Supplementary material

Materials and Methods

AgNP synthesis
The Ag nanoparticles (AgNPs) were synthesized by reduction of Ag⁺ to AgNPs with sodium borohydride (NaBH₄). The bifunctional ligands of the type XRSH (X = COOH and CH₃; R = undecyl or PEG) were added to provide an efficient stabilization. These syntheses were previously reported in the literature¹⁻³ with slight modifications and variations as indicated below. Note that the thiols used in these AgNP syntheses lose the thiol hydrogen atom upon coordination to the AgNP surface, presumably upon oxidative addition of the S-H bond onto surface Ag⁰ atoms followed by reductive elimination of two such hydride ligands to H₂, so that the final thiolate ligands are bonded to Ag¹ atoms of the AgNP surface. All the AgNPs were further purified by dialysis from aqueous nanoparticles solutions in pure water over a 7-day period during which the water was changed daily.

AgNP-thiolate-sodium 11-mercaptoundecanoate (AgCOONa NPs). 11-mercaptoundecanoic acid (654 mg, 3 mmol) in 30 mL of tetrahydrofuran (THF) was mixed with a silver nitrate solution (255 mg, 1.5 mmol) of THF/H₂O (180 mL). 15 mL of a solution of NaBH₄ in water (15 mg, 15 mmol) was added. The use of THF/H₂O (5:1) as solvent provides the right environment to allow the initial solubilization of silver nitrate and the fast precipitation of thiolate-11-mercaptoundecanoic-coated AgNPs after reduction. After precipitation of dark brown AgNPs, these AgNPs were purified three times by successive dispersion/precipitation cycles with water/THF, then dispersed in 30 mL of distilled water and annealed by refluxing for 24 h. After annealing the AgNPs, the population of small AgNPs practically disappeared. The absorption band experienced a blue shift and became narrower with the increase in the annealing time. Plasmon band: δₓₐₓ = 434 nm.

AgNP-thiolated PEG₅₅₀ (AgPEG NPs). 105 mg (0.6 mmol) of silver nitrate in 200 mL of water was added dropwise to a cold 600 mL aqueous solution of NaBH₄ (137 mg, 3.6 mmol) under stirring. At the end of the addition, stirring was stopped for 8 hr. CH₃-PEG-SH (348 mg, 0.6 mmol) in 20 mL of water was added under stirring. The solution was stored overnight. 200 mL of dichloromethane (CH₂Cl₂) was then added, and the aqueous suspension of AgNPs was salted out by addition of an excess of sodium chloride (NaCl) under stirring for 1 h. After phase separation, the organic phase was rota-evaporated to dryness, and the waxy product was dissolved in 50 mL of water. Plasmon band: δₓₐₓ = 410 nm.

Characterization of AgNPs
Determination of total Ag mass concentration in AgPEG and AgCOONa NPs stock suspension
The Ag mass concentration in AgNPs stock suspensions was determined by adding 3 mL of concentrated (68%) nitric acid (HNO₃) to 0.2 mL of 40-times diluted AgNPs suspension which was left at room temperature in the fume hood over the night. The next day, 2 mL of
concentrated (37%) hydrochloric acid (HCl) was added. An excess of chlorine (from HCl) was used to prevent the formation of insoluble AgCl precipitates (from potential traces of Cl in the suspensions) and to produce soluble AgCl₂. After digestion, the samples were diluted with 2% HNO₃ to a final volume of 10 mL, and further diluted 20-times with 2% HNO₃ prior to the determination of Ag mass concentrations by ICP-MS. Indium (PlasmaCAL, SCP Science, Baie D’Urfé, QC, Canada) was used as an internal standard at a concentration of 25 ng/mL. Instrumental parameters for the Agilent 8800 Triple Quadrupole ICP-MS instrument (Agilent Technologies, California, USA) used in single-quad mode are given in Table S3. Instrument tuning was performed prior to analysis by using a tuning solution according to the manufacturer’s recommendation. Since there are no AgNPs reference materials for ICP-MS measurements, the best indication that the digestion procedure and ICP-MS results were accurate was the comparison with the Ag concentration determined for 30 nm AgPVP NPs (104% agreement with the concentration provided by supplier).

