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The cytotoxicity of metal nanoparticles depends on their synergistic interactions

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ABSTRACT

With a steady growth in use of engineered nanoparticles (NP) in consumer products the unintended exposure to humans has increased. The risks associated with introduction of NPs in the environment have been widely investigated, but mostly for single type of NPs. Herein, we report a single NP and NP co-exposure study: the cellular effects of silver and platinum NPs on the main components of the blood–brain barrier (BBB), human cerebral microvascular endothelial cells (hCMEM/D3) and human primary astrocytes. We quantitatively evaluated the synergy as per the Chou–Talalay method. Co-exposure to two types of NPs synergistically inhibited proliferation of both cell types, to a greater extent for endothelial cells (where the combination index values for all tested concentrations were below 0.2, corresponding to strong or extreme synergism). In addition, NP-induced toxicity depended on the cell type; astrocytes were more tolerant to NPs. We further explored the mechanism of synergy with short-duration incubation time points (up to 30 min), where the cell metabolic activity decreased to approximately 60% of the controls. Although intracellular trafficking studies and quantitative assessments of NP uptake did not explain the mechanisms of synergistic cytotoxicity, a proteomics analysis suggests that it arises from activation of a immune modulating response and deregulation of the extracellular matrix organization. The substantial synergetic effects in our co-exposure studies highlight the importance of this work in relation to assessment of the health risks associated with nanomaterials.

Nanoparticles (NPs) have unique physicochemical properties and are a part of many consumer and biomedical products, including electronics, food packaging, textiles, cosmetics, and
automotive products. Based on the Nanodatabase online inventory, there are now more than 3,000 nanomaterial-containing products available on the market. Silver NPs are one of the most common nanomaterials added to consumer products. Among the many beneficial properties of these NPs (e.g., high catalytic activity, thermal conductivity, and chemical stability), antimicrobial potency and relatively low production cost contribute to their common use in consumer goods. The high surface area to volume ratio of NPs results in much higher catalytic activity compared to larger particles, rendering the former useful for bio- and electrochemical sensing as well as and automotive engineering and nanomedicine applications.

Despite remarkable nano-technological achievements, there are many concerns regarding toxicity and environmental risks associated with increased exposure to NPs. For example, NPs can enter the human body through several routes (including oral ingestion, inhalation, diffusion through the skin, and injection) and can be harmful depending on their accumulation site. At the molecular level, many NPs disturb cell homeostasis by generating reactive oxygen species (ROS), leading to oxidative stress. This imbalance might then provoke activation of a pro-inflammatory response, a decrease in mitochondrial membrane potential, an elevated level of lipid peroxide, an inhibited activity of antioxidants, and DNA damage and cell apoptosis. We recently demonstrated that a combined exposure of cadmium ions and silver NPs exert synergistic effects on a mammalian cell line. Despite the fact that the most likely human exposure is to more than one type of NP at the same time (co-exposure), the literature primarily focuses on exposure to a single type of NP. Few reports discuss the effect exerted on cells upon their co-exposure to more than one type of nanomaterial.

In our study, we explored NP co-exposure to two common artificial metal nanomaterials: silver and platinum NPs. The NP size and the surface properties were the same, 5 nm in diameter.
and citrate-coated, thus enabling a direct NP-type-based comparison of potential synergistic effects and to minimize the contribution of other factors (i.e., solubility, surface charge, and shape) that might significantly affect the experimental results. Since there is a longer half-life for NPs that accumulate within the central nervous system in comparison to other organs, we chose immortalized cerebral microvascular endothelial cells (hCMEC/D3) and primary astrocytes, both derived from the human brain. These cells help form a tight monolayer, the blood–brain barrier (BBB), which protects the human brain from toxins, maintains cellular homeostasis, and is thus pertinent to NP co-exposure studies. We performed a quantitative analysis of NP synergy using the Chou–Talalay method, adopted from drug combination studies. We then explored in detail the cellular mechanism involved in synergy using transmission electron microscopy (TEM), inductively coupled plasma mass spectrometry (ICP–MS), and quantitative proteomics. To our best knowledge, this is the first study describing the synergistic effect exerted by NPs on cells derived from the human blood–brain barrier. Our results can serve as a starting point for further investigation into this phenomenon and the study sheds light on previously under-appreciated nano–bio interaction risk that warrants further attention.

