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Validated *in vitro* models to quantify the effect of gastrointestinal digestion on MNPLs bioavailability and toxicity and of associated harmful contaminants and toxic additives

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PROJECT No. 965196

Innovative tools to study the impact and mode of action of micro and nanoplastics on human health: towards a knowledge base for risk assessment

HISTORY CHART

Issue	Date	Changed page(s)	Cause of change	Implemented by
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ACRONYMS AND ABBREVIATIONS

ABC	• Ammonium bicarbonate buffer
DLS	• Dynamic Light Scattering
MNPLs	• Micro and Nano Plastics
PEFAblocSC	• (2-aminoethyl)-benzene-sulfonyl fluoride, aminoethyl-benzene-sulfonyl fluoride, 4-2-, proteinase k inhibitor,
SDS-page	• sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	• Simulated gastric fluid
SIF	• Simulated intestinal fluid
SSF	• Simulated salivary fluid
TAME assay	• p-toluene-sulfonyl-L-arginine methyl ester assay to quantify trypsin activity
WST-1	• Water-Soluble Tetrazolium assay. Cytotoxicity test based on the cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenase

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1. Introduction

This deliverable report contains the protocols and the optimization thereof of *in vitro* models that are being used within Task 3.2.1. of the Plasticheal project to quantify the effect of gastrointestinal digestion on micro and nano plastics bioavailability and toxicity. For this work established (or validated) procedures are being used and modified according to the specific needs. Modification and optimization of existing *in vitro* digestion protocols were needed as these protocols were only developed to study the digestion of food ingredients and or chemicals and not specifically to study the influence of digestion on the fate (and toxicity) of micro and nano plastics.

2. Description of task 3.2.1 *In vitro* effects on the gastrointestinal model

Leader: WUR; Participants: WUR, UNIMAN, UAB; Months: M6–M30

Using *in vitro* models of the gastrointestinal barrier, the work will i) quantify the consequences of gastrointestinal digestion on the characteristics, bioavailability, and toxicity of the MNPLs, ii) quantify the translocation of digested MNPLs and selected associated harmful contaminants across the intestinal barriers, and iii) translate the *in vitro* results obtained to the *in vivo* human situation. To this end, we will use gastrointestinal incubation models and *in vitro* intestinal cell models available at WU-TOX. The gastrointestinal incubation model uses artificial saliva, gastric and intestinal juices. Samples that have passed through this incubation model can subsequently be transferred to *in vitro* intestinal epithelial co-culture models grown on transwells. Therefore, robust and easy-to-use bioassays to screen for i.e. estrogenic activity of the digested MPLs and NPLs will be performed. If technically feasible models using monolayers derived from human adult intestinal stem cells can be explored.

3. Summary of work performed

3.1. Optimization of *in vitro* digestion protocol

Several types of *in vitro* digestion methods are commonly used to study the fate of nutrients and chemicals present in food during gastrointestinal digestion. These models aim to simulate the physiological and biochemical conditions of the upper gastrointestinal tract, namely the oral, gastric, and small intestinal phases. Because of its simplicity static models which use a constant ratio of matrix to enzymes and electrolytes and a constant pH for each

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digestive phase have been widely used for food and pharmaceutical research purposes(Minekus et al., 1999; Oomen, Tolls, Sips, & Groten, 2003; Oomen, Tolls, Sips, & Van den Hoop, 2003; Van de Wiele et al., 2007; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005). Variants of static *in vitro* digestion models have been used before to predict the fate of engineered nanoparticles during transit of the gastrointestinal tract (Abdelkhaliq, van der Zande, Undas, Peters, & Bouwmeester, 2020; Lefebvre et al., 2014; Peters et al., 2012; Walczak et al., 2013).

In Task 3.2.1. the static *in vitro* digestion protocol developed by the INFOGEST2 project was used as a starting point(Brodkorb et al.), and modified to be able to use it to predict the gastrointestinal fate of micro and nano plastics. The final *in vitro* digestion protocol is included in annex 1. Several optimization steps were included to arrive at the final protocol.

