

Supplementary Material: Physicochemical Transformations of Silver Nanoparticles in the Oro-Gastrointestinal Tract Mildly Affect Their Toxicity to Intestinal Cells, *In Vitro*: An AOP-Oriented Testing Approach

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Table S1. Composition of simulated OGI fluids. ¹

| Title 1 | Simulated Saliva (pH 6.5±0.1) | Simulated gastric fluid (pH 1.4±0.1) | Simulated intestinal fluid (pH 8.1±0.1) |
|-------------------------------|--|--|---|
| Inorganic components (g/L) | KCl 0.90 KSCN 0.20 NaH ₂ PO ₄ x H ₂ O 1.02 Na ₂ SO ₄ 0.57 NaCl 0.30 | KCl 0.82 NH ₄ Cl 0.31 CaCl ₂ x 2H ₂ O 0.40 NaCl 2.75 NaH ₂ PO ₄ x H ₂ O 0.31 | MgCl ₂ x 6H ₂ O 0.05 KCl 0.94 KH ₂ PO ₄ 0.08 NaHCO ₃ 9.17 NaCl 12.27 CaCl ₂ x 2H ₂ O 0.42 |
| Organic components (g/L) | Urea 0.20 | Urea 0.09 D-Glucose 0.65 Glucuronic acid 0.02 D-Glucosamine hydrochloride 0.33 | Urea 0.35 |
| Active components (g/L) | Mucin 0.05 Uric acid 0.016 a-amylase 0.145 | Mucin 3 BSA 1 Pepsin 1 | Pancreatin 3.00 Lipase 0.5 Bile 6.00 BSA 2.8 |

These compositions are derived from previously published studies by Sohal et al. and Marucco et al. [1,2].

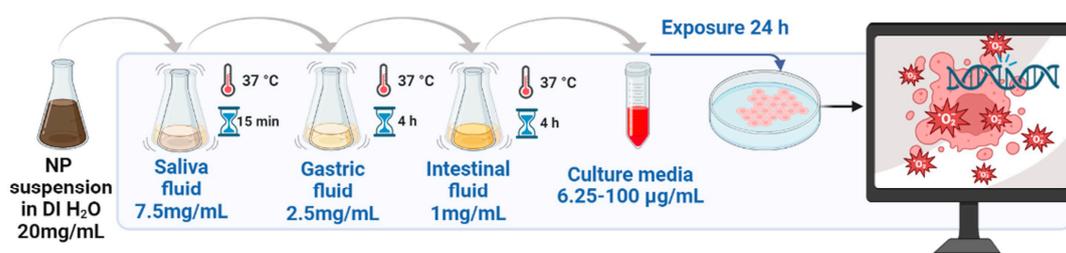


Figure S1. Schematic representation of the simulated digestion cascade. The three tested Ag NMs, as well as HEC powder were digested *in vitro* according to Sohal et al. and Marucco et al. [1,2]. Briefly, they were suspended in ultrapure water and vortexed and/or sonicated, then diluted in saliva fluid and incubated at 37 °C for 15 min, then diluted in gastric fluid and incubated at 37 °C for

4 h, then diluted in intestinal fluid and incubated at 37 °C for 4 h. At the end of this digestion procedure, samples were diluted in cell culture medium previously to cell exposure.

Table S2. Primer sequences for qPCR experiments.

| Gene | Reverse primer | Forward primer |
|--------|-------------------------------|---------------------------------|
| GAPDH | TTG-AT-TTG-GAG-GGA-TCT-CG | GAG-TCA-ACG-GAT-TTG-GTC-GT |
| CycloA | CG-AGT-TGT-CCA-CAG-TCA-GC | TCG-AGT-TGT-CCA-CAG-TCA-GC |
| CAT | TCC-AAT-CAT-CCG-TCA-AAA-CA | AGC-TTA-GCG-TTC-ATC-CGT-GT |
| SOD2 | TCT-TGC-TGG-GAT-CAT-AG-GG | TCC-ACT-GCA-AGG-AAC-AAC-AG |
| IL8 | CA-ACT-ACG-GT-GCC-AGA-TTT-AAC | GAA-TGG-GTT-TGC-TAG-AAT-GTG-ATA |
| GSR | CTT-AGA-ACC-CAG-GGC-TGA-CA | GAT-CCC-AAG-CCC-ACA-AA-GA |
| GCLM | ACA-CAG-CAG-GAC-GCA-AGA-TT | AGT-CCT-TGG-AGT-TGC-ACA-GC |
| HO-1 | GGC-ATA-AAG-CCC-TAC-AGC | TTC-TTC-GAT-GGG-TCC-TTA-CAC |
| MT-1 | TGA-CGT-CCC-TTT-GCA-GAT | GCT-TCT-CCT-TGC-CTC-GAA |
| MT-2 | TCT-TCA-GCT-CGC-CAT-GGA-T | TGC-ATT-GC-ACT-CTT-TGC-AT |