RESULTS AND DISCUSSION

NP stability as-prepared and in cell culture media. TEM images of NPs deposited on TEM grids from their stock solutions in 2 mM citrate buffer indicated monodisperse NPs, with an average size of approximately 5 nm (Figure 1a, 1b). That indicates good colloidal stability of both silver and platinum NPs dispersed in 2 mM citrate buffer. In contrast, NP stability in EndoGRO medium and Dulbecco’s modified Eagle medium (DMEM) used for culturing hCMEM/D3 and astrocytes, respectively, depended on the NP type. Whereas platinum NPs seem to be stable in either medium after 24 h of incubation at 37°C, silver NPs aggregated when
dispersed in EndoGRO media supplemented with 5% and 10% (v/v) fetal bovine serum (FBS; Figure 1 c, 1d).

**Figure 1. Metal NPs and their stability to cell culture media.** TEM images of (a) silver and (b) platinum NPs deposited on grids in 2 mM citrate buffer at 1 mg mL\(^{-1}\) stock concentration. Scale bar, 0.1 µm. Photographs of NPs re-suspended in (c) EndoGRO and (d) DMEM media supplemented with FBS at 5% (vial: left and third from left) and 10% (vial: second from left and fourth from left) concentrations (v/v). We first pre-coated NPs with FBS for 3 min at room temperature, and then added them to the cell culture media. Images obtained after a 24 h incubation period at 37°C, 5% CO\(_2\).

Based on available data regarding media formulation (Supporting Information, Appendix 1) and the literature,\(^{34-35}\) either ionic composition or additional supplements in EndoGRO media reduces NP colloidal stability by direct interaction with silver NPs, despite its citrate coating. Citrate-coated silver NPs dispersed in DMEM media supplemented with 10% FBS are stable.\(^{36}\)
Consequently, we performed all NP uptake experiments for both cell lines in DMEM media supplemented with FBS, at 5% and 10% (v/v), for hCMEC/D3 and astrocytes, respectively.

**Cytotoxicity studies.** To investigate the potential synergistic effect of silver and platinum NPs in hCMEC/D3 cells and astrocytes, we performed cell viability assays using 24 h exposures with solely silver NPs, solely platinum NPs, and both NP types. We chose the NP exposure doses based on a preliminary screening where we estimated IC50s for each (single) NP type for each cell line (Figure 2). There was a 2× lower IC50 for hCMEC/D3 cells, compared to astrocytes, to silver NPs. High tolerance of astrocytes to silver NPs may be caused by up-regulation of metallothioneins that bind and/or store silver ions potentially released from NPs into the intracellular milieu.\(^{37}\) Since metallothioneins in the human central nervous system are primarily expressed in astrocytes and there are very few reports showing that these proteins are present in the endothelium, the same mechanism may be responsible for the observed differences in nanotoxicity in our study.\(^{38-40}\)

Regarding platinum NPs, we detected much less sensitivity for either cell line. This agrees with the literature; platinum NPs feature excellent biocompatibility in various *in vitro* cell culture models.\(^{41-42}\) Since metallothioneins also have an affinity to platinum ions,\(^{43}\) their up-regulation in astrocytes should result in improved resistance to platinum NPs with respect to the endothelial cell line. However, there was a more pronounced effect for astrocytes (IC50 approximately 120 µg mL\(^{-1}\)) than for the endothelial cells (IC50 not reached), which indicates that the toxicity mechanism might derive from NP-cell interactions rather than from a release of ionic species into the intracellular milieu upon NP dissolution.

A quantitative analysis of NP toxicity based on the Chou–Talalay method showed strong and extreme synergism (CI < 1) for the hCMEM/D3 cell line, and a range of synergistic and
antagonistic interactions ($1 > CI > 1$) for astrocytes, depending on the applied combination doses (Figure 3). Although commonly considered inert, applying platinum NPs in combination with silver NPs significantly decreased cell viability to a few percent (Table 1). Obtaining similar low survival rates for cells exposed only to silver NPs would require very high NP doses ($> 500 \mu g mL^{-1}$ for hCMEM/D3 cells and $> 50 \mu g mL^{-1}$ for astrocytes, as estimated from cell proliferation curves (Figure 2a, 2c)). Our cell culture studies suggest that long-term, low-dose accumulation of both silver and platinum NPs in brain cells might be more toxic than exposure to only silver NPs.