Initial protocol:

Digestion was performed using 20 µL (0.5 mg) of micro/nanoplastics (MNPLs) in a total volume of 8 mL (2 mL of SSF, 2 mL of SGF and 4 mL of SIF) in 15 mL tubes. After digestion, particles were recovered by transferring the liquid to an ultracentrifuge tube and centrifuging at 30000 G for 30 min and resuspending them in 500 µL of serum-free medium. Two different digestions were performed, one based on the INFOGEST2 protocol, and one based on the UAB sterile digestion protocol.

1. Based on initial results the UAB sterile digestion protocol was discarded and the INFOGEST2 protocol was selected for further optimization.
2. In the first iteration recovery of particles was tested for centrifugation at 5000 G and 30000 G for 30 min, for 5000 G recovery was lower than 15% for all particles tested (PS spheres unmodified 50 nm, 100 nm, 200 nm, and 500 nm), for centrifugation at 30000 G recovery was high for particles sized 500 nm (between 50-80% recovery based on fluorescence); however, smaller particles had a significantly lower recovery (<10%-30%). Centrifugation at 30000 G was chosen
3. Given the previous results we assessed the recovery of micro and nanoplastics when adding 0.5 mg, 1 mg, 1.5 mg, 2 mg, and 2.5 mg to the digestion, the results showed that including 1.5 mg led to significant improvements in particle recovery and was chosen.
4. Initial WST-1 experiments showed high cytotoxicity and difficulty in assessing particle size with DLS which was believed to be due to undissolved bile and pancreatin fibers in the samples. In an attempt to reduce cytotoxicity, we centrifuged the SIF after suspending the bile and pancreatin at 3000 G and 30000 G for 30 min in an ultracentrifuge tube. Afterwards the trypsin activity of all three SIFs was measured using the TAME assay. Centrifuging at 3000 G led

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to no alterations in trypsin activity while centrifugation at 30000 G led to a reduction of trypsin activity from approximately 30 U/mL to 25 U/mLl. Both 3000 G and 30000 G centrifugation showed significant improvements in DLS size distribution and reduction in cytotoxicity with the biggest effects shown in 30000 G ultracentrifugation. An SDS-PAGE was performed to assess whether centrifugation at 3000 G or 30000 G led to an alteration in the protein corona. Significant alterations were seen for both 3000 and 30000 G compared to the uncentrifuged control; however, particles incubated in uncentrifuged control showed no difference from the digestion blank (background signal) while the 30000 G sample did show minor differences. **Based on these results the SIF was now centrifuged at 30000 G**

5. **The digestion protocol was adapted for use in 2 mL tubes since these require less material, led to a higher recovery, and can be ultracentrifuged at 30000 G.**
6. While the ultracentrifugation and washing of samples led to the reduction of matrix cytotoxicity and allowed us to have the exact same protocol for cell exposure and protein corona determination, issues with the resuspension of particles after ultracentrifugation and inaccuracies with particle number determination were noted. Especially charged particles could not be resuspended properly even after sonication in a sonication bath for over 10 min which led to difficulties in giving the same dose of MNPLs to different wells of a WST-1 assay. Furthermore, fluorescence based determination of particle recovery appeared inaccurate as often more than double the amount of particle added during digestion. **To avoid these issues, we decided to instead of the ultracentrifugation of the particles prior to cell exposure, to dilute the digested sample and directly add this to the cells.**
7. The digestate was shown to detach THP-1 cells and Caco-2 cells from 96-wells plates likely due to proteolytic activity, without the inclusion of serum. This was seen even at a 1% digestate concentration and after the addition of PEFAbloc SC, a serine-protease inhibitor. **We, therefore, decided to perform cell exposure in the presence of 10% serum at a 4% digestate concentration (the highest non-cytotoxic concentration).**
8. **To reach a concentration of 125 µg/mL in cell exposure systems we increased the SSF concentration to 2X and performed digestions with 3.125 mg/ml of MNPLs.**