**Determination of the dissolved Ag fraction for AgPEG and AgCOONa NPs in stock suspensions**

For determination of the dissolved Ag fraction, the AgPEG and AgCOONa NP stock suspensions were diluted 125- and 1000-times, respectively, with ultrapure water (UPW). In order to separate the Ag ions from particulate AgNPs, 500 µL of suspension were filtered through a micro filter with 10 kDa cut-off polyethersulfone (PES) membrane (VWR International, PA, USA) and centrifuged for 25 min at 11,000 x g centrifugal force with the use of a micro centrifuge (Eppendorf MiniSpin, Eppendorf AG, Hamburg, Germany). The filtrates were appropriately diluted with 2% HNO₃ prior the determination of Ag mass concentration by ICP-MS. The Ag mass concentration was also determined in the initial AgNPs suspensions (before filtration) in order to determine the percentage of Ag ions passing through the filters. All the experiments were performed in duplicates. To determine the recovery of the filtrations, 500 µL of 100 ng/mL ionic Ag standard (pH of 5) prepared in UPW was filtered in the same way as the AgNPs suspension and Ag concentration in the filtrate determined by ICP-MS. The recovery was calculated to be 96.1 ± 1.5%.

**TEM analysis**

The size of the AgNPs was determined by transmission electron microscopy (TEM) using a Tecnai G2 T20 instrument (FEI, Eindhoven, The Netherlands) operated at 200 kV accelerating voltage. A drop (10 µL) of diluted AgNPs suspensions (~ 20 µg/mL) was applied to a 200 mesh Formvar/carbon-coated copper grid (FCF-200-Cu, FORMVAR CARBON FILM, Electron Microscopy Science, USA). The size of constituent primary particles and agglomerates/aggregates was estimated upon visual inspection of the images.

**Measurement of zeta potential**

Prior to the zeta potential measurement by laser Doppler velocimetry (Zetasizer, Malvern Instruments, UK), using a disposable zeta cell (DTS1070, Malvern Instruments, UK), AgNP stock suspensions were diluted in UPW to an approximate Ag mass concentration of 50
µg/mL. The results are shown as mean ± 1 standard deviation (STD) of five repeated measurements of one sample.

Thermogravimetric analyses
Thermogravimetric analyses (TGA) were performed to characterize the functionalization of AgNPs. This technique depends on the thermal decomposition of the surface groups under inert atmosphere. TGA curves of AgNPs samples experienced two main weight loss downs through the TGA test. The first weight loss happened at 25-100°C due to the water evaporation and this was quantified as the % of humidity. The second main mass loss was due to the combustion of organic functional groups. TGA analyses of AgNPs were performed with TGA 4000 (Perkin Elmer) under a nitrogen (N₂) flow of 20 mL/min from 25°C to 995°C at a heating rate of 10°C/min.

Endotoxin content
Endotoxin content was evaluated using the QCL-1000™ Endpoint Chromogenic LAL Assay (Lonza) protocol and performed as described previously.