Figure 2. NP dose-response curves obtained for (a, b) hCMEC/D3 cells and (c, d) astrocytes treated for 24 h with silver (left) and platinum (right) NPs. Cell survival was as per a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Error bars correspond to the standard deviation ($n = 3$).
**Figure 3.** Fraction affected vs combination index plots obtained for hCMEC/D3 cells (left) and astrocytes (right) co-exposed for 24 h to silver and platinum NPs at different dose combinations (µg mL⁻¹). Plots prepared based on cell survival as per an MTS assay.

**TABLE 1.** Cell viability results obtained for hCMEC/D3 cells and astrocytes treated with silver and/or platinum NPs for 24 h, as per an MTS assay.

<table>
<thead>
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<th>Exposure dose [µg mL⁻¹]</th>
<th>Cell survival (%)</th>
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Astrocytes

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<td>9 ± 1</td>
<td>47 ± 11</td>
<td>8 ± 6</td>
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**Intracellular trafficking studies.** We performed high-resolution electron microscopy of NP uptake to understand whether co-exposing cells to NPs induces any changes to cell morphology or intracellular trafficking of NPs. TEM images of cells incubated with silver and/or platinum NPs (Figure 4) in various synergistic exposure conditions (Table 2) revealed trafficking of nanomaterials into multivesicular bodies, from where they might be sorted for either degradation inside lysosomes or excretion from the cell via exosomes.
**Figure 4.** TEM data obtained for hCMEC/D3 (left) and astrocyte cell lines (right) incubated for 15 and 30 min, respectively, with (a) citrate buffer vehicle (control), (b) silver NPs, c) platinum NPs, and (d) silver and platinum NPs. Scale bars, 0.2 µm.

The same intracellular trafficking of metal NPs into membrane-bound structures was reported in the literature for different cell lines, with some findings suggesting an induction of autophagy as a common cellular response to NPs.\(^{41, 44-45}\) Engulfment of NPs into lysosomal vacuoles might then result in release of ionic species into the cytosolic environment, increased ROS production, and initiation of apoptosis, a possible origin of metal NP-derive toxicity.\(^{46}\) Whereas He et al\(^{47}\) demonstrated a faster dissolution rate for silver NPs at low lysosomal pH, equivalent studies for
platinum NPs are unavailable. In our study, the fact that the NPs did not decrease in size after engulfment into endolysosomal compartments might indicate resistance to enzymatic degradation. This is consistent with the stability of platinum NPs in the intracellular milieu.\textsuperscript{41} However, silver NPs dissolve in cells, in a more pronounced manner at acidic pH.\textsuperscript{44, 46, 48} It should be noted that our experiments may have been too short to observe notable dissolution. Despite their small size, we did not detect NPs in the nucleus. Comparing single-type NP and co-exposure conditions, none of the cell lines featured differences in intracellular organelle morphology. A lack of nanomaterial aggregates inside vesicles suggests very good NP colloidal stability in the cell culture media. Overall, although TEM studies revealed the NPs short-term intracellular fate, they provided no clear insight on enhanced toxicity upon co-exposure.

**Quantification of NP uptake.** Changes in NP physicochemical properties caused by the interactions of silver and platinum NPs at the extra- and intracellular level, and competition of nanomaterials for cell membrane receptors when they are co-present in cell culture media, might result in an increased or decreased rate of uptake in comparison to single-type NP exposure. To investigate whether such alternations in the NP uptake occurred, we performed ICP–MS analyses for single-type and co-exposure conditions.
Figure 5. Quantitative analysis of NP uptake using ICP–MS obtained for (a) hCMEC/D3 cells treated for 15 min with 10 µg mL\(^{-1}\) of silver NPs and 70 µg mL\(^{-1}\) of platinum NPs, and (b) astrocytes treated for 30 min with the same exposure dose, 20 µg mL\(^{-1}\), for both silver and platinum NPs. Grey bars, silver abundance; black bars, platinum abundance.

There were no significant differences in metal abundance between single-type and co-exposure conditions for either cell line (Figure 5), which suggests no competition for uptake between different NP types. Lack of competition for receptors is indicative of a presence of either (1) two or more independent uptake mechanisms for these metal NPs, or (2) co-transport by the receptor(s) containing NP-type-specific unique binding sites. Clathrin-dependent endocytosis and macropinocytosis are the primary entry routes for silver NPs.\(^{49-50}\) Since the literature provides no pathway-specific uptake quantification study with platinum NPs, various cellular internalization mechanisms such as caveolin-dependent and clathrin- and caveole-independent endocytosis are conceivable. In addition, both cell lines demonstrate higher preferences toward silver NPs. ICP–MS data obtained for hCMEC/D3 cells, exposed to a 7:1 ratio of platinum to
silver NPs, demonstrate only a 4× increase in platinum compared to silver ions (Figure 5a). Astrocytes treated with both types of metal NPs at the same concentration (20 µg mL⁻¹) featured 57× greater silver concentration compared to platinum ions (Figure 5b). These results strengthen the hypothesis that the silver NP engulfment pathway is more efficient than that for platinum NPs.

The endocytic activity of hCMEC/D3 is elevated compared to astrocytes (approximately 7× and 246× higher silver and platinum content, respectively, corrected for differences in NP doses and number of cells per well). This agrees with the literature on cell-type-dependent uptake for different NPs. Preference toward silver NP uptake obtained in our study for hCMEM/D3 cells, together with increased secretory activity reported in the literature for astrocytes, support our cytotoxicity results of higher sensitivity to silver NPs observed for endothelial cells. Surprisingly, even though we also observed an enhanced endocytosis by hCMEC/D3 cells for platinum NPs, astrocytes seem to be more responsive to this nanomaterial. In summary, ICP–MS quantitative analysis provided improved insight into the cell-specific and NP-specific uptake. However, since co-exposure conditions did not result in enhance uptake of either NP type, it provided no clear insight on enhanced toxicity upon co-exposure.

**Proteome responses to NPs.** To more clearly understand the mechanism(s) underlying the NP-derived synergetic phenomenon, we performed quantitative mass spectrometry-based proteomic analysis. The scope was to evaluate the first response at the proteome level of cells exposed to NPs by using short exposure times, up to 30 min. There were 7,590 and 6,956 proteins quantified for hCMEC/D3 and astrocyte cell lines, respectively, among which 128 and 5 were regulated (with a p value set to be ≤ 0.05). This result confirms astrocytes to be less affected by the presence of Ag and Pt NPs, in alignment with the lesser extent of NP uptake established by ICP–
MS. However, results for endothelial cells (Figure 6) reveal the immediate response of cells co-exposed to NPs (up to a 4× and 10× higher number of down- and up-regulated proteins, respectively, in comparison to the single-type NP exposures). In addition, exposure of cells to platinum NPs seems to induce relatively small changes at the proteome level in comparison to silver NPs, taking into account the 1 silver to 7 platinum treatment concentration ratio. This agrees with the data obtained from the cytotoxicity studies, where we observed a smaller effect of platinum NPs on cell proliferation.

**Figure 6.** Venn diagrams generated from (a) down-regulated and (b) up-regulated protein lists obtained for the hCMEC/D3 cell line, exposed to silver, platinum, and both silver and platinum NPs.

Over-representation and pathway–topology analyses (Figure 7) revealed that NP co-exposure conditions in many cases result in enhancement and sometimes unique biological processes. One
example is detection of an immune modulating response in the co-exposure incubation condition (“Immune system” up-regulated; Appendix 2). The endothelial cells derived from the BBB have capability to influence the immune responses within the central nervous system by releasing various endogenous immune response modifiers in order to protect the brain. We found up-regulation of proteins involved in activation of the complement system. Although these proteins are primarily synthesized in the liver, there is evidence that the same proteins are also synthesized by cells of the BBB. In the BBB, complement proteins are activated upon injury or infections in order to protect the brain. Nevertheless, the balance of complement proteins is essential for brain function. Up-regulated level of these proteins can increase BBB permeability, rendering the brain vulnerable to toxins and antigens. The presence of immune activation is further highlighted by up-regulation of proteins involved in the cytokine immune response. A release of cytokines, specifically interleukin-6, to extracellular milieu from brain endothelial cells derived from monkey exposed to different noxious stimuli was reported by Reyes et al that further supports our findings. Surprisingly, single-type NP exposure conditions provoke immunosuppressive-mediated response in endothelial cells. Among metal NPs reported to induce this response, only gold NPs have considerable size dependence, favoring particles < 10 nm. Unlike gold NPs, silver NPs are generally recognized to raise the inflammation level in various human cells, including the endothelial cells of the human BBB. The reason(s) for this difference in immune response between single-type NP and NP co-exposure conditions are not clear from our study, but further underline the importance of considering synergetic effects when assessing NP impacts on cells.