3.2. Optimization protocol to study cellular uptake of *in vitro* digested and pristine micro and nanoplastics

The second important research question in task 3.2.1. is to quantify the translocation of digested micro and nano plastics across the intestinal barriers and to derive parameters to be used in pharmacokinetic modeling in other

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project tasks within Plasticheal. It is assumed that endocytic processes drive the cellular uptake and intestinal barrier translocation of micro and nanoplastics. Therefore, we have decided to use differentiated M0 THP-1 macrophages as a cell model to study the relationship between uptake and the role of *in vitro* digestion of micro and nanoplastics. In task 3.2.1. we have developed a protocol for the differentiation of THP-1 cells based on previously published work (Zhou et al., 2010). The final protocol used for the THP-1 differentiation, and the protocol to exclude cytotoxicity following the exposure to micro and nanoplastics (i.e. WST assay) have been included in Annex 2.

3.3. Optimization of protocol for proteomics study of protein corona associated with pristine and *in vitro* digested micro and nanoplastics

Several studies have been published in which the protein corona of nanoparticles have been characterized following incubation in cell culture media with serum. Uniquely for the current work in Task 3.2.1 we will perform a proteomic study of the protein corona following *in vitro* digestions of MNPLs. For this, we have optimized protocols based on previously developed methods and approaches (Abdelkhaliq et al., 2018). The final protocol is included in Annex 3.

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ANNEX 01: *In vitro* digestion protocols

In vitro digestion

Protocol based on INFOGEST2 model

Created by:	Hugo Brouwer
Modified by:	
Date:	03-01-2021
Version:	1.5

Materials:

- Micro and nanoplastics at a 2.5% mass concentration (Polysciences)
- Nanoplastics at a 10% mass concentration (Magsphere)
- Porcine Pancreatin 8X USP specifications (Sigma Aldrich)
- Porcine Pepsin >2500U/mL (Sigma Aldrich)
- RPMI 1640 medium with 4.5 g/L glucose, 2.383 g/L HEPES, 10 mM L-glutamine, 1.5 g/L sodium bicarbonate, 110 mg/L sodium pyruvate supplemented with 10% fetal calf serum and 1% Pen/Strep
- Tabletop centrifuge
- PBS 1X
- HCl 37%
- pH meter
- pH paper
- Sonication bath
- Rotating incubator (heads over heals)
- Sterile 2mL Eppendorf tubes
- Demi water
- Incubator (37 ˚C)
- Vacuum pump
- 500ml corning bottle-top filter(0.22µm)
- Autoclaved 500mL glass bottles with screw cap(duran)

reagents required:

- $CaCl_2(H_2O_2)_2$
- NaOH
- KCl
- KH_2PO_4
- $NaHCO_3$
- NaCl
- $MgCl_2(H2O)_6$
- $(NH_4)_2CO_3$
- $CaCl_2(H_2O_2)$
- p-Toluene-sulfonyl-L-arginine methyl ester (TAME)
- Bovine Bile (Sigma)

Note: perform all steps in a flow hood to prevent bacterial/fungi contamination of the plastic beads or the simulated digestion fluids.

Note: prior to the experiment you need to determine the trypsin activity of pancreatin, this can be done as described in the INFOGEST2 supplementary information using the TAME assay.

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Note: during digestion you need to alter the pH quite frequently, the amount of HCl that needs to be added to alter the pH is always the same so it may be helpful to perform pilot digestion without nanoplastics and record the amount of HCl required to adjust the pH at each step.

Note: enzymes tend to lose activity upon rigorous mixing or high temperature. Thereof, we should avoid sonication/vortexing of the enzyme solutions and keep enzyme solutions on ice as much as possible.