Determination of Ag concentration in perfusates by conventional ICP-MS
Sector field (SF)-ICP-MS analysis using a Sectorfield ICP mass spectrometer (Series 2, Thermo Fisher Scientific GmbH, Germany) was applied for determination of the total Ag mass concentration in the perfusates from all the perfusion experiments (samples I) except for the perfusates from one perfusion experiment with AgPEG NPs and two perfusion experiments with AgNO₃ (samples II), where an Agilent 7900 ICP-MS (Agilent Technologies, California, USA) was used. Instrumental settings for Thermo Fisher Scientific SF-ICP-MS and Agilent 7900 ICP-MS are given in Table S4. The analyses of samples I were performed as follows: 0.25 to 0.5 g of sample were weighted in a quartz vessel and 0.6 mL of HNO₃ and 1.8 mL of HCl were added. The samples were dissolved by microwave-assisted acid digestion (turboWAVE single reaction chamber, MWS GmbH, Heerbrugg, Switzerland). The samples were heated with the following program (2 min heating up to 80°C, 10 min at 250°C, 8 min at 250°C then cooling down to room temperature). The digested samples were then filled up to 10 mL with UPW. Prior to the quantification of Ag content by SF-ICP-MS against an external calibration curve with matrix matched standard solutions from 0 to 50 µg/L Ag, the digests were appropriately diluted with UPW. All measurement aliquots and stock suspensions were spiked with the internal standard Rh (final concentration: 10 µg/l) and diluted to approximately 9% HCl and 7% HNO₃ in solution. The quantification was carried out by external matrix matched six-point-calibration with internal standard correction. Silver has to show linearity (from limit of detection to 50 ng/ml) with a correlation coefficient higher than 0.995. The intra-assay and inter-assay inaccuracies, measured as the variation coefficient, should be under 10%. To address different performance characteristics and quality control aspects, three types of experiments were included: 1) To gather details about inhomogeneity in the perfusion medium, samples were analyzed as independent duplicates. Variations between duplicate samples were small (RSD< 5%). 2) The matrix effects were
studied by standard addition experiments (spiking of perfusion media with known concentrations of 5 – 10 μg/L of the silver standard solution) and calculation of the recovery. Determined recoveries were between 95-100% (data not shown). 3) Selected perfusion media were digested and measured in an interlaboratory comparison. Similar values were obtained in three different labs (data not shown).

The analyses of samples II were performed as follows: 0.25 g of sample was weighted in a Teflon vessel and 3 mL of HNO₃ (65%) and 3 mL of HCl (30%) were added. The samples were dissolved by microwave-assisted acid digestion (Mars 6, CEM, NC, USA). The samples were heated with the following program (15 min heating up to 140°C, 3 min at 140°C, 15 min heating up to 180°C, 15 min at 180°C then cooling down to room temperature). The digested samples were then filled up to 20 mL with UPW. Prior to the quantification of Ag content by ICP-MS against an external calibration curve with matrix matched standard solutions from 0 to 100 μg/L Ag and with the use of In as internal standard, the digests were appropriately diluted with UPW. All digestions were performed in duplicate. For evaluation of the accuracy of the analytical method (digestion procedure + ICP-MS analysis), 0.25 g of perfusion medium was spiked with ionic Ag standard solution to a concentration of 20 μg/g. Spiked samples were digested in exactly the same way as the perfusate samples and full recoveries (100.5% ± 1.1%, N=6) were achieved.

MTS viability assay
The in vitro cytotoxicity of the AgNPs was assessed in BeWo cells using the MTS viability assay. BeWo cells were used as a model of the syncytiotrophoblast which first gets into contact with AgNPs in the ex vivo placenta perfusion model. BeWo cells (b30 clone) were obtained from U. Graf-Hausner (Zurich University of Applied Sciences, Waedenswil, Switzerland) with permission from A.L. Schartz (Washington University School of Medicine, MO, USA). BeWo cells were cultured in Ham’s F-12K medium (Gibco, Thermo Fisher Scientific Inc.) supplemented with 1% penicillin-streptomycin and 10% fetal calf serum at 37°C and 5% CO₂ and sub-cultured twice a week using trypsin-EDTA solution. For MTS viability assays, BeWo cells were seeded in a 96-well plate (8000 cells per well) 24 h and subsequently treated with different concentrations of Ag NPs. As negative control, cells without treatment were used and as positive control 1, 10, 100 and 1000 μM CdSO₄ was applied. After 6 or 24 h of incubation at 37 °C and 5% CO₂, an MTS assay (CellTiter96® AQueous One Solution Cell Proliferation Assay; Promega) was performed according to the manufacturer’s instructions. Results were presented as mean percentage of the untreated control from three independent experiments.