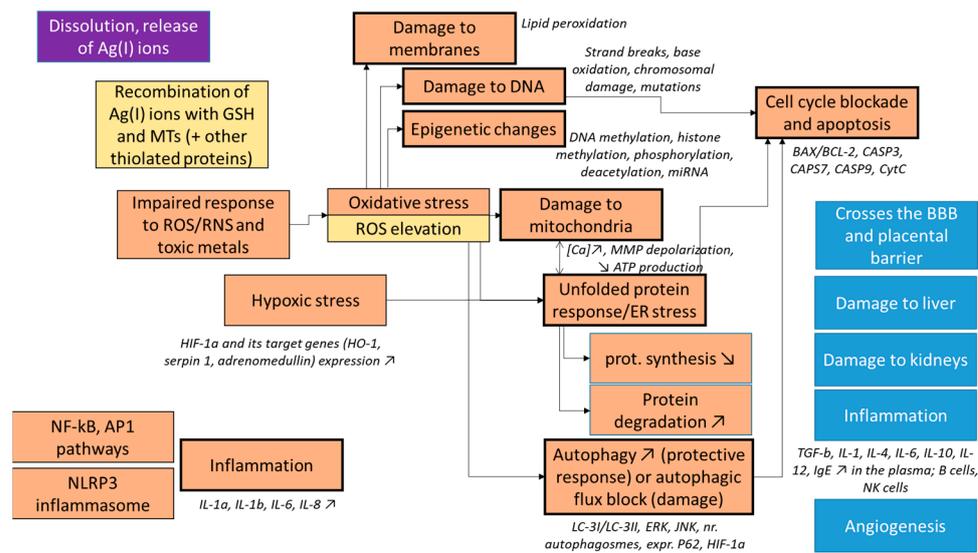


Figure S2. Toxicity mechanisms reported in the literature for Ag NPs. The potential molecular initiating event (MIE) is represented in purple, then molecular mechanisms are represented in yellow, cellular mechanisms in orange, and mechanisms at the organismal level in blue.

Table S3. Size distribution and zeta potentials of non-digested and digested Ag NPs in filtered fluids.¹

| | AgNKD | | | AgPVP | | |
|----------------------|-----------|-----------|-------------|-----------|-----------|-------------|
| | Size (nm) | PdI | ZP (mV) | Size (nm) | PdI | ZP (mV) |
| <i>Non-digested</i> | | | | | | |
| Saliva | 743±47 | 0.46±0.03 | -30±1.22 | 213±22 | 0.39±0.03 | -22.60±0.26 |
| Gastric Fluid | 979±137 | 0.50±0.01 | -0.54±0.13 | 606±105 | 0.50±0.08 | -4.03±0.27 |
| Intestinal Fluid | 1157±75 | 0.52±0.01 | -19.63±1.90 | 572±89 | 0.60±0.10 | -16.07±0.68 |
| Culture Medium t 0h | 936±127 | 0.60±0.02 | -9.27±0.57 | 536±50 | 0.61±0.07 | -8.93±0.28 |
| Culture Medium t 24h | 1024±129 | 0.76±0.10 | -9.31±0.69 | 324±86 | 0.58±0.20 | -8.98±0.43 |
| <i>Digested</i> | | | | | | |
| Saliva | 578±71 | 0.61±0.01 | -25.8±0.7 | 152±17 | 0.51±0.09 | -23.77±0.67 |
| Gastric Fluid | 1185±27 | 0.60±0.02 | -0.97±0.12 | 1289±07 | 0.59±0.04 | -0.95±0.11 |
| Intestinal Fluid | 274±18 | 0.64±0.01 | -23.17±0.31 | 212±10 | 0.39±0.01 | -8.97±0.78 |
| Culture Medium t0h | 454±30 | 0.61±0.05 | -8.78±0.72 | 367±23 | 0.58±0.01 | -9.44±0.21 |
| Culture Medium t24h | 286±14 | 0.57±0.03 | -7.89±0.85 | 231±21 | 0.47±0.02 | -10.17±0.31 |

