Responsible partner for this SOP: POSTNOVA	NanoCELL

In vitro study to investigate the fate of cellulose nanocrystals in the gastro-intestinal tract

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1. PURPOSE

This SOP describes the realization of an *in vitro* digestion study following potential oral exposure of cellulose nanocrystals (CNC) along a simulated gastro-intestinal tract (GIT) route. Purpose of this procedure is the preparation of CNC samples suitable for analysis by Transmission Electron Microscopy (TEM) and Asymmetrical-Flow Field-Flow Fractionation (AF4). However, the here described procedure may also be applicable to further oral exposure scenarios using different nanomaterials, additional food-matrix blends, and analytical methods.

2. OBJECTIVE

CNC, a cellulose with a crystalline nanostructure, is a promising raw material alternative to fossil-fuel-based polymers for e.g., biodegradable food packaging. However, the toxicological potential of CNC is not yet fully investigated and reliable and harmonized analytical strategies from sample preparation to advanced physico-chemical characterization particularly in complex matrices (such as saliva, gastric acid, intestinal fluid) are still missing. Therefore, a working procedure to enable the investigation of the behavior of CNC in simulated GIT fluids mimicking the oral exposure route was established.

3. REGULATORY BASIS, REFERENCE DOCUMENTS

EFSA (2018): Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health

https://doi.org/10.2903/j.efsa.2021.6768

Peters et al. (2012): Presence of nano-sized silica during in vitro digestion of foods containing silica as a food additive. ACS nano, 6(3), 2441-2451.

https://doi.org/10.1021/nn204728k

Versantvoort et al. (2005): Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food and chemical toxicology, 43(1), 31-40.

https://doi.org/10.1016/j.fct.2004.08.007

4. RELATED DOCUMENTS

Table 1 : References to documents needed to proceed according to this procedure.

Document ID	Document Title
n.a.	n.a.

5. DEFINITIONS

Table 2: Glossary of Terminology used in the SOP.

Term	Description
AF4	Asymmetrical-Flow Field-Flow Fractionation
CNC	Cellulose nanocrystal
EFSA	European Food Safety Authority
GIT	Gastro-intestinal tract
RT	Room temperature
SAS	Synthetic amorphous silica
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
UPW	Ultrapure water

6. PROCEDURE

a) Short description

In this *in vitro* study, the GIT digestion process is simulated in three consecutive steps mimicking the oral exposure route: the mouth, the stomach, and the intestine. Therefore, saliva, gastric juice, duodenal and bile juice have to be synthetically prepared. CNC suspensions are diluted to different concentrations. Then, food matrix is blended with each of these CNC suspensions. The digestion process is started by adding the saliva to the Food-CNC-Mix (step 1), followed by the gastric juice (step 2) and finished with the addition of the duodenal juice, bile juice and $NaHCO_3$ (step 3). Several parallel experiments are performed, with one experiment stopped after the first step, one experiment after the second step or the third step by sample collection.

Afterwards, the behavior and fate of CNC under these various digestion conditions can be investigated by analytical methodologies such as e.g., AF4 and TEM. However, subsequent sample preparation and physico-chemical characterization shall not part of this SOP.

b) Materials and devices

Table 3: Used devices.

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Devices
Several 50 mL or 75 mL glass containers, e.g., screw cap glass
Volumetric flask
Graduated cylinder
Magnetic stirrer with heating plate, IKA RT5, IKA, Germany
Milli-Q Integral 5 system, EMD Merck Millipore, MA, USA or similar

Preparation of digestive fluids

Digestive fluids were prepared one day prior to the *in vitro* study. The exact formulations are shown in Table 4 to Table 7.

Table 4: Formulation of saliva. A final volume and pH of approximately 333 mL and 6.8, respectively, should be achieved. To adjust the pH value 37 % HCl and 2 M NaOH can be used.

Materials	Description	Used amount
SALIVA inorganic components, Postnova	Formulation in Appendix # 1	Dissolve in 300 mL UPW
Urea 25 g/L (stored in bag at RT), Carl Roth	Use as solution (dilute 250 mg in 10 mL UPW)	2.67 mL
Alpha Amylase (from B. Subtilis 4 u/mg), Th. Geyer	n.a.	97 mg
Uric acid, Th. Geyer	n.a.	5 mg
Mucin, Carl Roth	n.a.	8 mg
UPW, Postnova	n.a.	30 mL

Table 5: Formulation of Gastric juice. A final volume and pH of approximately 333 mL and 1.3, respectively, should be achieved. To adjust the pH value 37 % HCl and 2 M NaOH can be used.

Materials	Description	Used amount
Gastric Juice inorganic components, Postnova	Formulation in Appendix # 2	Dissolve in 300 mL UPW
37 % HCl 37 %, Merck KGaA	n.a.	2.2 mL

Materials	Description	Used amount
D-(+)-Glucose, Sigma Aldrich	n.a.	217 g
Glucoronic acid 2 g/L (stored in bag at RT), Th. Geyer	Use as solution (dilute 20 mg in 10 mL UPW)	3.33 mL
Urea 25 g/L (stored in bag at RT), Carl Roth	Use as solution (dilute 250 mg in 10 mL UPW)	2.67 mL
Glucoseamin Hydrochloride, Th. Geyer	n.a.	110 mg
BSA, Sigma Aldrich	n.a.	333.3 mg
Pepsin (2500 u/mg), Th. Geyer	n.a.	833.3 mg
Mucin, Carl Roth	n.a.	1000 mg
UPW, Postnova	n.a.	26,7 mL

Table 6: Formulation of Duodenal Juice. A final volume and pH of approximately 333 mL and 8.1, respectively, should be achieved. To adjust the pH value 37 % HCl and 2 M NaOH can be used.