CytoViva Hyperspectral microscopy
The CytoViva® Hyperspectral Imaging (HIS) system was used to investigate the presence of AgNPs in the placental tissue. Dark field images were captured at 60x magnification using the CytoViva® 150 Unit integrated onto the Olympus BX43 microscope. Images were
captured using the Dagexcel X16 camera and DAGE Exponent software. HSI was performed at 60x magnification using the HSI system 1.1 and ENVI software.

To confirm if the particulates seen were AgNPs, a drop of AgNPs in UPW was placed onto ultra clean glass slides, covered with a coverslip and allowed to dry. Dark field images and HSI was performed as previously described. The HSI was then used to establish a spectral library by randomly selecting spectra of AgNPs, where the each spectrum represents a single pixel obtained from the HSI scan. These spectral profiles were compared to those of the AgNPs found within the tissue. The image classification algorithm SAM (Spectral Angle Mapper), an analysis tool of the ENVI software, was used to identify and confirm the presence of AgNPs visualised in the tissue. This was achieved by using the previously created spectral libraries, which was mapped to known spectra in HSI scans of placental tissue.
Fig. S1 Perfusion profiles of Ag during placental perfusion with AgPEG NPs and AgCOONa NPs. Ag mass concentrations were determined by splCP-MS (dashed lines) and conventional ICP-MS (continuous lines). Presented are Ag concentrations measured on the maternal (black color) and fetal side (gray color) of the placenta over a time period of 6 h. Data represent the mean ± STD of the independent experiments (N=3 (splCP-MS) and N=3 (ICP-MS) for AgPEG NPs, N=3 (splCP-MS) and N=3 (ICP-MS) for AgCOONa NPs). Asterisks (*) denotes statistically significant difference between Ag mass concentrations determined by two different ICP-MS methods at the same time point (p < 0.01).
Fig. S2 TEM images of AgPEG (top) and AgCOONa NPs (bottom) at two magnifications

Fig. S3 Size distributions of AgNPs determined in the perfusion medium from the fetal (A) and maternal side (B) of placenta after 6 h of perfusion with AgPEG NPs, AgCOONa NPs and AgNO$_3$. Size distributions were determined by spICP-MS and are representative of three independent perfusion experiments (N=3 for AgPEG NPs, AgCOONa NPs and AgNO$_3$).
Fig. S4 Dark-field images of placental tissue perfused with AgNPs. Dark-field images were captured at 60x magnification of placental tissue before perfusion (0h) or after 6 h of perfusion with perfusion media (control), AgPEG NPs and AgCOONa NPs. Scale bar represents 15 µm
**Fig. S5** Spectral profile of AgCOONa and representative images of SAM analysis. (a) Spectral profile of AgCOONa NPs collected from (b) HSI scan. Fifteen spectral profiles of randomly selected AgNPs, where each coloured line represents the spectrum from a single pixel. (c) HSI scan of placental tissue after 6 h of *ex vivo* perfusion with AgCOONa NPs. (d) SAM image indicating pixels of AgCOONa NPs that matched the spectral library.
**Fig. S6** Spectral profile of AgPEG and representative images of SAM analysis. (a) Spectral profile of AgPEG NPs collected from (b) HSI scan. Fifteen spectral profiles of randomly selected AgNPs, where each coloured line represents the spectrum from a single pixel. (c) HSI scan of placental tissue after 6 h of *ex vivo* perfusion with AgPEG NPs. (d) SAM image indicating pixels of AgPEG NPs that matched the spectral library.
**Fig. S7** DLS hydrodynamic size measurements of ionic Ag in PM. Presented are intensity-based size distributions of PM and different concentrations of ionic Ag in PM (100 µg/mL, 10 µg/mL and 1 µg/mL). Samples were analysed immediately after their preparation.

