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Inflammatory response of lung macrophages and epithelial cells after exposure to redox active nanoparticles: Effect of solubility and antioxidant treatment

Urner, Martin ; Schlicker, Andreas ; Z'graggen, Birgit Roth ; Stepuk, Alexander ; Booy, Christa ; Buehler, Karl P ; Limbach, Ludwig ; Chmiel, Corinne ; Stark, Wendelin J ; Beck-Schimmer, Beatrice

Abstract: The effects of an exposure to three mass-produced metal oxide nanoparticles-similar in size and specific surface area but different in redox activity and solubility-were studied in rat alveolar macrophages (MAC) and epithelial cells (AEC). We hypothesized that the cell response depends on the particle redox activity and solubility determining the amount of reactive oxygen species formation (ROS) and subsequent inflammatory response. MAC and AEC were exposed to different amounts of Mn3O4 (soluble, redox-active), CeO2 (insoluble, redox-active), and TiO2 (insoluble, redox-inert) up to 24 h. Viability and inflammatory response were monitored with and without coincubation of a free-radical scavenger (trolox). In MAC elevated ROS levels, decreased metabolic activity and attenuated inflammatory mediator secretion were observed in response to Mn3O4. Addition of trolox partially resolved these changes. In AEC, decreased metabolic activity and an attenuated inflammatory mediator secretion were found in response to CeO2 exposure without increased production of ROS, thus not sensitive to trolox administration. Interestingly, highly redox-active soluble particles did not provoke an inflammatory response. The data reveal that target and effector cells of the lung react in different ways to particle exposure making a prediction of the response depending on redox activity and intracellular solubility difficult.

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Inflammatory response of lung macrophages and epithelial cells after exposure to redox active nanoparticles: Effect of solubility and antioxidant treatment

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Supporting information

SUPPLEMENTARY FIGURES

Figure S1. Traces of metals according to ICP-OES measurements in the supernatants of macrophages (**A**) and epithelial cells (**B**) shown in mg kg^{-1} (corresponds to ppm). Values for titanium are not shown as they were below the limit of detection (2 ppb).

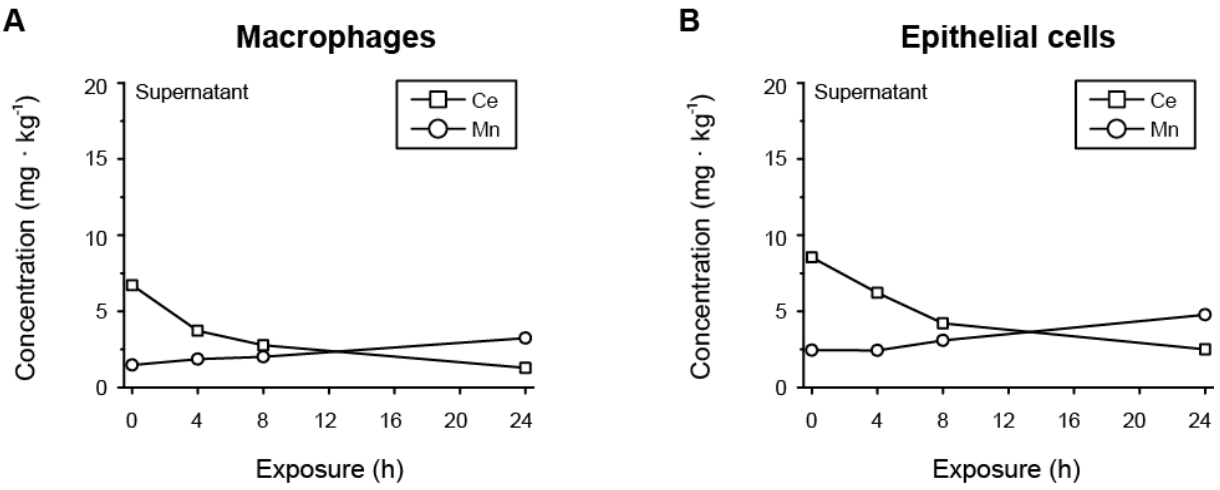


Figure S2. No significant influence on the amount of DNA in alveolar macrophages (**A**) and epithelial cells (**B**), determined after exposure to the three of the three metal oxide (6 μg/cm^2 cell surface area). Control was defined as 100% and the values are presented as mean ± SD.

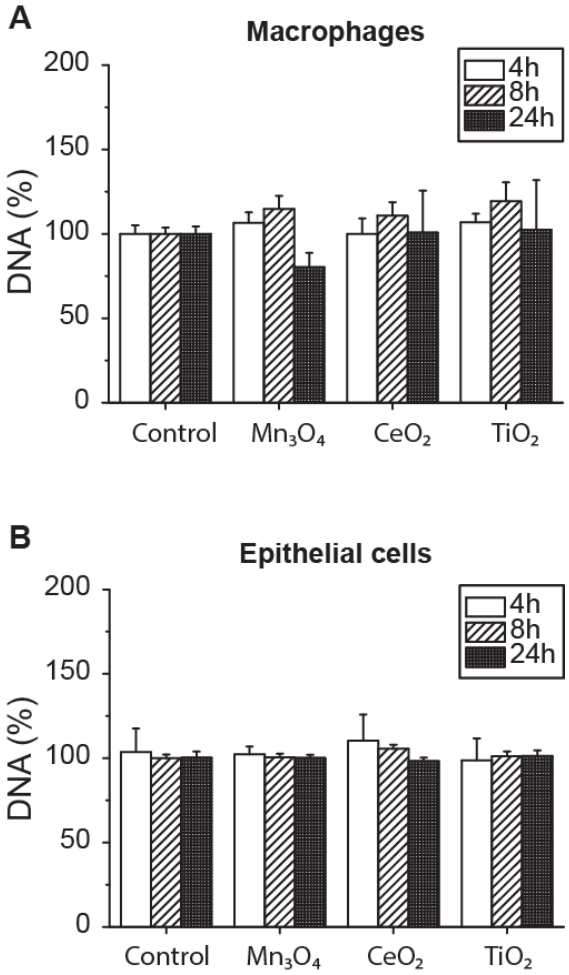


Figure S3. Cell-free experiments. To monitor for possible protein adsorption effects of particles, monocyte chemoattractant protein 1 (MCP-1) was added to culture medium (without cells), containing 20 mg kg^{-1} of the respective metal oxide particles. Thereafter, enzyme-linked immunosorbent assays (ELISA). The graph illustrates the amount of MCP-1 protein in culture medium after incubation with the particles, indirectly representing the remaining non-particle-adsorbed MCP-1 proteins.

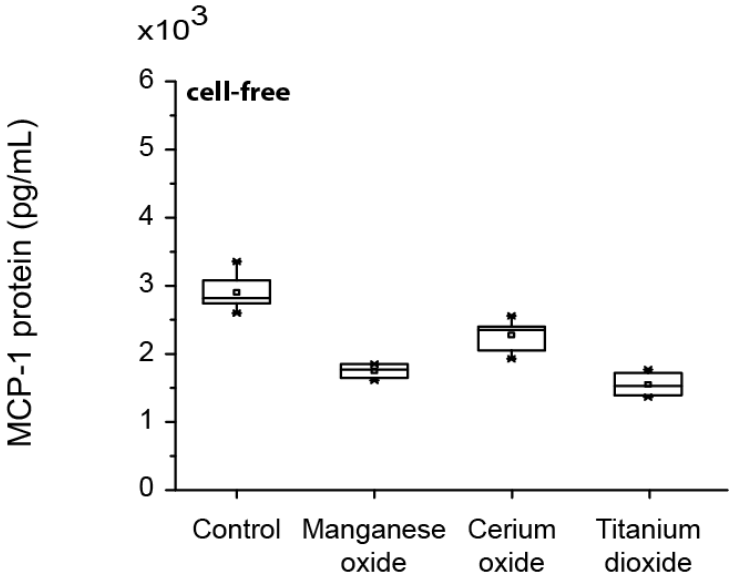


Figure S4. Cell death induced by Mn_3O_4 particles (6 µg/cm^2 cell surface) could be inhibited when 100µM Ac-Asp-Glu-Val-Asp-H (Aldehyde), an inhibitor of caspase-3/7/8, was added to the cell culture medium. The data shows mean ±SD.

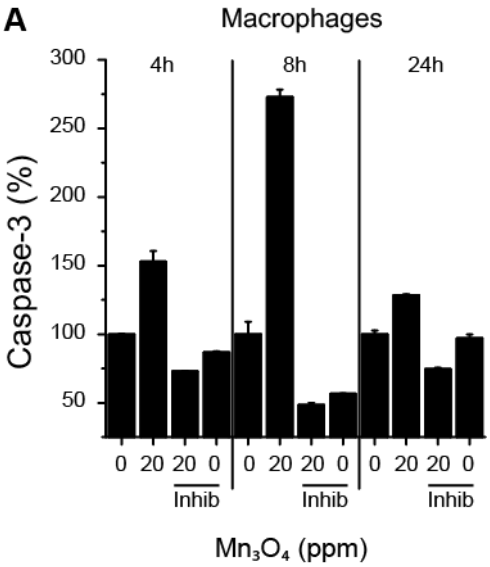


Figure S5. Influence of the antioxidant trolox on impaired viability and attenuated inflammatory mediator secretion in alveolar epithelial cells exposed to metal oxide particles (6 µg/cm^2 cell surface) over a time period of 24 hours (AEC). Trolox (a derivative of vitamin E) was used as free-radical scavenger to block reactive oxygen species (ROS) formation. Determination of NADPH oxidase-related metabolic activity (MTT, metabolic activity) (**A**). Determination of monocyte chemoattractant protein-1 (MCP-1) secretion (**B**). In MTT assays, control was defined as 100% and the values are presented as mean ± SD.

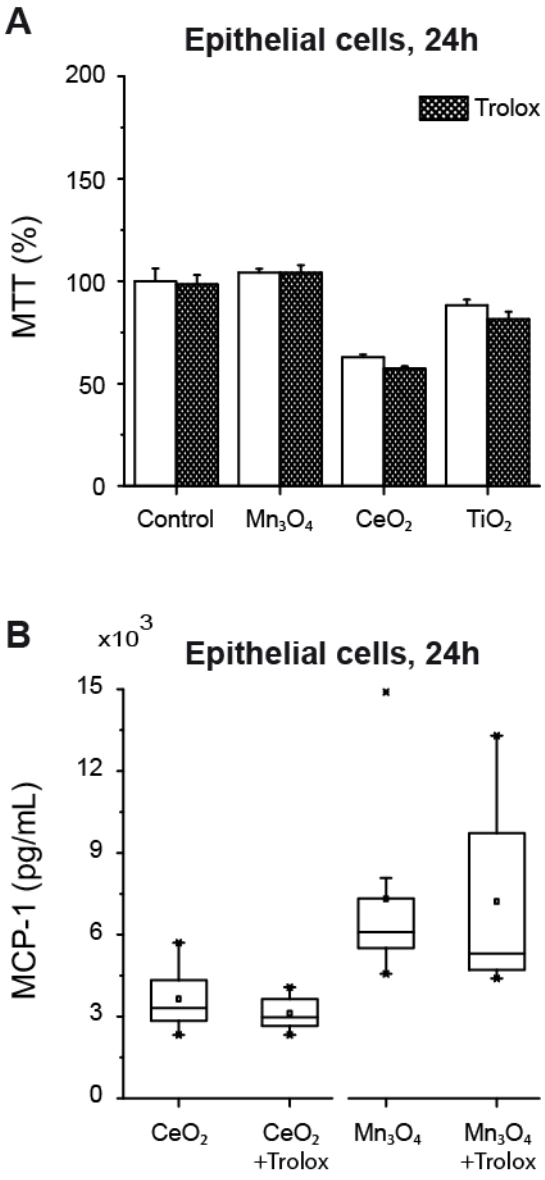
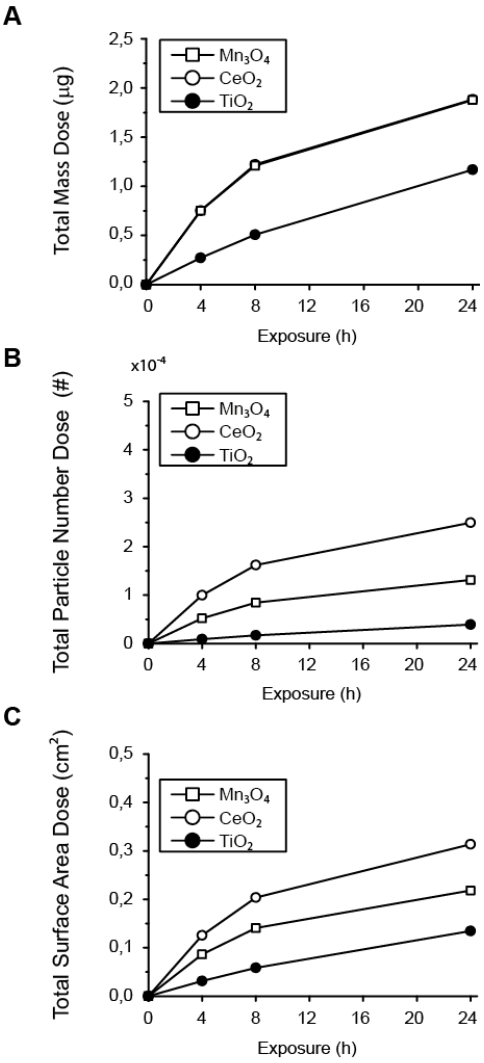


Figure S6. Estimated Relevant In Vitro Doses (RID) in our experimental settings were calculated for Total Mass Dose (**A**), Total Particle Number Dose (**B**), and Total Surface Area Dose (**C**) according to Cohen et al., Particle and Fibre Toxicology 2014, 11:20. Following assumptions were made: Mn_3O_4 has a deposition fraction constant of 0.118 h^{-1} and an effective agglomerate density of 1.8 g/cm^3; CeO_2 has a deposition fraction constant of 0.116 h^{-1} and an effective agglomerate density of 2.244 g/cm^3; TiO_2 has a deposition fraction constant of 0.0366 h^{-1} and an effective agglomerate density of 1.58 g/cm^3. We here use an effective agglomerate density corresponding to 20% of the respective material density and 80% of the density of water.



TABLES

Table S1. Specific surface area (SSA) of the used metal oxide nanoparticles from Brunauer-Emmett-Teller (BET) measurements.

Element	**SSA, m^2 g^{-1}**	**d, nm**
CeO_2	34	23
TiO_2	43	33
Mn_3O_4	61	20

Table S2. Linear regression on the response of alveolar macrophages and alveolar epithelial cells to different metal oxide particle exposure.

	Mn_3O_4	CeO_2	TiO_2	Time	R^2
Macrophages					
Reactive oxygen species – %	346 (290, 402) [a]	107 (51, 162) [a]	57 (2, 113) [c]	5 (3, 8) [a]	0.731
Metabolic activity – %	-23 (-33, -13) [a]	-8 (-19, 3)	-8 (-19, 3)	-1 (-1, -1) [a]	0.271
Amount of DNA – %	1 (-7, 8)	4 (-4, 12)	10 (2, 17)	-1 (-1, 0) [b]	0.154
MCP-1 – pg/mL	-9658 (-13849, -5467) [a]	-4361 (-10535, 1813)	-3783 (-10125, 2560)	375 (173, 578) [a]	0.155
Epithelial cells					
Reactive oxygen species – %	84 (74, 94) [a]	12 (2, 21) [c]	9 (0, 19) [c]	1 (0, 1) [a]	0.581
Metabolic activity – %	0 (-5, 5)	-22 (-26, -17) [a]	-9 (-14, -4) [b]	0 (-1, 0) [a]	0.583
Amount of DNA – %	0 (0, 1)	2 (0, 3)	1 (-1, 3)	0 (0, 0)	0.070[d]
MCP-1 – pg/mL	-781 (-1547, -16) [c]	-1675 (-2417, -934) [a]	-613 (-1378, 151)	149 (118, 181) [a]	0.430

The table shows the B coefficients (95% confidence intervals). Untreated control cells were used as reference category (defined as 100%). Exposures to Mn_3O_4, CeO_2, or TiO_2 particles were included as binary predictor variables. Significance in the regression model: [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$, [d] F-statistic not significant. Metabolic activity: NADPH oxidase-related metabolic activity; MCP-1: monocyte chemoattractant protein-1.

Table S3: Linear regression on the response of alveolar macrophages to manganese oxide particle exposure with or without the presence of the free radical scavenger trolox.

	Mn_3O_4	Mn_3O_4 + Trolox	Trolox	R^2
Macrophages				
Reactive oxygen species – %	677 (606, 749) [a]	96 (24, 167) [c]	-25 (-96, 47)	0.916
Metabolic activity – %	-68 (-74, -61) [a]	-55 (-61, -48) [a]	-6 (-13, 1)	0.960
Caspase-3 activity – %	49 (46, 53) [a]	18 (14, 22) [a]	0 (-3, 4)	0.961
MCP-1 – pg/mL	-8865 (-16062, -1668) [c]	436 (-7370, 8243)	868 (-6114, 7850)	0.093

The table shows the B coefficients (95% confidence intervals). Untreated control cells were used as reference category (defined as 100%). Exposures to Mn3O4 particles with or without Trolox were included as independent, binary predictor variables in the regression analysis. Significance in the regression model: a $p<0.001$, b $p<0.01$, c $p<0.05$. Metabolic activity: NADPH oxidase-related metabolic activity; Caspase-3: indirect marker for apoptosis rate; MCP-1: monocyte chemoattractant protein-1.

MATERIAL AND METHODS

ICP-OES analysis. Stock solutions containing nanoparticles (400 mg kg^{-1}) were prepared in distilled water by vortexing and sonification for 10 minutes (Branson Sonifier cell disruptor B15 80% duty cycle, pulsed). Furthermore the solutions were cooled in ice and diluted (1:10) with 1 % MAC medium. The resulting nanoparticle solution was added to cell containing solution in equal volumes. The cells were incubated within 4, 8 and 24 hours within in the pre-described nanoparticle containing solutions. The samples of nanoparticles dispersed in 10 ml of cell medium/water were analysed within 2 hours after the collection. The control medium contained 20 mg kg^{-1} initial concentration of the nanoparticles dispersed in 20 ml of medium. The samples were transported in the centrifuge tubes of 50 ml in the thermally insulated bag within T = 25 °C. Furthermore, the stock solutions of supernatants and lysed cells' solutions were centrifuged at 5000g (SIGMA, 3-30KS, Germany) for 5 min and filtered through a polyethersulfone syringe filter (Techno Plastic Products AG, Switzerland) with 0.22 μm pore size immediately after centrifugation. The collected supernatant was transferred to 10 ml vials and analyzed in the ICP. The composition of nanoparticles medium solutions was characterized by inductively coupled plasma optical emission spectroscopy (ICP OES, Arcos, SPECTRO Analytical Instruments GmbH, Kleve, Deutschland; plasma gas 12 l min-1 (Ar), auxiliary gas: 1 l min-1 (Ar), nebulizer gas: 0.78 l min-1 (Ar), pump rate: 1 ml min-1). There were 3 measurements per sample with intermediate system washing with deionized (Milipore) water. The ICP OES analysis was based on 9 calibration points (0, 0.05, 0.1, 0.5, 1, 2, 5, 10, 20 mg kg^{-1}) of the AAS standard solutions (Ti, Alfa Aesar, Ce and Mn, Fluka, all 1000 μg l-1) diluted in deionized water (Milipore).

Nanoparticle dispersions preparation. Experiments were performed using Mn_3O_4, TiO_2 and CeO_2 particles at a concentration ranging from 5 to 20 mg kg^{-1} in the assay medium. For treatment assays stock dispersions of 1000 parts per million (1 ppm, corresponds to 1 μg

particles per mL cell culture medium; further referred to as ppm) was prepared on a daily basis in double-distilled water. Dispersions were sonicated for 10 min (Branson B15; Branson Ultrasonics Corporation, Danbury, CT, USA) to break up agglomerates and diluted to the final exposure concentrations in assay medium prior to application. All the following measurement procedures were also performed in cell-free assays to monitor for effects of the particles on the respective assays.

Determination of oxidative stress. ROS formation was measured assessing the oxidation of non-fluorescent 2',7'-dichlorfluorescein-diacetate (=DCFH-DA) to the fluorescent DCF. For measurement of intracellular ROS generation in AEC, 20'000 cells were seeded in each well of a 96 well plate (Nunclon™; Fisher Scientific AG, Wohlen, Switzerland) and cultured for another 2 days to confluency. After removing the medium cells were loaded with 50 µM DCFH-DA by a 30 min incubation in Hanks´ Balanced Salt Solution (HBSS; Sigma-Aldrich, Buchs, Switzerland) supplemented with 30 mM D-(+)-glucose (Sigma-Aldrich Inc., St. Louis, MO, USA), pH 7.4 (= HBSS+) at 37°C in the presence of 5% CO2 in the dark. After exposure to the dye the cells were washed twice with HBSS+ to remove any probe from the cells external environment. Since the MAC used in our study have mixed growth properties (adherent and suspension) a different methodological approach was chosen. Alveolar macrophages were loaded in 50 mL tubes (Fisher Scientific AG, Wohlen, Switzerland) with 50 µM DCFH-DA as described above and washed twice with HBSS+ by centrifugation at 250 g for 5 min. For the detection assays 300'000 MAC were plated again in 96 well plates. Both cell types were then exposed to metal oxide particles at a concentration of 20 mg kg^{-1} in HBSS+. The amount of the fluorescent form of the dye was measured over time. To monitor possible interference of the nanoparticles with the dye of the ROS detection assay, we performed cell-free experiments with the intracellular produced intermediate of DCFH-DA, 2',7'-dichlorfluorescein (DCFH). Similar to cell experiments a suspension containing 20 mg kg^{-1} of the respective metal oxide particle was prepared in 96-well plates and mixed with 50

μM DCFH. The amount of the fluorescent form of the dye was measured over time. SIN-1 (3-morpholinosydnonimine hydrochloride; Sigma-Aldrich, Buchs, Switzerland), a potent ROS stimulator, was used at a concentration of 200μM as positive control.

Viability and cytotoxicity. The tetrazolium bromide (MTT) assay, determining the amount of NADPH oxidase-related metabolic activity, was used to measure the cells' viability in vitro. Yellow tetrazoliumsalt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide is converted by living cells into purple formazan crystals. The lipid soluble formazan product was extracted with organic solvent (80% EtOH) and estimated by spectrophotometry. The apoptosis rate of MAC and AEC was determined using fluorometric assays for caspase-3 activity as previously described [19]. Briefly, caspase-3 activity was assessed by measuring the cleavage of the fluorogenic caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC (Calbiochem, Läufelfingen, Switzerland) at an excitation wavelength of 360 nm. To evaluated the results, untreated control cells were defined as reference category (=100%). Ac-Asp-Glu-Val-Asp-H (Aldehyde) (from PeptaNova, Sandhausen, Germany) at a concentration of 100μM was used to as inhibitor of caspase-3.

Statistical analysis. SPSS Version 20 (SPSS Inc, Chicago, Ill) was used to perform all statistical analyses. All experiments were at least performed 3 times each with six independent samples per group. Figure data are summarized as mean ± standard deviation. Boxplot figures show medians and quartiles. Whiskers represent 5% and 95% confidence intervals, * represent 1% and 99% confidence intervals. Linear regression was used to evaluate the influence of metal oxide particle exposure on MAC and AEC (dependent variables: formation of ROS, NADPH-oxidase related metabolic activity, amount of DNA, and MCP-1 expression). Unexposed control cells were used as reference category. Metal oxide particle exposures were included in the linear regression model as binary, independent predictors (Mn_3O_4, TiO_2, or CeO_2). The different measurement time points were addressed using a categorical predictor variable. Results are illustrated as B coefficients and

corresponding 95% confidence intervals. Using binary independent predictor variables, the resulting B coefficients represent the mean effect difference in relation to untreated control cells (reference category). Spearman's rank correlation analysis was performed to describe the relation between ROS formation and inflammatory mediator secretion. The influence of a co-incubation with the free radical scavenger trolox and metal oxide particles in MAC and AEC was evaluated in two separate linear regression analyses in which treatment with trolox was coded as binary, independent variable. Spearman's rank correlation analysis was used to correlate changes in inflammatory mediator expression and the amount of adsorbed inflammatory mediators by metal oxide particles. To demonstrate the dose-dependence in oxidation of DCFH to DCF by Mn_3O_4 particles in cell-free experiments, as well as the dose-dependency of changes in MTT and MCP-1 assays also spearman's rank correlation analysis was performed. For all analyses, we considered $p < 0.05$ to be statistically significant.