology between rods and needles (“blunt-ended needles”)</td>
<td>95 × 740</td>
<td>Albrecht et al. (TIV-D-08-00441), Fig. 1d</td>
</tr>
</tbody>
</table>
after calcination corresponds to water and protein. The water content was determined with the Moisture Analyzer. The protein content was calculated based on both analyses. Each sample was analyzed in duplicate. Results are listed in Table 1b.

2.2.3. Calcium/phosphorous ratio
The ratio of calcium and phosphorous of HPC, HA-NP and HA-NR was analyzed with Inductively Coupled Plasma (ICP) spectrometry using a Perkin Elmer Optima 3000 spectrocope. The samples were dissolved in 6 wt% hydrochloride acid and diluted 1:100 with deionized water. Calcium was measured at 316, 318, 397 and 423 nm wavelength. Phosphorous was measured at 213 and 214 nm wavelength. Each analysis was done in duplicate. Results are listed in Table 1b.

2.2.4. Specific surface
Specific surfaces of HPC, HA-NP and HA-NR were analyzed by the Brunauer–Emmet–Teller-method (BET, Brunauer et al., 1938). The wet sediments of HPC, HA-NP and HA-NR were shock frozen with liquid nitrogen, freeze dried and ground manually thereafter. The specific surface was determined with a Quantachrome Autosorb-3B. Prior to nitrogen adsorption the dried samples were heated at 100 °C for 24 h. The measurement was based on five points. Results are listed in Table 1b.

2.2.5. Particle size and morphology
Particle morphology and size were analyzed with a Philips CM12 transmission electron microscope (TEM). Wet sediments of HPC, HA-NP and HA-NR were dispersed in ethanol, sprayed onto a copper grid and investigated at 120 kV acceleration voltage.

ESEM was performed using a FEI Quanta FEG 200 device. Samples were mounted on a carbon taped sample holder, vaporized with gold and scanned with 15 kV acceleration voltage in an atmosphere of 1 mbar water vapor. Results are listed in Table 1b.

2.3. Controls
Quartz dust particles, DQ12, a frequently used toxicological standard, were provided by DMT GmbH, Essen, Germany. DQ12 was chosen as positive control since it is known to induce inflammation and respiratory burst in macrophages. The particles have sharp crystalline edges; the particle size of the batch used by the manufacturer of the ELISA.

2.3.2. Preparation of dilutions of test substances and controls
Test substance dispersions were diluted in cell culture medium (see Section 2.2) in concentrations of 50, 100, 500, 1000 and 5000 µg/ml calculated according to solid matter content. DQ12 quartz was first dispersed in assay medium, and concentrations of 10, 75, 200 and 400 µg/ml have been adjusted. LPS was adjusted to concentrations of 5 and 10 µg/ml. This range of concentrations was determined in a pilot experiment performed to obtain an appropriate range of non-cytotoxic to cytotoxic compound concentrations (data not shown).

2.5. Cell culture
The murine macrophage cell line RAW 264.7 (ATCC No. TIB 71; Institut für angewandte Zellkultur, Dr. Toni Lind GmbH, Munich, Germany) has been used as biological test system to characterize compound derived immunoreactions. Cells were suspended in growth medium (cell culture medium as described in Section 2.2, supplemented with 10% v/v FCS) at a density of 2 × 10^4 cells/ml. Cells were seeded into 96-well microtiter plates (Sarstedt, Nümbrecht, Germany) in a density of 2 × 10^5 cells per well. The cells were subsequently pre-incubated for 4–5 h at 37 °C and 5% v/v CO₂ and allowed to form adherent monolayers. After preincubation and removal of the cell culture medium, 100 µl of test substances and controls were applied (see Section 2.3). The cells were exposed to test compounds and controls for 18 and 42 h, respectively. The culture conditions were set to 37 °C, 5% v/v CO₂ and maximum humidity.

2.6. Determination of cell viability
For the examination of cell viability, an XTT assay was performed (Cell Proliferation Kit II, Ref No. 11465015901, Roche Diagnostics GmbH, Mannheim, Germany), which has been proven to be a sensitive routine application for assessing cell viability and proliferation. The assay is a non-invasive colorimetric method based on an enzymatic metabolism of the vital dye XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) to a water-soluble formazan. The reaction is catalyzed by mitochondrial succinyl dehydrogenase and requires NAD(P)H, derived from cell metabolism. Any impairment or change of proliferation rate, cellular function, energy state or metabolic capacity caused by the test substances results in decreased or increased XTT reduction. After incubation with the test substance, treated cells were washed with 100 µl culture media without FCS and incubated for 4 h with 100 µl culture medium and 50 µl XTT reagent at 37 °C and 5% v/v CO₂. After incubation the absorbance of formazan was measured with a microtiter plate reader (Spectraflour, Tecan Austria Ges.m.b.H., Grödig, Austria) at a wavelength of 450 nm (reference wavelength 620 nm). The cell viability (%) of the treated cells was calculated in relation to the negative controls (100% viability).

2.7. TNF-α release
TNF-α release from exposed RAW 264.7 macrophages was determined using a quantitative mouse TNF-α ELISA (Enzyme Linked Immunoabsorbent Assay, BMS607INST, Bender MedSystems GmbH, Vienna, Austria). The Limit of detection (LOD) was 4.3 pg/ml. In order to prevent the collected samples from biodegrading, a protease inhibitor (Complete® Roche Diagnostics GmbH, Mannheim, Germany) was added to the cell supernatants. All samples were collected and stored at −20 °C prior to the assay. To eliminate solid material (detached macrophages, dispersed particles), the supernatant containing the inflammatory mediators was collected from the cells and subsequently centrifuged for 10 min at 163 g. The supernatant was diluted 1:2 with the cell culture medium (DMEM). Cell culture supernatants were then frozen at −20 °C prior to determination of TNF-α. The quantitative calculation of TNF-α release was performed by following the advice of the manufacturer of the ELISA.

2.8. NO release
RAW 264.7 macrophages were suspended in growth medium at a density of 1 × 10⁶ cells/ml. A volume of 0.1 ml of the cell suspension was seeded into 96-well dishes and was pre-incubated for 18 h. NO release was measured with the Griess reaction (Griess reagent, Sigma, Munich, Germany) at 540 nm wavelength.
4–5 h at 37 °C and 5% CO₂ for adherence. NO production of cells was determined using the method of Griess (Verdon et al., 1995). In general, nitrate resulting from released NO molecules is reduced to nitrite by nitrate reductase. Nitrate is determined in a two-step reaction to allow for the indirect quantification of NO.

For this, 50 μl supernatant of particle-treated cells were transferred to a 96-well microtiter plate. Volumes of 10 μl NADPH (10 μM; Roche, Mannheim, Germany) and 40 μl of a mixture of 4.4 ml phosphate buffer (40 mM), 100 μl nitrate reductase (10 U/ml; Roche, Mannheim Germany), 500 μl glucose-6-phosphate (12.5 mM; Roche, Mannheim, Germany) and 6 μl glucose-6-phosphate dehydrogenase (350 U/ml; Roche, Mannheim, Germany) were added to each well. The mixture was incubated for 45 min at room temperature, and color dye formation was started subsequently by adding 100 μl sulfanilamide (1%; Sigma–Aldrich, Taufkirchen, Germany; diluted in 5% phosphoric acid; Merck KGaA, Darmstadt, Germany) and 100 μl naphthylethylenediamine (0.1% aqueous solution; Sigma–Aldrich, Taufkirchen, Germany). After 10 min at room temperature the azo dye formation was measured with a microtiter plate reader (Spectraflour, Tecan Austria Ges.m.b.H., Grödig, Austria) equipped with a 550 nm filter. Nitrite was used as standard (Merck KGaA, Darmstadt, Germany). An additional nitrate standard (Merck KGaA, Darmstadt, Germany) served as a control for the enzyme reaction producing nitrite from nitrate in the cell culture medium.

2.9. Calculation and statistics

GraphPad Prism Version 4.00 (Reg. No.: GPW4-010007-RLA-8644, GraphPad Software Inc., San Diego CA, USA) was used to pre-
pare the standard curve for the quantitative TNF-α determination. The standard curve for NO was created on the basis of Microsoft Excel 2002 (Product ID: 54187-640-2034411-17829). This program was also used to calculate the amount of NO and TNF-α and to evaluate the cell viability (XTT-test). As curve fit model non-linear regression was used. All samples were measured in triplicate (n = 3) with the exception of quartz (n = 4) and standard deviations (SDs) were determined. The statistical relevance was calculated on the basis of a Student t-test. Statistically significant results yield p < 0.05. The raw data were assessed for outliers using the method of Nalimov (Kaiser and Gottschalk, 1972). ED50 values for cytotoxicity were calculated according to the genetic function algorithm (GFA).