Note: For THP-1 cells the digestion matrix needed to be diluted down to 4% to avoid cytotoxicity. The maximal concentration to be tested is limited by nanoparticle concentration and SSF concentration, at a 2X SSF and 2.5% MNPLs concentration the maximal amount to be added to cells is 125 µg/mL, if more is required increase the SSF to 5X concentration.

Method

Preparation (at least 1 day prior to digestion):

1. On the day prior to the digestion autoclave 4 empty 250 mL containers, at least two 2 mL Eppendorf vial for each of your samples and for the digestion blank.

2. Prepare 1 M of sterile HCl by filling a container with 840 ml of demi-water and carefully pouring 120 mL of 37% HCl into the same container. Then using a vacuum pump filter and sterilize the solution using a 500ml corning bottle-top filter(0.22µm) into an autoclaved 500ml glass bottle(duran).

3. Prepare the simulated saliva, gastric fluid and intestinal fluid as follows:

4. Weigh 441 mg $CaCl_2(H_2O)_2$ in a sterile 15 mL tube to prepare a 10 mL of (0.3 M) in demi water

5. Weigh 340 mg of KH_2PO_4 in a sterile 15 mL tube to prepare 10 mL (0.25 M) in demi water

6. Weigh 152 mg $MgCl_2(H_2O)_6$ in a sterile 15 mL tube to prepare 5 mL of (0.15 M) in of demi water

7. Weigh 144 mg of $(NH_4)_2CO_3$ in a sterile 15 mL tube to prepare 0.5 M in 3 mL of demi water

8. Weigh NaCl, KCl and $NaHCO_3$ according to the schedule below in three sterile 250 mL containers

Reagent	Saliva (2X) (SSF)	Gastric fluid (SGF)	Intestinal fluid (SIF)
KCl	559 mg	128.6 mg	126.7 mg
$NaHCO_3$	572 mg	525.6 mg	1787.25 mg
NaCl	-	689.6 mg	561 mg

9. Add the KH_2PO_4, $MgCl_2(H_2O)_6$, and $(NH_4)_2CO_3$ to the containers according to the table below

Reagent	Saliva(2X)	Gastric fluid	Intestinal fluid
KH_2PO_4	7.4 mL	0.9 mL	0.8 mL
$MgCl_2(H_2O)_6$	0.5 mL	0.2 mL	0.55 mL
$(NH_4)_2CO_3$	0.06 mL	0.25 mL	-
H_2O	To end volume 250 mL	To end volume 250 mL	To end volume 250 mL

10. Dissolve all the salts using a stirring bean and set the pH to 7 for simulated saliva and intestinal fluid and set the pH to 3.0 for simulated gastric fluid using 1M HCl

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11. Top SSF, SGF, and SIF off to 250 mL using demi H_2O

12. Autoclave the $CaCl_2(H_2O)_2$ 0.3 M solution and the SSF, SGF, and SIF

13. The solutions made in the preparation can be kept for at least 3 months if they are sterile

Prepare for digestion:

14. Prepare and label one 2 mL Eppendorf tube for each type of MNPLs and one additional 2 mL tube for the digestion blank.

15. One hour prior to starting the digestion, weigh 0.8 mg/mL of pepsin in a 50 ml tube and add SGF. Prepare at least 750 µl of pepsin containing SGF per sample. Dissolve by rotating heads over heals for 15 min then keep on ice till use.

16. Calculate how much SIF is needed (at least 1.25 ml/sample) and weigh enough bovine bile to reach 4 mg/mL. Then take a 15 mL tube and add 0.75 ml of SIF/sample then add the weighed bovine bile and incubate by rotating heads over heals for at least 1 h at 37C.

Digestion of nanoplastics

17. Briefly, invert the MNPLs suspensions and check for aggregates/pellets, if it does not look homogenous, turn on a sonication bath with a low water level and hold the container in the area where the highest disturbance of the water surface is observed (the area of maximum sonication intensity) for 10-30 sec.

18. Pipette 6.25 mg of MNPls suspension (250 µL of 2.5% suspension, 62.5 µL of 10% suspension) into a sterile 2 mL Eppendorf vials and top off to 250 µl using sterile demi H_2O.

19. Add 250 µl of 2X SSF followed by the addition of 2.5 µl $CaCl_2$ and incubate for 1-2 min at room temperature by manually rotating heads over heels.

    note: the SSF will rapidly precipitate when you add $CaCl_2$ so it cannot be present prior to addition to the MNPLs.

20. Add 500 µL of SGF with pepsin and adjust the pH of each sample to 3 with 1 M HCl, and check pH using pH paper.

21. Incubate at 37 °C for 2 h while rotating heads over heals at 20 rpm.

22. During this incubation weigh 100 trypsin U equivalent/mL of pancreatin (14 mg/mL in our case) and add this to the SIF with dissolved bovine bile, dissolve pancreatin by rotating heads over heals for 2 h @ 37C.

    Note: The pancreatin never fully dissolves at these high concentrations and it is common to see fibers or flakes of pancreatin in the SIF.

23. Centrifuge the prepared pancreatin at 3000G for 1-2 min and move the supernatant to a new container to remove large undissolved pancreatin aggregates.

24. Add 1 mL of SIF containing pancreatin and bile and adjust pH to 7.0 using HCl and check pH using pH paper.

25. Incubate for 2 h at 37 °C while rotating heads over heels.

26. Digestion is finished and the particles can either be used to analyze the protein corona or for cellular exposure (see relevant protocols).

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Note: after digestion, the sample must be used on the same day because the samples cannot be kept at -20 ° due to precipitation of the particles and thus protein degradation may slowly occur.

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ANNEX 02: THP-1 differentiation and WST-1 cytotoxicity test

THP-1 differentiation and WST-1 cytotoxicity test

Created by:	Hugo Brouwer
Modified by:	
Date:	15-11-2021
Version:	1.4

Materials:

- PMA (Sigma)
- THP-1 cells (ATCC TIB202)
- RPMI 1640 medium with 4.5 g/L glucose, 2.383 g/L HEPES, 10 mM L-glutamine, 1.5 g/L sodium bicarbonate, 110 mg/L sodium pyruvate, 10% fetal calf serum and 1% Pen/Strep
- PBS (sigma)
- Nanoplastics (polysciences/magsphere) digested/undigested (see "digestion protocol WUR")
- 96 well plates
- T75 culture plates
- Cell counter
- WST-1 reagent

Note: THP-1 cells dedifferentiate after more than 48 h without PMA so you need to do the measurement before the end of the second day of recovery.

Note: Use non-fluorescent MNPs since the fluorescence may interfere with the absorption measurements of WST-1.

Note: Heavier MNPLs may be poorly suspended in the stock solution. Always vortex briefly for about 10-30 sec and spin down for ~1-3 for all particles prior to the experiment. If visual debris, a pellet or a color gradient is observed prior to addition to the cells sonicate the particles for 10 min in a bath sonicator after vortexing.

Note: It is unknown how stable the protein corona on nanoparticles is and therefore digested particles are used in cell experiments right as they have finished digestion (samples are never stored overnight for cell experiments).

Note: PMA is toxic and penetrates gloves and nanoparticles are potentially toxic and penetrate gloves, work carefully and change gloves/wash hands if PMA/nanoparticles fall on it (takes about 1-5 min to penetrate nitrile gloves).

Method

Maintenance of THP-1 cells:

Maintain cells between 2-10^5 and 8-10^5 cells/mL in RPMI 1640 medium and split the cells twice a week to 2-10^5 cells/mL. Keep the cells in a standing T75 flask at 5% CO_2 and 37 °C in a humidified incubator.