Changes in protein expression related to disassembly of the extracellular matrix (“Extracellular matrix organization” down-regulated; Appendix 2) are relatively higher for the NP co-exposure
condition compared to single-type NP exposure. Disassembly of the extracellular matrix can negatively influence cell proliferation, adhesion, and migration. In the BBB, astrocytes and endothelial cells secrete extracellular matrix proteins to maintain the basement membrane, which is pertinent to BBB tightness. Dysfunction of these vital extracellular matrix proteins and their receptors disturbs the formation of tight junctions between BBB endothelial cells, since surface extracellular matrix receptors function as signal transducers in BBB organization. The influence of NPs on tight junction integrity in \textit{in vitro} BBB models has been described in literature, with some recent reports documenting disruption of the barrier upon exposure to silver NPs for several hours. A follow-up study, including measurements of transepithelial/endothelial electrical resistance (TEER) as measure for the integrity of the tight junctions between endothelial cells, can reveal if the co-exposure conditions indeed promote deterioration of the tight junctions, as suggested by the proteomics results.

The enhanced effect on extracellular matrix organization are further substantiated by a similar enrichment of the term cellular communication (“Cell-Cell communication” down-regulated; Appendix 2). Prominent in that term are down-regulation of proteins involved in cell junction organization, which is also enriched for single-type NP exposure conditions. Further analyses of proteome response revealed more pronounced inhibition of cellular metabolism, intracellular trafficking, and signal transduction for cells exposed only to platinum NPs, compared to other exposure conditions (“Metabolism”, “Metabolism of proteins”, “Vesicle-mediated transport”, and “Signal Transduction” down-regulated; Appendix 2). The metabolic processes negatively affected by only platinum NPs exposure and co-exposure conditions include lipid/glycogen homeostasis and carbohydrate metabolism/respiratory electron transport, respectively. These disturbances in energy metabolism are accompanied by down-regulation of cellular pathways
involved in cytoskeletal organization, intracellular trafficking, and cell division [i.e., WNT, TNF, MAPK1/MAPK3, G(12/13), NOTCH, receptor tyrosine kinases, and mTOR signaling], some of them also detected for only silver NP exposure condition. With respect to astrocytes, a lack of a significant proteome response to NP exposures might be caused by a lesser concentration of nanomaterials as discussed. This observation is also supported by studies reporting no influence, at the protein expression level, on astrocytes exposed to silver NPs grown in 3D cell cultures.39
Figure 7. Over-representation and pathway-topology analyses generated from (a) down-regulated and (b) up-regulated protein lists, obtained for the hCMEC/D3 cell line exposed to silver (green), platinum (blue), and silver/platinum (red) NPs. Relative protein expression levels displayed as a scaled colored overlay.
CONCLUSIONS

Our study provides evidence of silver/platinum NP synergistic toxicity to microvascular endothelial cells and astrocytes, both derived from the human BBB. Platinum NPs, considered to be relatively inert, greatly enhanced an overall toxic effect when added to cell cultures together with silver NPs, raising concerns on how a long-term accumulation of these nanomaterials might affect brain homeostasis. Qualitative and quantitative analyses of NP uptake did not reveal any differences in neither the intracellular trafficking nor uptake rate of nanomaterials between the single-type and co-exposure conditions. Both types of NPs were engulfed into multivesicular bodies that direct them either for enzymatic degradation inside the lysosomes or clearance from the intracellular milieu by exocytosis. The synergy effect in the endothelial cells induced by the metal NPs was accompanied by the responses at the proteome level. NP co-exposure triggered both an enhanced immune modulating response and disassembly of the extracellular matrix. In contrast, single-type NP exposure conditions induced immunosuppressive-mediated response in endothelial cells, which should be further investigated. Other changes at the proteome level include disruption in energy metabolism (for only platinum and NP co-exposure conditions), and a decrease in the rates of intercellular communication, intracellular trafficking, and cell proliferation (common to all conditions). Overall, our study raises the possibility that otherwise relatively inert NPs may induce enhanced synergistic toxicity. Further studies in this direction are clearly warranted.

MATERIALS AND METHODS

Materials. Silver and platinum NPs, citrate-coated, Biopure, 5 nm in diameter, were obtained from Nanocomposix, USA. 300-mesh formvar/carbon-coated copper grids were purchased from Agar Scientific Ltd, UK. Modified trypsin and the CellTiter 96 AQueous One Solution Cell
Proliferation assay were purchased from Promega, USA. Poros Oligo R3 reverse-phase resin was purchased from Applied Biosystems, USA. iTRAQ 8plex was obtained from ABSciex, USA. cComplete protease inhibitor cocktail was acquired from Roche, GE. 3M empore C18 disks were purchased from 3M Bioanalytical Technologies, USA. Immortalized human brain capillary endothelial cell line (hCMEC/D3), EndoGRO-MV Complete Culture Media kit (Cat. No: SCME004), fibroblast growth factor (Cat. No: GF003AF-MG), and Collagen Type I, Rat Tail (Cat. No: 08-115) were purchased from Millipore, USA. Primary human astrocytes and Poly-L-Lysine (PLL), 1 mg/mL (Cat. No: 0403) were obtained from ScienCell, USA. Astrocyte Medium (Cat No: A1261301) and Nunc 6- and 96-well plates were acquired from Thermo Fisher Scientific, USA. T75 cell culture flasks were purchased from VWR, Denmark. All the remaining reagents and materials used in the experiments were obtained from Sigma–Aldrich, Denmark.

**NP characterization.**

**TEM.** NPs dispersed in 2 mM citrate buffer at a concentration of 1 mg mL$^{-1}$ were dried on a 300-mesh formvar/carbon-coated copper grid. Imaging was performed using a JEM 1400 Plus TEM (JEOL, Germany) equipped with the bottom-mounted digital camera, Quemesa (Olympus, UK). iTEM (Olympus, UK) and Fiji (National Institute of Health, USA) softwares were used for the data acquisition and processing, respectively.

**Colloidal stability studies.** NPs at a concentration of 10 µg mL$^{-1}$ were added to EndoGRO and astrocyte cell culture media supplemented with FBS at 5% and 10%, respectively. The samples were left in the cell culture incubator at 37°C for 24 h. NP colloidal stability was then evaluated based on a visual inspection.

**Cellular experiments.**
**Cell culturing.** hCMEC/D3 cells were grown in EndoGRO-MV Complete Culture Media kit supplemented with 5% FBS (v/v) and fibroblast growth factor at 1 ng mL⁻¹. Astrocytes were cultured in astrocyte medium consisting of DMEM, N-2 supplement, and FBS (10% v/v). Endothelial cells and astrocytes were grown on the surfaces of T75 flasks pre-coated for 1 h at 37°C with collagen (219 µg mL⁻¹) and PLL (15 µg mL⁻¹), respectively. Cell cultures were maintained at 37°C, 5% CO₂, and passaged in accordance with the manufacturer’s instructions. Cells used for the experiments corresponded to passage numbers from 25–32 and 2–5 for hCMEC/D3 cells and astrocytes, respectively. Three biological replicates per condition were prepared in each cellular experiment.

**Cytotoxicity assay.** Cells were seeded in 96-well plates pre-coated with collagen or PLL at densities of 5×10³ or 7×10³ cells per well for hCMEC/D3 and astrocytes, respectively. After reaching 80% confluency, cells were exposed to NPs in the following manner: NP stocks (1 mg mL⁻¹) were sonicated for 5 min in an ultrasonication bath, and different dilutions of NPs (10× more concentrated than used for the final exposure) in 2 mM citrate buffer were prepared. NPs were then pre-coated with FBS (at 5% and 10% depending on the cell line) for 3 min and the astrocyte cell culture media was added at the end (Table 2).

**TABLE 2.** Experimental design used for testing the synergy effect derived from silver and platinum NPs on hCMEC/D3 and astrocyte cell lines.

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2.5 | 50
10<sup>a</sup> | 70<sup>a</sup>
25 | 90
50 | 100

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</table>

<sup>a</sup>The combination of synergistic NP concentrations (c<sub>s</sub>) chosen for further synergy effect investigations performed under short-term exposure conditions (t<sub>e</sub>); 15- and 30-min incubation period for hCMEM/D3 cells and astrocytes, respectively.

The cell culture media used for culturing cells was removed from the plate and new media containing NPs was added to the wells. Cells were incubated with NPs for 24 h at 37°C, 5% CO<sub>2</sub>. The shorter exposure times (t<sub>e</sub>; up to 1 h) were additionally tested for the synergistic NP concentration (c<sub>s</sub>) condition to establish the time points with approximately 60% cell viability (Table 1). After incubation, cells were washed 1× with PBS, and 100 µL of the cell imaging media was added to the wells. The background readout of the absorbance was then performed at
490-nm wavelength using a FLUOstar Omega microplate reader (BMG Labtech, USA). Next, 20 µL of MTS solution, as recommended by the manufacturer, was added to the wells for 90 min and 4 h for hCMEC/D3 cells and astrocytes, respectively. The final absorbance readout for the samples was then conducted. Each condition was prepared in triplicate and the experiment was repeated independently 3 times. The synergistic effect was calculated as per the Chou–Talalay method with minor changes. Briefly, absorbance measurements were recalculated into the survival rate and the parameters for the dose response curves of individual methods were calculated. All data processing was performed using Python (3.6) scripting language.

**Intracellular trafficking studies.** Cells were seeded on a surface of a 6-well plate pre-coated with collagen and PLL at a density of 2.5 × 10⁵ and 3.5 × 10⁵ cells/well for hCMEC/D3 cells and astrocytes, respectively. Co-exposure to NPs pre-coated with FBS was performed when cells reached approximately 80% confluency (Table 2). The total volume of the cell culture media containing NPs was 1 mL. Cells were then washed 1× with PBS, detached from the wells with trypsin, and centrifuged at 200× rcf for 5 min in the cell culture media corresponding to each cell line supplemented with FBS. Cells were re-suspended in 1 mL of media and transferred into Eppendorf tubes. After centrifugation at 100× rcf for 5 min, supernatants were carefully removed and 1 mL of glutaraldehyde solution (2 % w/v in 0.04 M phosphate buffer) was added dropwise above the cell pellets. After 1 h fixation at room temperature, the glutaraldehyde was removed and the cell pellets were stored in 0.1 M phosphate buffer at 4°C overnight. Cells were then centrifuged at 3000× rcf for 5 min, placed for 1 h in 2% glutaraldehyde solution, and rinsed 1× in 0.1 M phosphate buffer. After rinsing, cells were post-fixed with osmium tetroxide (1% w/v in 0.1 M phosphate buffer) for 90 min at 4°C. After post-fixation, samples were rinsed 1× in 0.1 M phosphate buffer and 2× with sterile water at 4°C, dehydrated through a series of ethanol
gradients at 4°C to 20°C, infiltrated with a graded mixture of acetone and Epon at 20°C, and then embedded in Epon at 30°C. Then, the Epon was polymerized at 60°C overnight and cut into thin sections (60 nm) with a ultramicrotome (Leica, Germany). Images were acquired in the same manner as described in the NP characterization / TEM section.

**Sample preparation and chemical labeling for proteomics analysis.** Cells were seeded in 6-well plates pre-coated with collagen and PLL at a density of $2 \times 10^5$ and $3 \times 10^5$ cells/well for hCMEM/D3 cells and astrocytes, respectively. NP exposure was initiated when cells reached 80% confluency (Table 2). The total volume of the cell culture media containing NPs was 1 mL. Cells were then washed 1× with PBS, scraped from wells in a presence of protease inhibitor cocktail (1 mL/well, cOmplete, Roche) on ice, and pelleted by centrifugation at 200× rcf for 5 min. The protein extraction and solubilization for both cell lines were performed using the lysis buffer (6 M urea, 2 M thiourea, 10 mM dithiothreitol, and 1% protease inhibitors cocktail stock solution). The cell pellets were kept in the lysis buffer for 3 h at room temperature. Next, the lysates were diluted 10× with 2 mM triethylammonium bicarbonate adjusted to pH 7.5, and cells were further lysed using probe-sonication on ice. Next, iodoacetamide was added to a final concentration of 20 mM and incubated for 30 min in the dark. After the incubation, sequential digestion of samples with LysC (Wako) and then trypsin (Promega) was performed at room temperature for 2 h and overnight, respectively. The enzyme to protein ratio was 1:50 in both cases. The peptides were purified using C18 microcolumns filled with POROS oligo R3 and R2 bulk resins (at a 1:1 v/v ratio), and dried before labeling with iTRAQ 8plex kit. The samples were labeled as follows: astrocytes: 113, Control; 114, AgNP; 115, PtNP; 116, AgNP+PtNP; endothelial cells: 117, Control; 118, AgNP; 119, PtNP; 121, AgNP+PtNP. Three replicates were labeled in parallel using one iTRAQ kit for each. Labeled peptides from both cell lines were
fractionated by high-pH reverse-phase HPLC. An UltiMate 3000 UPLC system (Thermo Scientific, Germering, Germany) equipped with an Acquity CSH C18 column (300 μm × 100 mm, 1.7 μm) was employed. Samples were fractionated with a gradient from 2% to 69% B over 160 min (phase A: 20 mM ammonium formate, adjusted to pH 9.2 by NH₄OH; phase B: 80% acetonitrile 20% 20 mM ammonium formate, adjusted to pH 9.2 by NH₄OH). Forty preliminary fractions were pooled into 20 final fractions. Peptides eluted with 2% B (flow-through) were collected as a separate fraction. Peptide fractions were lyophilized in a vacuum centrifuge prior to LC–MS analysis.

**LC–MS/MS analysis.** Each peptide fraction was concentrated on a pre-column (μ-Precolumn C18 PepMap100, 5 μm, 300 μm i.d. × 5 mm, 100 Å, Thermo Scientific). Peptides were then separated with the analytical column (33 cm × 75 μm i.d.) home-packed with 2-μm C18 beads (Inertsil ODS-3, GL Sciences) with a gradient of 2% to 47% of buffer B over 60 min (A: 0.1% formic acid; B: 95% acetonitrile, 0.1% formic acid), 275 nL min⁻¹. An Ultimate 3000 UPLC system (Thermo Scientific, Germering, Germany) connected to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher scientific, Bremen, Germany) was employed for the analysis. The mass spectrometer was operated in Top Speed mode with a 3-s cycle time. Acquisition parameters were as follows. MS1: mass range, 350 to 1500; resolution: 120,000 at 200 Th; maximum injection time: 50 ms; AGC target: 4e5. MS2: resolution, 30,000 at 200 Th; maximum injection time, 54 ms; AGC target, 5e4. Data dependent acquisition parameters: isolation window width, 0.7 Th with 0.1 Th offset; activation type, HCD; stepped collision energy, 40 ± 5 NCE units; dynamic exclusion duration, 20 s.

**Database search.** Raw files were searched against the human proteome database (NCBIInr) using Mascot (v 2.4) and Sequest HT as implemented in Proteome Discoverer 2.1 (Thermo
Fischer, Germany). The search parameters were: carbamidomethylation of cysteines, iTRAQ 8plex labeling of N-terminus and lysine as fixed modifications, and oxidation of methionine as variable modification; maximum of 2 missed cleavage; 10-ppm precursor mass tolerance, 0.02 Th fragment mass tolerance. Search results were validated using Percolator, as supplied with Proteome Discoverer 2.1. PSMs, peptides, and proteins were filtered for FDR < 0.01. Quantification was performed by Proteome Discoverer using default settings for reporter ion quantitation.

**Statistical analysis.** The PSM quantitation data was exported to InfernoRDN software. Quantitative values for unique peptides were calculated as median values of the corresponding PSMs. Peptide abundancies were log2-converted and normalized using the median. Normalized peptide abundancies were submitted to the RRollup module of InfernoRDN to merge the peptides into proteins using the mean summarization with a minimum of 3 peptides per protein. The significance of the protein abundance changes was calculated using the LIMMA package with a 0.05 FDR cutoff. Venn and pathway–topology diagrams were then generated using InteractiVenn and Reactome software, respectively.

**Quantification of NP uptake.** Cells were seeded on a surface of a 12-well plate pre-coated with collagen and PLL at a density of $1.5 \times 10^5$ and $2 \times 10^5$ cells/well for hCMEM/D3 cells and astrocytes, respectively. When cells reached 80% confluency, they were exposed to NPs in accordance with the co-exposure conditions described in Table 2. The total volume of the cell culture media containing NPs was 0.5 mL. Next, cells were washed 3× with PBS, detached from the wells with trypsin, and centrifuged at 200× rcf for 5 min in the cell culture media corresponding to each cell line supplemented with FBS. After removing the supernatants, cells with NPs were dissolved in 69% nitric acid (v/v), overnight at 4°C. Next, samples were further
diluted in DI water up to 1% nitric acid (v/v) and analyzed using an ICP–MS (Bruker 820-MS) coupled to an SPS 3 autosampler.

ASSOCIATED CONTENT

Supporting Information

Composition of EndoGRO and DMEM cell culture media with the supplements (Appendix 1). Significantly enriched reactome pathways with corresponding gene names, Uniprot IDs and reactions following NP single-type and co-exposure conditions (Appendix 2). List of all proteins identified in the study (Appendix 3). This material is available free of charge via the Internet at http://pubs.acs.org

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The authors declare no competing financial interest.

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REFERENCES


36. Casals, E.; Pfaller, T.; Duschl, A.; Oostingh, G. J.; Puntes, V. F., Hardening of the nanoparticle-protein corona in metal (Au, Ag) and oxide (Fe3O4, CoO, and CeO2) nanoparticles. *Small* 2011, 7 (24), 3479-86.