3. Results

3.1. Cell viability

With all test substances, i.e., HPC, HA-NP, HA-NR and HA-NN, the viability of RAW 264.7 macrophages was not impaired up to a concentration of 500 μg/ml in the XTT-test after 18 h and decreased at 1000 μg/ml (Fig. 1a). A similar situation was observed after 42 h, although a slight decrease in viability following treatment with HA-NP and HA-NN was observed from 50 μg/ml; decreased viability was again most marked at 1000 μg/ml (Fig. 1b). Overall, the test substances proved to be about one order of magnitude less toxic towards the macrophage cells than the positive control DQ12 quartz, which showed a significant cytotoxic effect from 75 μg/ml after both treatment periods. As expected, LPS treatment resulted in strong increase of cell metabolism (234% at 18 h and 105.7% at 42 h). A slight increase of cell metabolism was noticed at low concentrations with all test substances after 18 h and with some test substances after 42 h, but not with DQ12 quartz.

3.2. TNF-α release

As shown in Table 2, a significant TNF-α release of 187.1 pg/ml was detected after 18 h following the application of 75 μg DQ12/ml. Increasing DQ12 concentrations resulted in a decreased release of TNF-α, which correlates with the marked cytotoxic effect of the substance; no TNF-α release was observed after 42 h (Table 2b). None of the test samples reached as strong a signal as the positive control, LPS, applied at a single concentration of 10 μg/ml (3230.1 pg/ml (18 h) and 4278.8 pg/ml (42 h) were detected in the cell culture medium). TNF-α release of the cell culture control was 29.8 pg/ml after 18 h and was below the detection limit after 42 h.

Treatment with HA-NR, HPC, HA-NP and HA-NN led to relatively low activation, with the significant (p < 0.01) exception of HA-NR after 18 h at a concentration of 100 μg/ml, resulting in an induction of 900.1 pg/ml TNF-α, which was 4.8 times higher than the induction by DQ12 at 75 μg/ml, and about 28% of the TNF-α release induced by 10 μg/ml LPS. At 500 μg/ml, the signal induced by HA-NR was significantly (p < 0.01) reduced compared to 100 μg/ml, and further decreasing towards the cytotoxic range. After 42 h, a slight TNF-α secretion was found at the lowest concentration.

A more detailed look at HPC shows that the most marked response of 99.3 pg/ml was observed after 18 h following application of 100 μg/ml. This value is about half of the effect of DQ12 quartz at 75 μg/ml, and a factor of 32 below LPS-induced TNF-α secretion. Remarkably, TNF-α release was still detectable at clear cytotoxic concentrations (5000 μg/ml), whereas no TNF-α release could be detected after 42 h.

For HA-NP, in contrast to HA-NR and HPC, the highest TNF-α release after 18 h was observed at a concentration of 500 μg/ml, i.e., 122.3 pg/ml, being slightly but significant (p < 0.05) lower than for DQ12 quartz at 75 μg/ml and 26 times lower than with LPS. After 42 h, detectable induction through HA-NP could only be found at 1000 μg/ml (93.4 pg/ml), 46 times lower than LPS induction.

A dose-dependent increase was observed for HA-NN after 18 h with the peak-level of 172.5 pg/ml reached at 1000 μg/ml, comparable to the DQ12 signal at 75 μg/ml, and 19 times lower than the LPS-induced TNF-α secretion. A dose-dependent increase of TNF-α release up to 1000 μg/ml was observed after 42 h, with a peak-level of 139.4 pg/ml, i.e., 30 times lower than the LPS-induced TNF-α secretion.

Overall, HA-NR seems to have the highest inflammatory potential amongst the test samples, whereas HPC displayed the lowest potential. Compared to HA-NN, all other test samples did not exhibit persistent TNF-α release at 42 h.

3.3. NO formation

A significant response was only observed with the positive controls. Treatment with DQ12 resulted in the release of 9.0 nmol/ml

Table 2

<table>
<thead>
<tr>
<th>Test samples</th>
<th>50 μg/ml</th>
<th>100 μg/ml</th>
<th>500 μg/ml</th>
<th>1000 μg/ml</th>
<th>5000 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>43.9 (±14.8)</td>
<td>99.3 (±13.1)*</td>
<td>46.2 (±9.4)</td>
<td>54.4 (±10.9)</td>
<td>41.8 (±0.3)</td>
</tr>
<tr>
<td>HA-NP</td>
<td>105.8 (±23.2)*</td>
<td>63.6 (±2.1)*</td>
<td>122.3 (±8.7)*</td>
<td>62.7 (±26.2)</td>
<td>0.0 (±0.0)</td>
</tr>
<tr>
<td>HA-NR</td>
<td>0.0 (±0.0)</td>
<td>900.1 (±246.8)*</td>
<td>51 (±59.5)</td>
<td>42.9 (±5.2)</td>
<td>0.0 (±0.0)</td>
</tr>
<tr>
<td>HA-NN</td>
<td>11.9 (±8.5)</td>
<td>39.2 (±53.3)</td>
<td>46.8 (±13.7)</td>
<td>172.5 (±13.1)*</td>
<td>58.7 (±24.0)</td>
</tr>
<tr>
<td>Controls</td>
<td>0 μg/ml</td>
<td>10 μg/ml</td>
<td>75 μg/ml</td>
<td>200 μg/ml</td>
<td>400 μg/ml</td>
</tr>
<tr>
<td>DQ12</td>
<td>11.4 (±36.1)</td>
<td>187.1 (±4.1)*</td>
<td>108.7 (±24.6)</td>
<td>65.1 (±44.8)</td>
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<td>LPS</td>
<td>3230.1 (±10.4)*</td>
<td>3230.1 (±10.4)*</td>
<td>3230.1 (±10.4)*</td>
<td>3230.1 (±10.4)*</td>
<td>3230.1 (±10.4)*</td>
</tr>
<tr>
<td>Cell culture control</td>
<td>29.8 (±3.91)</td>
<td></td>
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<table>
<thead>
<tr>
<th>Test samples</th>
<th>50 μg/ml</th>
<th>100 μg/ml</th>
<th>500 μg/ml</th>
<th>1000 μg/ml</th>
<th>5000 μg/ml</th>
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<tbody>
<tr>
<td>HPC</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
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<tr>
<td>HA-NP</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>93.4 (±1.5)*</td>
<td>0.0 (±0.0)</td>
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<td>HA-NR</td>
<td>46.6 (±39.2)</td>
<td>2.5 (±3.5)</td>
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<td>HA-NN</td>
<td>49.6 (±27.5)</td>
<td>64.9 (±7.8)*</td>
<td>113.3 (±22.1)*</td>
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<td>DQ12</td>
<td>0.0 (±0.0)</td>
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<tr>
<td>LPS</td>
<td>4278.8 (±154.1)*</td>
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</tbody>
</table>
NO after 18 h (Table 3a) and 6.7 nmol/ml NO after 42 h (Table 3b) at a concentration of 75 µg/ml, and decreasing amounts at higher concentrations. As expected, the response towards DQ12 was low in comparison to LPS. LPS, applied at a concentration of 10 µg/ml, strongly stimulated NO formation; 53.4 nmol/ml NO were detected after 18 h, and 119.2 nmol/ml after 42 h. NO release of the cell culture control was in the range from 1.1 nmol/l (18 h) to 3.7 nmol/l (42 h).

NO release in RAW 264.7 macrophages after 18 h was within the range between 0.4 and 8.4 nmol NO/ml (w/o SD) for HA-NR, HCP, HA-NP and HA-NN, and was thus an order of magnitude below the LPS-induced effects. After 42 h, the responses were in a very similar range between 0.8 and 8.4 nmol NO/ml (w/o SD). Incubation of macrophages with HA-NR and HA-NN for 42 h resulted in a slight increase in NO release relative to the other test substances, in particular at a concentration of 1000 and 5000 µg/ml, respectively. The NO release is comparable to the release observed with DQ12 at 75 µg/ml. This again indicates that the potency of DQ12 is about 10 times higher than that of these two test samples. NO release induced by the other two substances, HPC and HA-NP, was still lower. The NO release induced by the test substances was overall very low. As a consequence, relatively high standard deviations complicated the statistical evaluation. None of the averaged values of the test samples reached the averaged value for DQ12.