**Fig. S8** Effects of Ag NPs on BeWo cell viability. Cell viability of BeWo cells was assessed by MTS assay 6 and 24h after treatment with the indicated concentrations of NPs. CdSO\(_4\) was used as a positive control. Optical density was measured at 490 nm and values are blank corrected as well as normalized to the untreated samples. Data represent mean ± SEM of 3 individual experiments.
Tables

Table S1  ICP-MS settings for single particle analysis

<table>
<thead>
<tr>
<th>Parameter/ICP-MS type</th>
<th>Thermo Fischer Scientific iCAP Q</th>
<th>Agilent 8800 Triple Quadrupole/7900</th>
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<tr>
<td>Plasma power</td>
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<tr>
<td>Plasma gas flow rate</td>
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<tr>
<td>Carrier gas flow rate</td>
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<tr>
<td>Sample uptake flow rate</td>
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<tr>
<td>Isotopes monitored</td>
<td>¹⁰⁷Ag, ¹⁹⁷Au</td>
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</tr>
<tr>
<td>Integration time per isotope</td>
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<tr>
<td>Analysis time</td>
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<td>Nebulizer type</td>
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<td>MicroMist</td>
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<td>Spray chamber type</td>
<td>Quartz cyclonic type, Peltier-</td>
<td>PFA Scott chamber, Peltier- cooled</td>
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<tr>
<td></td>
<td>cooled</td>
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\(^a\) Determined on daily basis

Table S2  List of samples, collected from perfusion experiments, with dilution factors and total measurement times for spICP-MS analysis

<table>
<thead>
<tr>
<th>Perfusion experiment</th>
<th>Ag mass concentration dosed into maternal circulation</th>
<th>Dilution factor</th>
<th>Total measurement time (min)</th>
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<tr>
<td></td>
<td>maternal perfusates</td>
<td>fetal perfusates</td>
<td>maternal perfusates</td>
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<tr>
<td>AgPEG NPs</td>
<td>12.5 µg/mL</td>
<td>100,000</td>
<td>100</td>
</tr>
<tr>
<td>AgCOONa NPs</td>
<td>39.3 µg/mL</td>
<td>500,000</td>
<td>100</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>1.0 µg/mL</td>
<td>10,000</td>
<td>100</td>
</tr>
<tr>
<td>Control(^a)</td>
<td>0 µg/mL</td>
<td>/</td>
<td>/</td>
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\(^a\) Samples from the control experiment were not measured by spICP-MS because the results from conventional ICP-MS showed that no Ag (< 0.001 µg/mL) was present.
Table S3  ICP-MS settings for the determination of Ag mass concentration in the digested AgNPs

<table>
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<th>Parameter</th>
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<tr>
<td>Plasma gas flow rate</td>
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<tr>
<td>Carrier gas flow rate</td>
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<tr>
<td>Sample uptake flow rate</td>
<td>1.0 mL/min</td>
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<tr>
<td>Isotopes monitored</td>
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</tr>
<tr>
<td>Integration time per isotope</td>
<td>300 ms</td>
</tr>
<tr>
<td>Nebulizer type</td>
<td>MicroMist</td>
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<td>Spray chamber type</td>
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Table S4  ICP-MS settings for the determination of total Ag mass concentration in the digested perfusates

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<th>Parameter/ICP-MS type</th>
<th>Thermo Fisher Scientific SF-ICP-MS</th>
<th>Agilent 7900 ICP-MS</th>
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<td>Sample gas flow rate</td>
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<tr>
<td>Sample uptake flow rate</td>
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<tr>
<td>Isotopes monitored</td>
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<tr>
<td>Integration time per isotope</td>
<td>150 ms</td>
<td>300 ms</td>
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<tr>
<td>Nebulizer type</td>
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<td>MicroMist</td>
</tr>
<tr>
<td>Spray chamber type</td>
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<td>PFA Scott chamber, Peltier-cooled</td>
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References: