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In vitro toxicological screening of nanoparticles on primary human endothelial cells and the role of flow in modulating cell response

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Abstract

After passage through biological barriers, nanomaterials inevitably end up in contact with the vascular endothelium and can induce cardiovascular damage. In this study the toxicity and sublethal effects of 6 types of nanoparticle, including 4 of industrial and biomedical importance, on human endothelial cells was investigated using different *in vitro* assays. The results show that all the particles investigated induce some level of damage to the cells and that silver particles were most toxic, followed by titanium dioxide. Furthermore endothelial cells were shown to be more susceptible when exposed to silver nanoparticles under flow conditions in a bioreactor. The study underlines that although simple *in vitro* tests are useful to screen compounds and to identify the type of effect induced on cells, they may not be sufficient to define safe exposure limits. Therefore, once initial toxicity screening has been conducted on

nanomaterials, it is necessary to develop more physiologically relevant *in vitro* models to better understand how nanomaterials can impact on human health.

1. Introduction

The unique toxic properties of nanomaterials is inextricably linked with their attractive physico-chemical features and size (The Royal Society, 2004). We know that NPs (nanoparticles) can be very reactive or catalytic (Ying, 2001) because of their high surface to volume ratio. Furthermore they have access to transport mechanisms not open to larger structures, overcoming biological barriers and penetrating the interior of cells (Isidoro, 2012; Hillaireau, 2009; dos Santos, 2011). For this reason much concern has been expressed as the safety of many nanomaterials has not yet been demonstrated and there are still a large number of unanswered questions regarding both toxicity mechanisms and methods of assessment. Toxicity studies are therefore essential to gain new insights into the hazards of NPs and to define new standards for testing as well as constructing databases which provide information for material safety data sheets and basis for potential NP risk assessment and risk management. The gold standard for toxicity testing is still based on *in vivo* experiments. However, due to the large variety of different NPs and the difficulty of extrapolating animal studies to humans, it is desirable to start toxicity assessment using *in vitro* assays, which are cheaper and ethically less questionable than animal testing.

No matter what the port of entry of a nanomaterial, if NPs are able to cross biological barriers (skin, intestines, lungs, etc.) and to enter the general circulation, they end up in contact with the vascular endothelium. The endothelium is the first internal layer of blood vessels and is distributed throughout the body. It is of primary importance for

transport and act as a selective barrier between the circulation and the surrounding tissue, controlling the passage of molecules in and out of the bloodstream. Moreover the endothelium modulates a number of pathways such as lipid metabolism and also releases signals involved in vascular inflammation and the modulation of vascular tone. Alterations in these pathways are correlated with the onset of cardiovascular dysfunction leading to cardiovascular disease, the number one cause of mortality in the West (Wu, 2006). Considering the importance of the endothelium in human physiology, and the very high probability that NPs come into contact with the vascular system after crossing biological barriers, this study is focused on the toxic effects of engineered nanomaterials on primary endothelial cells.

Endothelial cells are the only adherent cells in the human body exposed to direct and continuous tangential fluid flow. They are known to be highly responsive to flow, modifying their morphology, cytoskeletal organization, and gene expression in the presence of fluid dynamic forces (Li, 2005). Therefore we initially assessed the effects of exposure of a range of engineered nanomaterials using microwell based cultures; then the material identified as the most hazardous was tested in the presence of flow using a fluidic chamber.

Although the flow dependency of nanoparticle uptake in endothelial cells has recently been the object of investigation (Samuel, 2012; Kusunose, 2012), there are very few studies on the effects of flow and nanomaterial induced toxicity. Only Kim et al. (Kim, 2011) have studied the cytotoxic effects of silica nanomaterials in the presence of flow, reporting a flow rate dependent toxicity.

In this work, 6 different nanomaterials (15 nm and 80 nm Au, NM 101 TiO₂ (7 nm), NM 300 Ag (20 nm) and 50 nm and 200 nm fluorescent labeled (with fluorescein isothiocyanate or FITC) polystyrene NPs) were first characterized in cell culture medium. HUVEC (Human Umbilical Vein Endothelial Cells) were then exposed to the

materials and ranked for their toxicity. The most toxic NP was then further assessed using a fluidic system to better simulate physiological conditions. The materials were chosen because of their relevance to the nanomaterial industry and in particular the TiO_2 and Ag are categorized as OECD reference manufactured nanomaterials. Some of them have not been tested on endothelial cells and others have yielded controversial results (Peters, 2004; Brammer, 2008; Peng, 2009; Montiel-Dávalos, 2012). Our contribution is thus assessment of damage induced by well-characterized metals, metal oxides and polystyrene beads in primary endothelial cells (Peters, 2007; Gojova, 2007; Meng, 2012; Oesterling, 2008) and an evaluation of the effects of flow on nanomaterial induced toxicity. The results of the study can be used to establish a minimal set of sensitive markers and endpoints for evaluating NP cytotoxicity on endothelial cells and underline that flow can affect the toxicity of potentially hazardous nanomaterials.

2. Materials and methods

Experimental workflow

The aim of this paper is to screen NPs within a set of standard assays commonly used by nanotoxicology community, and then determine the effect of flow on toxicity by exposing HUVEC to a significantly toxic material in a bioreactor.

In order to reach this goal, we first characterized each nanoparticle in the HUVEC medium, then we tested them in standard static conditions within a large concentrations range to determine the LC_{50} . This value was taken to classify nanoparticles in 2 groups, low toxicity and high toxicity, in order to identify an appropriate range of concentrations to be further assessed for sublethal effects. Finally we selected the most toxic NP for testing in dynamic conditions demonstrating that even the application of gentle flow can change the susceptibility of cells to toxic insult from nanomaterials.

Reagents

All the reagents were purchased from Sigma-Aldrich (St. Louis, USA) unless otherwise specified.

Culture of endothelial cells

HUVEC were isolated, passaged and cultured as described in SI.1. All the experiments, including those involving NP characterization were performed in EMEM with supplements (see SI.1) henceforth referred to as HUVEC medium.

For the toxicity experiments HUVEC were seeded on 1% gelatin coated wells (static tests) or 13 mm diameter plastic slides (NUNC, Denmark) (dynamic tests) at a concentration of 20,000 cells/cm² and allowed to reach confluence (typically 24 h) before exposure to NPs.

Nanoparticle preparation and characterization

The types and sources of the nanoparticles tested are listed in table 1.

All the dispersion and dilution protocols are reported in SI.2.

Particle hydrodynamic diameter was determined via dynamic light scattering using Malvern Zetasizer nano (Malvern Instruments, Herrenberg, Germany). Size and polydispersity were determined at time points 0, 1, 2, 4, 6, 8 and 24 hours after preparation of the respective dilution. Between measurements, dilutions were kept at room temperature and protected from light. Diluted samples were again sonicated for 16 minutes in an ultrasonic bath. For each time point, two independent samples were measured three times each and mean and standard deviation were reported.

Due to interference with the zeta sizer measurement, Ag NP could not be evaluated using dynamic light scattering in our work. Therefore in the case of Ag NP, size

distribution and stability were investigated using NanoSight LM10 instrument (NanoSight Limited, Amesbury, UK) which uses single particle tracking analysis. Two concentrations were tested and mean particle diameter (out of 3 independent measurements) and width of the distribution are reported. Changes in Ag NP agglomeration and Ag loss through adsorption or deposition in the bioreactor were monitored by comparing optical absorption spectra before and after 24 hours of circulation in the fluidic system.

Static Set up

The viability evaluation was performed in a 96 well plate with 200 μL of HUVEC medium containing a range of ten concentrations (from 625 $\mu\text{g}/\text{cm}^2$ to 1.2 $\mu\text{g}/\text{cm}^2$) for each NP, performed as simple serial dilutions from a stock using the HUVEC medium.

All other assays were performed testing 3-5 significant concentrations in a 24 well plate. In each case the assay was performed as an endpoint after 24 hours exposure of the cells to the NPs. Control samples were seeded and analyzed in the same conditions and time of treated samples. They were untreated HUVEC (negative control) and 0.1% Triton™ X-100 (named Triton) treated HUVEC (positive control). Micrographs for the controls and flow experiments are reported in figure 1 A-C. Figure 1 A shows the typical cobble-stone morphology of endothelial cells *in vitro*. For each particle concentration the interaction with the assay was also tested in the absence of cells in order to avoid false negative or positive results.

Dynamic Set up

Dynamic experiments were carried out in the Quasi-Vivo bioreactor (Kirkstall Ltd, UK), a modular system which allow chambers to be connected in series or parallel as required (Mazzei, 2010). The chamber has approximately the same dimensions as a 24-well (13

mm diameter and 11 mm height) as shown in figure 2 A. The main features of the bioreactor are the absence of air bubbles, high oxygen transport through convection, and the possibility of connecting additional chambers in series or parallel. In this work a single bioreactor was connected in a closed loop to a pump (IPC4 Ismatech, CH) and a mixing bottle (Vozzi, 2011). According to the manufacturer the optimal range of flow rates, to guarantee laminar conditions (figure 2 B), is between 100 and 500 $\mu\text{L}/\text{min}$. Higher flow rates result in turbulence. HUVEC vitality was first tested between 100 and 500 $\mu\text{L}/\text{min}$ (see Supplementary Information, figure S1), and as there was little difference between viabilities all dynamic experiments were carried out at 100 $\mu\text{L}/\text{min}$. At this flow rate the shear stress at the base of the bioreactor, calculated using fluid dynamic models as described in Mazzei et al., is about 10^{-6} Pa (figure 2 C) and the fluid residence time in the chamber is about 15 min. Nanoparticles (Ag) were added to the media (15 mL in total) to give final concentrations ranging from 0 to 50 $\mu\text{g}/\text{mL}$. One endothelial cell coated slide was placed in the bottom of the bioreactor and filled with 500 μL of media in order to avoid drying of cells during assembly of the system. The rest of the media was added to the mixing bottle after which the circuit, shown in figure 2 D, was closed. After 24 h in the incubator the bioreactor circuit was disassembled; medium was recovered and the HUVEC slide was moved to a 24 well plate for analysis.

Assays

At the end of the experiment cells were assayed for an array of markers commonly used for nanotoxicity screening (Kroll, 2009), as summarized in table 2. The experimental details for all the assays are reported in S1.4.

Data processing and statistical analysis

Results were calculated from at least three different experiments while the dosing was performed in duplicate. They were expressed as means $\pm$ standard deviation of the mean. In the case of image analyses, the intensity data were averaged over at least 5 representative images per replicate.

Data were analyzed by two tails Student's t-test and statistical significance was set at $p < 0.05$ (indicated with *) and high significance was set at $p < 0.01$ (marked with **). Significance of mRNA expression results was defined as the fold-increase with a cut-off level of 1.8-fold relative to control (Cheng, 2007; Gaiser, 2013). LC_{50} was calculated using Sigma Plot 11.0 (Systat Software, Inc., Erkrath, Germany).

Results

Nanoparticle Characterization in HUVEC medium

Au 15 and 80 NPs were synthesized as described in Pan et al. (Pan, 2007) and their characterization has been fully reported (Hirn, 2011; Schleh, 2012). Briefly the hydrodynamic diameter from DLS analysis was 21 nm and 85 nm while the polydispersity index (PDI) was 0.10 and 0.12. The surface was negatively charged thanks to triphenylphosphinemonosulfonate (TPPMS) group showing a ζ potential of -22.8 ± 3.1 mV and -22.3 ± 1.6 mV respectively.

We found the two Au NPs and PS 50 easy to disperse in the HUVEC medium over the whole concentration range tested. For each of these NP the measured hydrodynamic diameter fit with the nominal diameter given by the manufacturer (table 3). A minor increase in size could be observed for Au likely due to serum protein absorption on the particle surface. The PDI was found to be < 0.1 even at high concentrations of 1 mg/mL. In contrast, a significant increase in size could be observed for PS 200: at 0.1 mg/mL big agglomerates of ~ 900 nm size were formed and agglomeration increased during 24 h storage. Agglomeration was less pronounced for concentrations of 0.01 and 0.001

mg/mL and showed a size of ~420 nm and PDI < 0.2 together with a monomodal distribution of the particle population.

Being a reference material, TiO₂ was well characterized both from JRC and in Kermanizadeh et al. (Kermanizadeh, 2012). Briefly TEM (Transmission Electron Microscopy) analysis showed two structures with size of 4-8 nm and 50-100 nm. Surface area (BET) was 322 m²/g and size (from Dynamic Light Scattering analysis) in the medium was 185 and 742 nm.

In our study, TiO₂ dispersability was very poor in plain water (data not shown) but was improved in HUVEC medium likely due to the high serum/protein concentration and steric stabilization of the particle dispersion. Nevertheless, agglomerates were formed varying in size between 700 and 1200 nm with a high PDI (>0.4) and two to three subpopulations could be identified in the size distribution profile at concentrations of 0.1 and 0.01 mg/mL, confirming TEM data from Kermanizadeh's group. In the most diluted concentration of 0.001 mg/mL, hydrodynamic diameter was improved varying between 200 and 300 nm, although the sample was still highly polydispersed (PDI ~0.25).

Ag was also characterized both from JRC (Klein, 2011) and Kermanizadeh (Kermanizadeh, 2012). They found an average size at TEM of 17.5 nm with euhehedral morphology. Size in the medium measured with DLS was 12, 28 and 114 nm.

Using the Nanosight system, Ag presented mean hydrodynamic diameters of 120 nm hinting at particle cluster formation together with serum proteins. Size distribution was monomodal in single particle tracking analysis and no further agglomeration was observed within 24 h of storage at room temperature. After 24 h of circulation in the bioreactor system we detected a slight decrease (between 10 and 30% depending on the concentration) in Ag concentration due to adsorption onto the walls of the bioreactor

chamber and tubing, but no wavelength shifts, characteristic of particle agglomeration, were recorded.

Further details about nanoparticle's characteristics in HUVEC media can be found in the Supplementary Information.

NP toxicity screening (static conditions)

Cell Viability Screening

Dose dependent toxicity was assessed using two different assays since NPs can interfere with standard assays leading to confounding or even conflicting data (Krug, 2011). Therefore a comparison between results of two independent assays should exclude cross-reactions with NPs and give us a higher level of confidence when quantifying toxic doses.

Metabolic activity was evaluated using Alamar blue added directly to treated cells while membrane integrity was assayed measuring LDH (Lactate dehydrogenase) levels in the medium. Figure 3 shows the dose response curves of the NPs; in both assays high toxicity was shown for Ag and TiO₂ with an LC₅₀ of 24.5±7.5 µg/cm² (Alamar) and 12.2±2.6 µg/cm² (LDH) for Ag and an LC₅₀ values of 25.0±7.9 µg/cm² (Alamar) and 47.0±9.5 µg/cm² (LDH) for TiO₂ (figure 3 A and B). However preprocessing of the LDH raw data was required in order to correct values for particle interference as a reduction in enzyme availability was noticed in the presence of Ag. This interference is thought to be due to the adsorption of the enzyme onto the particle surface, reducing enzyme activity (Clift, 2008). The positive control value (Triton treated) was 0.74±0.11% with Alamar assay and 0±0.9% with LDH assay. The toxicity of the Ag dispersant was also investigated, no effects in the range of dispersant concentrations used was observed (data not shown).

There was no significant toxicity with PS 50 and PS 200 (figure 3 C and D), besides some SD fluctuation, which may be due to primary cell variability, while significant toxicity was shown for both Au particle sizes at the highest tested concentration (figure 3 E and F), however, the LC₅₀ was not reached with the concentrations used in this study.

Inflammation

Inflammation was assessed measuring HUVEC cytokine release after NP treatment. The cytokines selected were IL-8, a pro-inflammatory protein stored in the Weibel-Palade bodies and released in case of stress and inflammation, it is involved in the inflammatory cascade and is directly synthesized by endothelial cells (Middleton, 1997); sICAM-1, the soluble version of the transmembrane protein ICAM-1, typically expressed in endothelial cells, that is associated with activation of recruited inflammatory cells (Gearing, 1992; Languino, 1993; Springer, 1995) and TNF- α a cytokine involved in systemic inflammation and in the acute phase reaction of the endothelium, it stimulates the ubiquitous transcription factor NF- κ B, activating the other proatherogenic molecules (Cines, 1998).

Three concentrations for each NP type were tested, from 160 to 5 $\mu\text{g}/\text{cm}^2$ for low-toxicity NPs and from 20 (or 40 $\mu\text{g}/\text{cm}^2$ for TiO₂) to 1 $\mu\text{g}/\text{cm}^2$ for high-toxicity ones.

PS NPs of both sizes induced IL-8 expression comparable to controls with a slight decrease at the highest concentration (figure 4 A). On the contrary Ag, TiO₂ and Au of both sizes showed a significant increase with respect to the negative control (untreated). The increase was found to be concentration dependent for all particles. For comparison, the IL-8 level for Triton treated cells value was extremely low, 68.59 pg/mL $\pm$ 22.57.

sICAM-1 was only detected at the highest concentration of PS 50 (figure 4 B) while in all concentrations of PS 200 it was under the limit of detection. TiO₂ and Au showed highly significant ($p < 0.01$) increases of sICAM-1. In these experiments the highest sICAM-1 levels (28 fold higher than in controls) were found in the presence of 20 $\mu\text{g}/\text{cm}^2$ of Au 80. Interestingly, sICAM-1 was detected neither in Ag treated samples, nor in the Triton treated cells.

TNF- α was below the limit of detection in all samples (data not shown), although investigations using other cells types have reported measurable levels (Kaur, 2013; Xue, 2012).

Apoptosis

Fas ligand (Fas-L or CD95L) is a transmembrane protein. When the receptor binds it activates the caspase cascade with a consequent induction of apoptosis. Fas-L was only detectable in a few analyzed samples (in TiO₂, Au 80 and Au 15 samples it was below the limit of detection). Ag treated HUVEC showed a concentration dependent high apoptosis marker level; surprisingly PS 50 had the highest increase at a concentration of 160 $\mu\text{g}/\text{cm}^2$ (figure 5). This concentration did not show toxic effects in Alamar and LDH Assays. Fas-L in Triton treated samples were below the limit of detection.

von Willebrand Factor expression

vWF expression was analyzed at the mRNA level for all NPs and the significantly toxic NPs (Ag and TiO₂) as well as one type of the least toxic particles (PS 50 and PS 200) were used to verify expression at the protein level.

RTqPCR showed that TiO₂, Au as well as PS NPs did not differ significantly in the mRNA expression with respect to untreated control. Conversely, significant higher

levels of vWF expression were found for Ag at low concentration, indicative of endothelial injury (figure 6). Analyses could not be performed on Triton treated cells due to the low mRNA availability.

vWF protein levels were also assessed and the fluorescence images of vWF staining were quantified using image processing. PS NPs of both sizes had a strong interference with the visualization of the secondary antibody as the FITC emission (green) overlapped with the excitation wavelength of the Alexa Fluor labeled antibody used to stain vWF. To discern the Alexa Fluor signal from that of FITC, only zones where FITC and vWF signals were not co-localized were used to quantify vWF. In the image analyses, the background was set at the same level as Triton treated cells.

The results show a constant and concentration independent expression of vWF in PS treated cells with respect to untreated samples independent of the concomitant concentration dependent FITC signal from both the PS NPs (figure S6). A bright field micrograph of PS NP 200 treated HUVEC morphology is reported in figure 1 D, confirming the normal morphology of the cells (shown in figure 1 A) despite the presence of NPs clustered around the cell membrane.

vWF expression from Ag treated HUVEC showed a peak of more than 250% fluorescence at 3 $\mu\text{g}/\text{cm}^2$ with respect to controls, while at higher concentrations the fluorescence dropped, either because of cell detachment or because the vWF expression machinery was compromised (figure 7 A). The same trend was found with TiO_2 treated cells (figure 7 B), albeit at lower intensity levels.

The data obtained in these studies are summarized in table 4, and represent a first insight into NP toxicity, inflammation, apoptosis and stress on HUVEC cells.

Clearly Ag is far more toxic to HUVEC than the other materials studied and merits further investigation as it could represent a potential hazard.

Dynamic tests on HUVEC with Ag NP

The typical elongated morphology of HUVEC under flow conditions is shown in figure 1 C. In the absence of NPs, the viability of HUVEC in the bioreactor was not affected, showing comparable values with respect to cells cultures in static conditions (data shown in the Supplementary Information). Figure 8 shows the viability data with reference to the volumetric Ag concentration, comparing the dose response curves in static and dynamic conditions.

Note that in static conditions the volumetric and predicted surface concentrations are almost identical because the surface to volume ratio of the wells is approximately 1. Comparative dosage in the 2 systems does merit further investigation, although it is beyond the scope of this toxicology study. As a first approximation, we can assume that the NP volumetric concentrations in static and dynamic conditions are comparable because I) we did not observe a significant decrease in Ag NP media concentration after 24 hours of circulation in the fluidic system, II) a recent report on gravitational sedimentation of NP in a cuvette by Alexander et al. (Alexander, 2013) shows that small NP do not deposit in significant amounts over 24 hours. We reproduced the same experiment with our Ag NPs confirming this result (data not shown).

In this context, the LC_{50} value in the dynamic system ($12.5 \pm 3.9 \mu\text{g/mL}$) is significantly lower ($p=0.02$) than the LC_{50} in static conditions ($40.4 \pm 12.4 \mu\text{g/mL}$), indicating that the cells are more susceptible to Ag induced toxicity in the presence of flow. This is even more evident considering the viability curves: dynamic viability values are consistently lower with respect to the static values at the same concentration.

SICAM-1 and Fas-L were undetectable in the dynamic experiments maybe due to the higher media volume required to fill the bioreactor circuit. IL-8 was detected in flow conditions, but only at Ag concentrations lower than about $10 \mu\text{g/mL}$ (figure 9 A): at

higher concentrations the cells were not viable, as confirmed by the data in figure 8. In static samples measureable levels of IL-8 were found up to 50 µg/mL indicating a shift of pro-inflammation towards lower concentrations under flow.

Further confirmation of increased susceptibility was obtained analyzing vWF stained HUVEC using image processing; although the inverted u-shaped trend with increasing concentration (figure 9 B) remained similar, the peak in vWF specific fluorescence was observed at lower concentrations in dynamic conditions.

Discussion

Besides providing preliminary data, well-based cell culture experiments definitely represent a first, if somewhat simplistic, insight into the effects of NPs on HUVEC. Knowing the effect of chosen NPs on a simple culture also serves to better orient subsequent hazard testing and safety classification using more appropriate testing methods.

The cardiovascular system is considered an important site of nanomaterial induced toxicity and it has been suggested that NPs can alter the endothelium integrity impairing vascular function (Mann, 2012). As a consequence, several investigations on toxicity of different NPs on HUVEC have been conducted. Solarska et al. (Solarska, 2012) showed that nanodiamond particles to lead to cell death in a time and concentration dependent manner. Carbon black NPs, were also found to increase expression of endothelial intracellular adhesion molecules and reactive oxygen species (Vesterdal, 2012). Magnetic particles were found to inhibit HUVEC proliferation through an increase of eNOS activity and NO production (Su, 2012).

As different endpoints and results have been described in the literature for a range of NPs, in this study the toxicity and effects of sub lethal exposure of a range of well-

characterized industrial nanoparticles to HUVEC were assessed at different concentrations with different assays. Both Alamar and LDH assays confirmed that Ag and TiO₂ NPs were highly toxic for these cells (figure 3 A and B). Although the LC₅₀ values were very low, they were not identical as already found in Gaiser et al. (Gaiser, 2012), indicating that the two assays have slightly different sensitivities for the two nanoparticles or that the mechanism of cell death induction differs. In fact cytotoxicity is first manifested by an increase in membrane permeability in the case of Ag, while TiO₂ appears to compromise the metabolic machinery before inducing membrane damage mirroring that observed for carbon black nanoparticles (Vesterdal, 2012). The toxicity of TiO₂ on HUVEC is particularly interesting since the same nanoparticles (NM 101) were tested on a hepatocyte cell line and no toxic effects were observed (Gaiser, 2012).

PS 50 and PS 200 were not toxic across the concentration range tested while slight toxicity was observed with Au NPs of both sizes.

However, NPs have many other effects besides cell death (Foldbjerg, 2009). For instance carbon black, TiO₂, silver NPs are known to induce oxidative stress or inflammation (Hussain, 2010; Lee, 2011; Park, 2010). In fact inflammation is one of the most common sublethal reactions to occur in cells after exposure to NPs (Oberdörster, 2009; Peters, 2007). Therefore, to better understand the effects of lower and more physiologically relevant NP concentrations, we also measured IL-8, sICAM-1, and TNFα levels in the media. We underline that none of these markers were expressed at high levels in the Triton treated cells, which were set as our positive control. In fact while Triton is a good positive control for viability, it is not suitable for stress markers since cells probably died before the onset of inflammatory signaling. TNFα was undetectable while high IL-8 levels were found in all samples except for PS NPs treated cells, which showed a small decrease at the highest concentration (figure 4 A). There was a slight

increase in sICAM-1 levels at the highest PS 50 concentration (figure 4 B), indicating that only high amounts of the smaller PS 50 initiate a low level pro-inflammatory signal. The most toxic NPs, Ag and TiO₂, released large amounts of IL-8, but interestingly, only in TiO₂ treated samples were sICAM-1 levels also increased. In fact, sICAM-1 may be involved in a different pathway as it is known to be stimulated by IL1 and TNF α in hepatocytes (Satoh, 1994), endothelial cells (McHale, 1999), and vascular smooth muscular cells (Couffinhal, 1993). This result suggests that inflammation can actually be triggered through different pathways and involve different markers depending on the type of NPs.

Increased cytokine expression after exposure to TiO₂ NPs was also observed by Montiel-Davalos et al. (Montiel-Davalos, 2012) in HUVEC cells, confirming our result; they also showed that cell death was mainly through necrosis, which may explain the absence of Fas-L in our experiments.

Au NPs also induced high levels of IL-8 and sICAM-1 release (figure 4 A and B), with higher sICAM-1 for Au 80 than Au 15 and higher levels of IL-8 for the smaller Au. Therefore gold NPs seem to activate both pathways, but in a size dependent manner.

In summary, Ag triggered inflammation activating an IL-8 dependent pathway and did not have any effect on sICAM induction. Both TiO₂ and Au 15 triggered inflammation by IL-8 and sICAM related pathways, but principally that involving IL-8, while Au 80 evidently activates the sICAM to a greater extent. Whatever the pathway, the data shows that almost every NP induces inflammation in cells, even at low concentrations as described in literature for many NPs (Peters, 2007; Montiel-Dávalos, 2012; Kermanizadeh, 2012; Gaiser, 2013).

Although we did not observe any toxicity and only slight proinflammatory effects on cells (figure 4 B) in the presence of PS, the apoptosis marker Fas-L increased on exposure

to PS 50. These low-toxicity NPs induce low inflammation at very high, un-physiological doses that appears to lead to the initiation of apoptotic signaling already described for endothelial and lung epithelial cells by other groups (Solarska, 2012; Park, 2010). Finally, the von Willebrand Factor mRNA and protein expression data, correlate quite well in Ag exposed HUVEC; after an increase in vWF expression at low concentrations of Ag, as already described for other NPs (Bauer, 2011), the expression dropped. The decrease can be ascribed to a decrease in cell number due to Ag toxicity which results in fewer viable cells being stained (figure 7 A). On the contrary there was no correlation between mRNA and protein expression in TiO₂ exposed cells. Although the vWF immunohistochemical staining for the TiO₂ treated HUVEC (figure 7 B) first increased and then decreased at higher concentrations (albeit to a lesser extent than with Ag), mRNA expression did not change increasing TiO₂ concentration. In the case of Au NPs, mRNA expression data showed that both Au were not able to affect HUVEC vWF production (figure 6). No relevant vWF expression from PS NPs was observed, both at mRNA and protein level, confirming that these NPs do not trigger inflammation, despite the increase in the FITC signal from uptake/binding to the membrane with increasing concentration (figure S6).

As summarized in table 4, of the different particles used, the most toxic was Ag. Although there was little loss in viability, at very low concentrations there was significant inflammation (figure 4) as well as pro-apoptotic signaling (figure 5) probably leading to cell death (figure 3) at higher concentrations. One hypothesis about Ag NP toxicity is that is partially due to toxic Ag ions released from the NP (Klein, 2011). However any Ag ions will tend precipitate due to the presence of chloride ions in the medium. Thus a more complex mechanism must be the root of Ag NP toxicity.

As the endothelium is exposed to tangential flow *in vivo* and the presence of a fluidic environment has been reported to increase endothelial susceptibility to toxicity from silica nanoparticles (Kim, 2011), we also probed the effects of Ag exposure in dynamic conditions. The bioreactor used applies low shear stress to cells, while ensuring high flow rates and oxygen transport. Although endothelial cells *in-vivo* are generally subject to much higher shear ($1\text{-}10^{-3}$ Pa, (Caro, 2011)) than in our experiments, the studies confirm that Ag toxicity and Ag induced inflammatory stress increases even in the presence of very low shear stress conditions, highlighting the sensitivity of HUVEC to shear (Vozzi, 2011). The LC_{50} as well as peaks from IL-8 and vWF release shifted to lower concentrations, while maintaining the general trend of the cell responses in static conditions. This indicates that the organism is likely to be more susceptible to NP induced injury than standard *in vitro* tests would suggest and that even NP with only slight toxicity in static tests (table 4) should be assessed under flow. A few investigations have shown that cells such as hepatocytes and myoblasts are more sensitive to drug induced injury in dynamic conditions (Vinci, 2011; Tirella, 2008), albeit the mechanism by which this occurs has yet to be elucidated. Although endothelial cells are known to undergo significant changes, such as increased membrane permeability and ruffling, elongation (as observed in our experiments), stiffening and expression of adhesion and vasodilative factors under flow (Samuel, 2012; Kusunose, 2012), it is still a question of debate as to whether fluid mechanical forces directly influence cell response to flow, or if the increased mass transport brought about by convection is actually responsible for most of the flow dependent phenomena observed in endothelial and other cells (Vandragi, 2012). Certainly these studies show that simple *in vitro* tests may not be sufficient to screen compounds and define safe exposure limits and that once initial toxicity screening has been conducted on nanomaterials, it is necessary to

develop more physiologically relevant *in vitro* models to better understand how nanomaterials can impact on human health.

Conclusion

In this paper we studied the toxicity and sublethal effects of a panel of industrial nanoparticles on primary human endothelial cells, finding the highest toxicity with Ag treatment. The general trend in the inflammation and apoptosis cells induction to our NPs well reflects literature data on HUVEC and other cell types, confirming that particles can be toxic at different levels. The NP toxicity screening as performed in this paper and several other studies in the literature are a useful but purely a qualitative analysis. We demonstrate that moving a small step closer to *in vivo* environment, just by adding tangential flow to cells, results in the same qualitative response, but the sensitivity of cells is markedly different. Therefore, to better quantify nanomaterial hazard, especially for the estimation of toxic thresholds, without resorting to animal tests, physiologically relevant cell culture methods more representative of the *in vivo* milieu are necessary.

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Table

Nanoparticle	Acronym in text	Source
Polystyrene (PS) fluorescein isothiocyanate (FITC) Fluoresbrite® Microparticles 0.05 µm (~50 nm)	PS 50	Polysciences Inc. (Germany)
Polystyrene (PS) fluorescein isothiocyanate (FITC) Fluoresbrite® Microparticles 0.2 µm (~200 nm)	PS 200	
Ag NM 300, 20 nm	Ag	Ras GmbH
TiO ₂ NM101 anatase, thermal, 7 nm	TiO ₂	Hombikat UV100
TPPMS-Au NP 15 nm	Au 15	Kind donation from Dr W.G. Kreyling (Helmholtz Zentrum Munich)
TPPMS -Au NP 80 nm	Au 80	

Table 1: Nominal dimensions of NPs and their sources.