Differentiation to macrophages

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Day 1:

1. Spin down THP-1 cells at 200 g 5 min and resuspend in RPMI 1640 growth medium with 20 ng/mL of PMA, 10% serum to a final concentration 5-10^5 cells/mLl (assessed with cell counter).

2. Seed 100 uL THP-1 cells in a 96 well plate at (50000 cells/well); leave the outside rim of the well empty and wrap the plate in parafilm.

3. Incubate for 2 days.

Day 3:

4. Check the THP-1 cells under the microscope to see if they have differentiated properly to M0 macrophages (the cells become adherent and get a morphology seen in the figure below)

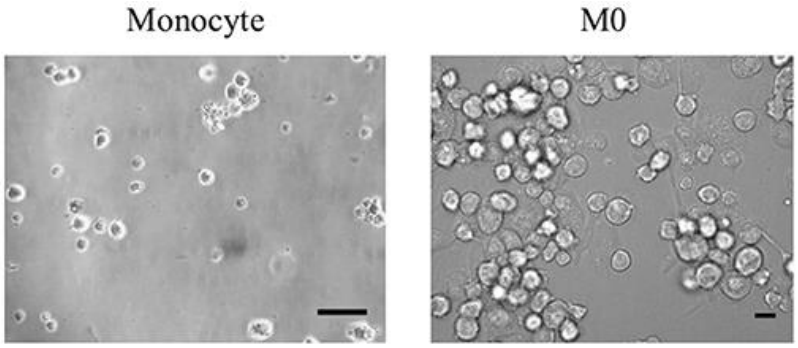

Figure: morphologies of THP-1 taken from *"A THP-1 Cell Line-Based Exploration of Immune Responses Toward Heat-Treated BLG"*

5. Aspirate the supernatant, wash with PBS and replace with 100 µL RPMI 1640 medium without PMA

Day 4:

6. Perform "digestion protocol WUR" to obtain digested MNPLs for cell exposure.

7. Briefly hold the digested particles in a low-water volume sonication bath to resuspend the nanoparticles.

8. Dilute the digestion blank and digested nanoparticles to a final concentration of 4% with serum-containing medium to avoid cytotoxicity.

9. Make serial dilutions of the digested and undigested MNPLs to a final concentration of 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL, and 7.813 µg/mL.

10. Aspirate the cell medium and add RPMI 1640 medium containing the compounds of interest and add 100 µL/well of the MNPLs suspensions.

11. Incubate the plate for the planned exposure time (usually 24 h) in the incubator at 37 °C, 5% CO_2 and 100% humidity.

Day 5: WST-1 measurement

12. Assess whether the cells look normal (see figure above) under the microscope.

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13. Remove MNPLs containing medium and wash with 100 µl of PBS.

14. Add 100 µl of 90% RPMI medium/10% WST-1 proliferation reagent.

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ANNEX 03: Extraction of the protein corona and SDS-PAGE/LC-MS-MS based proteomics

Extraction of the protein corona and SDS-PAGE/LC-MS-MS based proteomics

Protocol based on Sample preparation for proteomics by WUR (Sjef Boeren)

Created by:	Hugo Brouwer
Modified by:	
Date:	11-02-2021
Version:	1.1

Materials obtained from in vitro digestion:

- Digested nanoplastics 2 mL, 3.125 mg/mL
- Digestion blank 2 mL

Additional materials required:

- RPMI 1640 medium with 4.5 g/L glucose, 2.383 g/L HEPES, 10 mM L-glutamine, 1.5 g/L sodium bicarbonate, 110 mg/L sodium pyruvate supplemented with 10% fetal calf serum and 1% Pen/Strep (Sigma Aldrich)
- PBS
- C18 empore disk (Thermo Fischer)
- Lichroprep C18 column material
- Ammonium bicarbonate buffer 50 mM (ABC)
- Ammonium bicarbonate buffer 1 M
- Ammonium bicarbonate buffer 2 M
- Acetonitrile
- Methanol
- Cysteine (24 mg/mL ABC)
- Dithiotreitol (23 mg/mL ABC)
- Iodoacetamide(23 mg/mL ABC)
- Sequencing grade trypsin
- TriFluoro-acetic acid
- Leamlli buffer 2X (Biorad)
- MOPS SDS-PAGE gels (Genscript)
- MOPS SDS-PAGE running buffer (Genscript)
- Mini-Protean Tetra gel cassette (Biorad)
- SDS-PAGE protein ladder (Biorad)
- RC-DC protein kit

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- Fixation solution (40% methanol, 10% glacial acetic acid in demi water)
- Bio-Safe Coomassie stain
- Formic acid
- Tabletop centrifuge
- Sonication bath
- Rotating incubator (heads over heals)
- 10 mL syringe
- Sterile 2 mL Eppendorf tubes
- SDS-PAGE premade gels (Genscript)
- Blunt Gauge 14 hollow needle
- 200 µL pipette tips

Note: first digest the nanoplastics as indicated in the *in vitro* digestion protocol

Method Protein sample preparation for SDS-PAGE

1. Spin the digested sample down at 30000 G on a tabletop centrifuge for 30 min.
2. In a flow-hood discard the supernatant and add 1 mL of PBS to each sample to wash them.
3. The nanoplastics are not easily resuspended and it is recommended to put them in a sonication bath with low water volume for 15 sec-1min or until you see the pellet resuspend in the PBS.
4. Once again pellet the sample at 30000 G for 30 min and discard the supernatant.
5. Repeat steps c-e until you have washed the sample thrice with PBS.
6. After discarding the PBS supernatant for the third time resuspend the pellet in 80 µL of 2X Laemlli buffer.
7. Sonicate the sample in a low-volume sonication bath to resuspend nanoplastics and subsequently boil the sample at 95 °C for 5 min to release proteins.
8. Spin down the protein sample for 1 min at 30000 G to pellet the now non-protein-bound nanoparticles and take an aliquot of 20 µL for protein concentration determination

   Note: Most protein kits like the DC or Bradford kits are not compatible with reducing agents or detergents, therefore use a compatible kit such as the RC-DC kit.
9. Determine the protein concentration according to the user manual of the protein determination kit.
10. Calculate the amount of sample that needs to be loaded to have an equal amount of protein in each of the lanes of the SDS-PAGE gel and dilute the samples accordingly.
11. Load the SDS-PAGE gels into an electrophoresis chamber and fill the chamber with a MOPS-SDS PAGE buffer to the indicated level for 2 gels.

    Note: Always run 2 gels even if you have to include a blank gel since most electrophoresis devices do not work with just a single gel

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12. Run the gel on 120 v for approximately 1-1.5 h

13. Once the electrophoresis has finished, discard the running buffer and wash the tank with clean water.

14. Remove the gels from the tank and crack open the protective casing.

15. Gently remove the gel from the casing using a spatula or comparable object, and transfer to a suitable container where the gel can be uncurled and fully extended without damaging the gel.

16. Remove excess SDS by washing the gel twice, fully submerged in demi water, for 10 min each.

    Note: Better results are seen when the gel is shaken at 350cRPM during washing steps.

17. Fix the gel by adding a fixation solutionc(40% MeOH, 10% glacial acetic acid) and incubating the gel for 20 min in a fume hood.

18. Discard the leftover fixation solution in the chemical waste and submerge the gel in bio-safe Coomassie stain for 1.5 h or overnight.

19. Remove the Coomassie and wash with water, replacing the water once every hour until most of the background Coomassie staining has faded.

20. Measure the gel on an Odyssey gel imaging system and quantify the bands using either the odyssey integrated software or by using ImageJ/ image labs lite.