¹ Average Hydrodynamic Size, Polydispersity Index (PDI) and ζ Potential (ZP) of AgNKD, and AgPVP, in the three simulated, filtered human OGI fluids (saliva pH 6.5, gastric fluid pH 1.4, intestinal fluid pH 8.1) and culture medium (McCoy's 5A media 1%FBS, pH 7.4) at final concentrations of 50 $\mu\text{g}/\text{mL}$. DLS measurements are the mean of at least 3 runs each containing 20 submeasurements. All data are presented as mean of three independent characterizations \pm SD. The size distribution and zeta potential of AgHECp in filtered fluids could not be measured due to limited amount of sample.

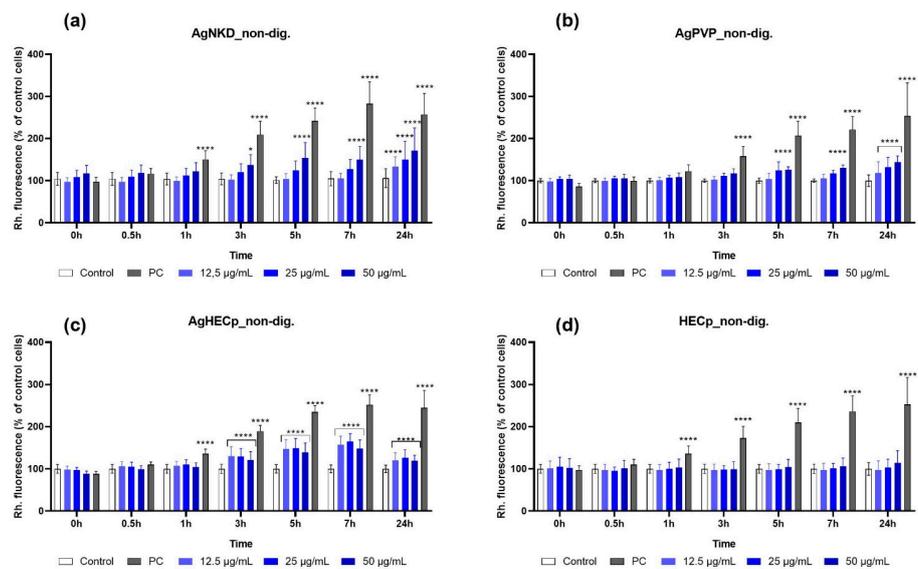


Figure S3. ROS intracellular accumulation of non-digested Ag NMs treated conditions. ROS intracellular content was assessed using DHR123 assay 24 h after HCT116 cells were exposed to non-digested Ag NKD (a), AgPVP (b), AgHECp (c) and HECp (d) at the indicated concentrations. Positive control (PC) refers to Luperox 250µM. Values are the mean ± SD of three independent experiments with 5 replicates per experiment. Statistical significance, exposed vs. control, (*) $p < 0.05$, (***) $p < 0.0001$.

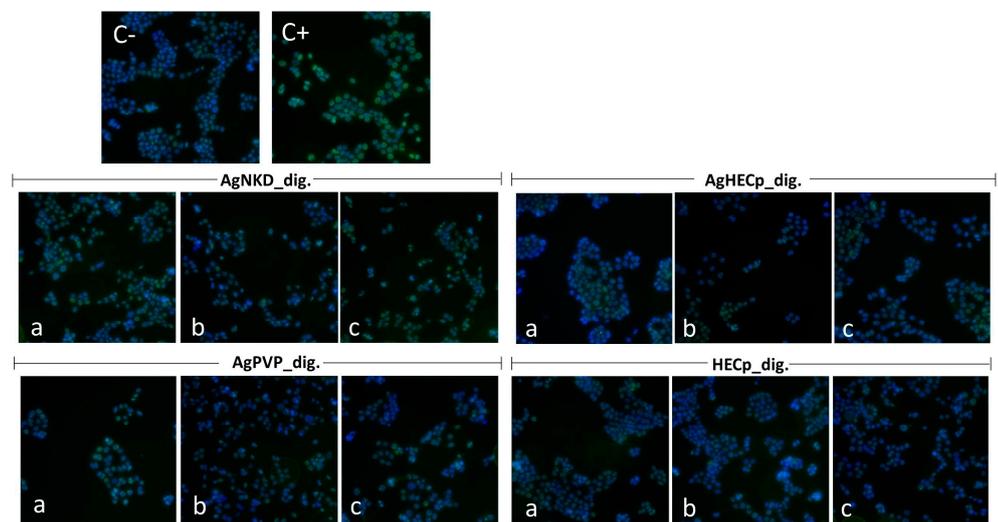


Figure S4. Double strand break level measured via 53BP1 immunostaining and foci count, using high content analysis. Double strand breaks in DNA were assessed via immunostaining and counting of 53BP1 foci, in control cells (C-), cells exposed to 50 µM of etoposide (C+), or 12.5 µg/mL (a), 25 µg/mL (b) and 50 µg/mL (c) of indicated samples for 24 h. Blue fluorescence corresponds to staining of nuclei, and green fluorescence corresponds to staining of 53BP1. 53BP1 foci counting, assessing double-strand DNA breaks.

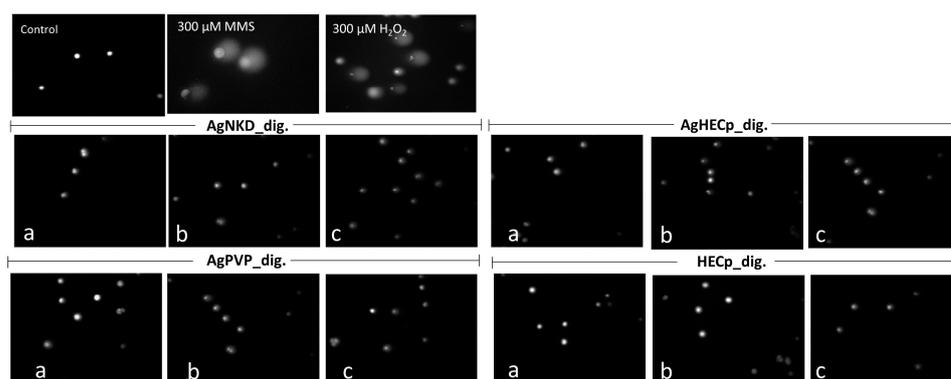


Figure S5. Comet assay representative images. Images were captured at 100× magnification on HC116 cells exposed to digested samples at 12.5 µg/mL (**a**), 25 µg/mL (**b**) and 50 µg/mL (**c**) for 24 h. As positive controls of the comet assay, cells were exposed to 300 µM of methyl methanesulphonate (MMS) for 24 h and comet slides were exposed to H₂O₂ for 10 min.

References

1. Marucco, A.; Prono, M.; Beal, D.; Alasonati, E.; Fiscaro, P.; Bergamaschi, E.; Carriere, M.; Fenoglio, I. Biotransformation of Food-Grade and Nanometric TiO₂ in the Oral-Gastro-Intestinal Tract: Driving Forces and Effect on the Toxicity toward Intestinal Epithelial Cells. *Nanomaterials* **2020**, *10*, doi:10.3390/nano10112132.
2. Sohal, I.S.; O'Fallon, K.S.; Gaines, P.; Demokritou, P.; Bello, D. Ingested engineered nanomaterials: state of science in nanotoxicity testing and future research needs. *Particle and Fibre Toxicology* **2018**, *15*, doi:10.1186/s12989-018-0265-1.

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