FUNCTION	ASSAY	SOURCE	REF
Viability	Metabolic activity: Alamar Blue	CellTiter-Blue®, Promega	Kroll, 2009
	Membrane integrity: LDH assay	Cytotoxicity Detection Kit, Roche	
Inflammation	IL-8 sICAM-1 TNF-alpha	BD FLEX kit bead based ELISA, BD Biosciences	Middleton, 1997 Gearing, 1992; Languino, 1993; Springer, 1995 Cines, 1998
Apoptosis	Fas Ligand	BD FLEX kit bead based ELISA, BD Biosciences	Solarska, 2012; Park, 2010
Endothelial specific marker stress: vWF	mRNA expression	DyNAmo™ FlashSYBR® Green qPCR Kit, Finnzymes	Sumpio, 2002
	Immunostaining	Monoclonal Mouse Anti-hvWF, Dako	

Table 2: Chosen assays for the screening of NP toxicity. Sometimes more than one assay was performed to evaluate the same biological end point in order to exclude cross-reactions between NPs and assay (Kroll, 2009).

NPs	Nominal diameter	Mean hydrodynamic diameter in HUVEC medium	PDI in HUVEC medium
PS 200	211 nm	419 ± 23 nm	0.14 ± 0.03
PS 50	55 nm	55 ± 4 nm	0.02 ± 0.00
Au 80	80 nm	116 ± 5 nm	0.04 ± 0.02
Au 15	15 nm	51 ± 6 nm	0.04 ± 0.01
Ag	<20 nm	120 ± 4 nm	NA
TiO ₂	7-10 nm	896 ± 133 nm	0.42 ± 0.14

Table 3: Dispersion behavior of engineered nanomaterials in HUVEC cell culture medium at 0.1 mg/mL after 1 h of incubation; mean ± SD, n = 6.

NPs effect on HUVEC						
	Toxicity (Alamar)	Toxicity (LDH)	IL 8 release	sICAM-1 release	Fas L release	vWF release
PS 50						
PS 200						
Ag						
TiO ₂						
Au 15						
Au 80						

No effect Strong effect

Table 4: Summary of determined toxicity levels of tested nanoparticles on HUVEC cells; light grey = not toxic over the determined concentration range (response below detection limit or only at very high concentrations); grey = slight toxicity (significantly higher than controls) and black = high toxicity at low concentrations (<20 µg/mL).

FIGURE

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FIGURE CAPTIONS

Figure 1: HUVEC morphology in the negative control (untreated cells in static conditions) (A), positive control (Triton X 100 treated cells in static conditions) (B), under flow conditions (untreated) (C) and PS 200 ($156 \mu\text{g}/\text{cm}^2$) treated cells (D). Scale bar $50 \mu\text{m}$.

Figure 2: The Quasi-Vivo chamber (A), velocity streamlines in the central xz plane of the chamber ($100 \mu\text{L}/\text{min}$, dimensions in m). Cells are seeded at the base of the bioreactor (B), shear stress map at the base of the chamber (C), schematic diagram for the experimental set up used in dynamic conditions (D). B and C were generated using COMSOL Multiphysics applying the Navier-Stokes equations for an incompressible fluid with the same density and viscosity as water at 37°C as described in Mazzei, 2010.

Figure 3: Viability dose response curve for NP treated HUVEC. Viability was assessed both at the level of metabolic activity (Alamar, black line) and membrane integrity (LDH, white line). Data are expressed as percentage of untreated HUVEC viability; mean $\pm$ SD, $n=3$.

Figure 4: IL-8 release (A) and sICAM-1 release (B) at different concentrations of NPs treated HUVEC. Data are expressed as concentration of cytokine (pg/mL) in the medium; control sample is untreated HUVEC; mean $\pm$ SD, * $p<0.05$ and ** $p<0.01$ with respect to control, $n=3$.

Figure 5: Fas Ligand release at different concentrations of NPs treated HUVEC. Data are expressed as concentration (pg/mL) in the medium; control sample is untreated HUVEC; mean $\pm$ SD, * $p<0.05$ and ** $p<0.01$ with respect to control, $n=3$.

Figure 6: vWF mRNA expression at different concentrations of NPs treated HUVEC. Data are expressed as samples/control (untreated HUVEC) ratio; mean $\pm$ SD, $n=3$.

Figure 7: Dose response curve of HUVEC treated with Ag (A) and TiO_2 (B) NPs at different concentrations. vWF expression is represented as the % of control red pixels after processing fluorescent micrographs, mean $\pm$ SD, $n=3$. Scale bar $100 \mu\text{m}$. A fluorescent micrograph for each concentration and

controls (untreated and triton treated cells) is also shown (for matching bright field images see Supplementary Information).

Figure 8: Viability dose response curve for Ag NP treated HUVEC in static (black line) and dynamic (grey line) conditions. Viability was assessed using Alamar. Data are expressed as percentage of untreated static HUVEC viability; mean $\pm$ SD, n=3.

Figure 9: IL-8 concentration (A) and vWF expression (B) in the Ag NP treated HUVEC media in static (black bar) and dynamic (grey bar) conditions. Data are expressed as percentage of respective untreated controls; mean $\pm$ SD, n=3. * p<0.05 and ** p<0.01 with respect to the controls.