4. Discussion

The aim of this study was to evaluate cytotoxicity, TNF-α induction and NO release in macrophages in response to different hydroxyapatite dispersions. The observed low toxicity towards murine RAW 264.7 macrophages in our investigations correlates with other investigations using HA and composites thereof, which made use of a wide spectrum of different specifications, test systems and testing conditions, with some of the studies focusing on efficient biodegradation (Cui et al., 1996; Trofimov et al., 1996; Liu et al., 1997; Bloebaum et al., 1998; Guo et al., 1999; Wenisch et al., 2003; Rumpel et al., 2006). It should be noted that cytotoxicity as observed at high concentrations does not necessarily represent a compound-related effect, and may at least partly be due to physical coverage of the cells by the test substance (microscopic evaluation, data not shown), or impairment of contact to the cell culture medium or of gas exchange. After 42 h, a reduction in cell viability started at lower concentrations with HA-NN and HA-NP than with HA-NR and HPC. Even though subtle changes could be observed, no unambiguous trend related to variations in morphology and size of the test samples could be assigned in our test system. None of the test samples was impairing cell viability more than commercially available HA-NN, which is marketed as a medical product and can therefore be regarded as a benchmark with officially approved safety.

All tested materials, including composite HPC with particles sized in the low micrometer range, did elicit a significant TNF-α response at one or more concentrations after 18 h, but significantly lower than the one induced by LPS, for which TNF-α is considered as a dominant mediator of its pathophysiological effects (Bauss et al., 1987; Tracey et al., 1988). A certain level of TNF-α release can be regarded as a typical indicator of macrophage activation upon particle exposure which in our study largely correlates with viability as a necessary prerequisite for effective degradation, and is therefore not considered to represent an adverse effect per se. Previous studies demonstrated that TNF-α is not the main mediator of the inflammatory and pathogenic effects of quartz that occur following high and chronic exposures, but rather the combination of cytotoxic effects, several cytokines and oxidative stress response driven by surface reactivity (Fubini, 1998; Albrecht et al., 2004, 2007).

Physical particle properties like surface area, size and morphology were also reported to influence cytokine production of HA materials (Evans, 1991; Laquerriere et al., 2003; Grandjean-Laquerriere et al., 2004, 2005; Ramesh et al., 2007), and a variety of other materials (Duffin et al., 2007; Singh et al., 2007). Needle-shaped HA particles, for instance, induced greater production of cytokine IL-18 in human monocytes than spherical or irregularly shaped particles (Grandjean-Laquerriere et al., 2004). In our test system, TNF-α release was declining after 42 h for all test samples except for to needle-shaped HA (HA-NN), while after 18 h rod-like HA-NR led to the most prominent effect, with overall no clear trend related to morphology alone.

The transience of the response observed with most samples in our study might be regarded as an indication for a good biocompatibility, while it needs to be acknowledged that cell cultures

<table>
<thead>
<tr>
<th>Test samples</th>
<th>50 µg/ml</th>
<th>100 µg/ml</th>
<th>500 µg/ml</th>
<th>1000 µg/ml</th>
<th>5000 µg/ml</th>
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<tbody>
<tr>
<td></td>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPC</td>
<td>4.4 (±2.38)</td>
<td>2.7 (±0.91)</td>
<td>4.5 (±0.95)</td>
<td>4.0 (±1.88)</td>
<td>5.0 (±1.44)</td>
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<tr>
<td>HA-NP</td>
<td>3.2 (±1.45)</td>
<td>3.3 (±1.33)</td>
<td>2.8 (±0.67)</td>
<td>3.2 (±0.72)</td>
<td>3.8 (±1.67)</td>
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<tr>
<td>HA-NR</td>
<td>3.4 (±0.25)</td>
<td>3.4 (±0.52)</td>
<td>3.3 (±0.42)</td>
<td>5.7 (±0.66)</td>
<td>3.8 (±1.17)</td>
</tr>
<tr>
<td>HA-NN</td>
<td>2.6 (±0.97)</td>
<td>1.7 (±1.15)</td>
<td>0.4 (±2.0)</td>
<td>8.4 (±0.94)</td>
<td>4.3 (±1.49)</td>
</tr>
<tr>
<td>Controls</td>
<td>0 µg/ml</td>
<td>10 µg/ml</td>
<td>75 µg/ml</td>
<td>200 µg/ml</td>
<td>400 µg/ml</td>
</tr>
<tr>
<td>DQ12</td>
<td>1.8 (±0.17)</td>
<td>9.0 (±3.72)</td>
<td>3.7 (±2.26)</td>
<td>1.5 (±1.07)</td>
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</tr>
<tr>
<td>LPS</td>
<td>53.4 (±9.78)*</td>
<td></td>
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<td></td>
<td>b</td>
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</tr>
<tr>
<td>HPC</td>
<td>2.1 (±0.31)</td>
<td>1.5 (±0.60)</td>
<td>1.5 (±0.94)</td>
<td>2.5 (±1.25)</td>
<td>4.8 (±1.10)</td>
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<td>HA-NP</td>
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<td>2.7 (±0.54)</td>
<td>2.1 (±0.30)</td>
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<td>5.1 (±2.78)</td>
<td>7.6 (±1.60)</td>
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<td>Controls</td>
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<td>10 µg/ml</td>
<td>75 µg/ml</td>
<td>200 µg/ml</td>
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</tr>
<tr>
<td>DQ12</td>
<td>4.8 (±3.42)</td>
<td>6.7 (±1.01)</td>
<td>2.7 (±0.50)</td>
<td>2.2 (±0.93)</td>
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</tr>
<tr>
<td>LPS</td>
<td>119.2 (±2.6)*</td>
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<tr>
<td>Cell culture control</td>
<td>3.7 (±2.7)</td>
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</tbody>
</table>
are not expected to adequately reflect subchronic or chronic exposures in vivo. HA-NN was previously reported to be biocompatible and safe in preclinical and clinical studies (Huber et al., 2006, 2007), which clearly demonstrates that a significant response in macrophages after 42 h does not necessarily correlate with adverse effects in vivo.

Regarding the influence of size, it has been shown by others that mid-sized particles of about 10 μm recruit more macrophages than fine or ultrafine or larger particles, indicating that an intermediate particle size might favor the elicitation of inflammatory and immune reactions (Ramesh et al., 2007). On the other hand, nano-sized HA-NN has been shown to effectively recruit macrophages and osteoblasts in vivo resulting in an efficient bioresorption and degradation (Heraeus and Kulzer, 2005; Arts et al., 2006). A number of studies have also been performed with HA ceramics for bone substitution or implants, indicating that surface properties have an impact on the recruitment and growth of osteoblasts; a nanostructured surface of sintered HA was shown to recruit osteoblasts more efficiently than micro-structured material (Guo et al., 2007) and human osteoblast-like cells attached more efficiently to substrates sprayed with HA nanoparticles compared to untreated glass substrates (Huang et al., 2004). The protein content in HPC may represent the standard measurement technique, should not directly be considered in the toxicological interpretation of the results. Samples need to be dried and ground for the measurement which inevitably changes their properties compared to the dispersions as they were used for the macrophage tests.

Another aspect to be considered is the pH-dependent solubility of HA upon phagocytosis (Bloebaum et al., 1998). HA is soluble under acid conditions, as e.g. found in endosomes, which contributes to its ready biodegradation and non-sustained inflammation. Cellular stress responses as measured by NO release were overall very low following treatment with test samples. For rod-like and the needle-shaped morphologies (HA-NR and HA-NN), a slight increase in NO production can be observed after 42 h, but not after 18 h, corresponding to the results of TNF-α re-lease after 42 h. This can be explained by an induction cascade: iNOS is induced by TNF-α, which in turn leads to enhanced NO production. The data also indicate that DQ12 is a more potent inducer of oxidative stress than the test samples, particularly at lower concentrations where the cytotoxic effect is not yet too deleterious to the cells, but no clear trends related to physical properties of the test samples could be deduced. In addition, the high standard deviations were an obstacle to obtaining statistically valid conclusions. Nevertheless, the overall low induction of NO raises no concerns relating to an adverse oxidative stress response and may even represent a background level of untreated cells.

Regarding the test system itself, RAW 264.7 macrophages have been widely used to investigate particle effects, including those of nano-sized materials (Diociaiuti et al., 2001; Kagan et al., 2006). Up to now, no test has been officially validated and accepted within a hazard assessment context as true for all types of macrophage incubation assays. Since no official testing strategy and agreed tests are in place for investigating specific effects of small particles, appropriate test systems have to be chosen according to the state-of-the-art. In this context, the present results are considered useful as screening elements for hazard evaluation and to meet the needs of the current discussion within the scientific community.

In summary, the combined results of the performed screening assay support the conclusion that the tested materials are safe to use. Further standardization and validation of in vitro test sys-

tems will be required to assess their relevance for the in vivo situation.

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References