Method Protein sample preparation LC-MS-MS

Day 1: protein corona extraction

21. Spin down the digested sample at 30000 G on a tabletop centrifuge for 30 min.

22. In a flow-hood discard the supernatant and add 1mL of PBS to each sample to wash them.

23. The MNPLs are not easily resuspended and it is recommended to put them in a sonication bath with low water volume for 15 sec-1min or until you see the pellet resuspended in the PBS.

24. Once again pellet the sample at 30000 G for 30 min and discard the supernatant.

25. Resuspend the MNPLs in 2M of ammonium bicarbonate buffer to remove any alternate counter ions that might interfere with LC-MS-MS proteomics.

26. Resuspend by sonication and repeat steps e-f once with 1M ammonium bicarbonate buffer.

27. Redissolve the MNPLs in 50 µL of 50 mM ABC and move to a proteomics lab.

28. Add 10% (5 µL) of the prepared 150 mM DTT solution and let the reduction proceed by incubating for 30 min at 45 °C while shaking at 350 RPM.

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29. After exactly 30 min take the sample out and let it cool to 20 °C.

30. Add 10% (5.5 µL) of 200 mM Iodoacetamide, mix and incubate for 30 min at room temperature in the dark.

31. Add 4 µl of 200 mM cysteine dissolved in 50 mM ammonium bicarbonate buffer and mix.

32. Dilute 5 µL of sequencing grade trypsin (500 ng/µL) 20 times in 50 mM ammonium bicarbonate buffer to reach a concentration of 25 ng/mL.

33. Incubate the sample at room temperature overnight while shaking at 350 RPM.

Day 2: protein sample cleanup using µcolumns:

Note: never let µcolumns fall dry as the quality of the sample will drop

Note: it is not uncommon for air bubbles to appear in the µ column when switching between organic and aqueous buffers. In this case, hold the µcolumn in your hand, lift your arm, and as fast as you can swing your arm down in a semi-circular motion to push the air out through the centrifugal force.

34. Add 2.5 µLl of TFA 10 times diluted in ABC to inactivate the trypsin.

35. Take a sheet of C18 column material and use a Gauge 14 hollow needle to obtain 2 slices that fit in a 200 µL column to make a µcolumn.

36. Using a thin metal rod tap the C18 material firmly, but not forcefully to obtain a fit that does not block the flow through of liquids but does not allow liquid to flow around the C18 material.

37. Add 200 µL of methanol to the µcolumn and see if the µcolumn leaks, if so discard the µcolumn and make a new one following steps b-d.

38. Make a slurry of 50% w/v Lichoprep in methanol and pipet vigorously to homogenize the slurry.

39. Add 4 µL of the prepared slurry to the 200 µL of methanol already present in the µcolumn and elute the methanol/column material slurry using a 10 mL syringe or using a vacuum manifold but be careful not to let the columns fall dry.

40. Wash the µcolumn with 100 µL methanol.

41. Equilibrate the columns by adding 100 µL of 1 mL/L of formic acid in water and elute.

42. Dilute the sample 1:1 with 2 mg/mL of formic acid in water and add the sample to the µcolumn and elute.

43. After adding the sample manual elution becomes impractical due to the nanoparticles so a vacuum manifold should be used.

44. Wash the µ column with 100 ml/L formic acid in water.

45. Transfer the µ column to a clean 1.5 mL Eppendorf tube.

46. Add 50 µL of 50% acetonitrile/50% formic acid of 1 mg/mL formic acid in ddH_2O.

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47. Manually elute the sample completely (let the column fall dry) and collect the flow through in the 1.5 mL Eppendorf.

48. Discard the µcolumn and remove the remaining acetonitrile by rotary evaporation at 45 °C for 30 min to 1 h.

49. Redissolve the sample in 50 µL of 1 mL/L formic acid in ddH_2O, if the sample has fallen dry sonicate for 30 sec in a low-water-volume sonication bath.

50. The sample is ready for LC-MS-MS analysis.

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