Materials	Description	Used amount
Duodenal Juice inorganic components, Postnova	Formulation in Appendix # 3	Dissolve in 300 mL UPW
37 % HCl 37 %, Merck KGaA	n.a.	60 µL
Urea 25 g/L (stored in bag at RT), Carl Roth	Use as solution (dilute 250 mg in 10 mL UPW)	1.33 mL
$CaCl_2$ x $2H_2O$ 22.2 g/L (stored in bag at RT), Carl Roth	Use as solution (dilute 222 mg in 10 mL UPW)	3 mL
BSA, Sigma Aldrich	n.a.	333.3 mg
Pancreatin (hog), Th. Geyer	n.a.	3 g
Lipase (hog), Th. Geyer	n.a.	500 mg
UPW, Postnova	n.a.	29 mL

Table 7: Formulation of Bile juice. A final volume and pH of approximately 333 mL and 8.2, respectively, should be achieved. To adjust the pH value 37 % HCl and 2 M NaOH can be used.

Materials	Description	Used amount
Bile Juice inorganic components, Postnova	Formulation in Appendix # 4	Dissolve in 300 mL UPW
37 % HCl 37 %, Merck KGaA	n.a.	50 µL
Urea 25 g/L (stored in bag at RT), Carl Roth	Use as solution (dilute 250 mg in 10 mL UPW)	3.33 mL
$CaCl_2$ x $2H_2O$ 22.2 g/L (stored in bag at RT), Carl Roth	Use as solution (dilute 222 mg in 10 mL UPW)	3.33 mL
BSA, Sigma Aldrich	n.a.	600 mg
Bile salt, Th. Geyer	n.a.	10 g
UPW, Postnova	n.a.	26,6 mL

Table 8: Further used materials.

Materials	Description	Used amount
$NaHCO_3$, Merck KGaA	Use as 1 M solution (dilute 8401,0 mg in 100 mL UPW)	2 mL

c) Sample preparation

CNC suspensions are prepared at different concentrations (depending on the analysis method applied subsequently, having a maximum concentration of 5 mg/mL) by diluting with UPW. Afterwards the suspensions are split in 3 aliquots each.

All prepared digestive juices are heated to 37 °C ± 2 °C prior to analysis.

d) Detailed description of the procedure

The experiment was conducted according to Peters et al. (2012, ACS Nano) and Versantvoort et al. (2005, Food and Chemical Toxicology). A schematic representation of the experimental setup is shown in Figure 1. In detail, CNC suspension can be used pure (worst-case) or mixed with any kind of food matrix (the simplest food matrix is drinking water). In any case, 4.5 mL CNC or CNC-Food matrix-mix has to be provided in a 50 mL glass bottle. After equilibrating at 37 °C in a first step (simulating the digestion in mouth) 6 mL of tempered saliva is added. The mix is stirred for 5 min at 37 °C with a pH of 6.5. In a second step 12 mL of tempered gastric juice is added, and the mixture is

stirred for 2 h at 37 °C. The pH can be adjusted with 1 M NaOH or 37 % HCl to a value of 2.0 ± 0.5. In the final step, 12 mL of duodenal juice, 6 mL of bile juice (both tempered) and 2 mL of $NaHCO_3$ are added and stirred again for 2 h at 37 °C. The pH rises to a value of around 6 to 8.

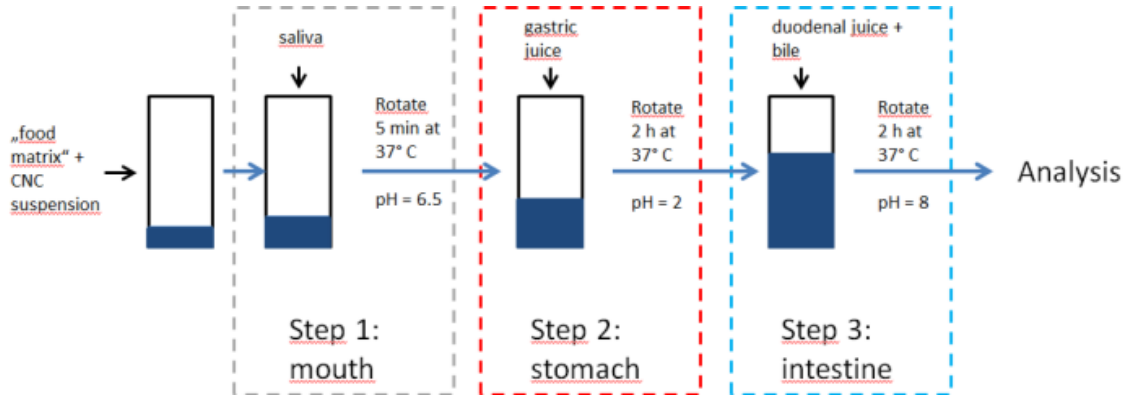

Figure 1: Experimental setup of *in vitro* digestion study with CNC.

Samples are collected after the first, the second and the third step. In any case, the experiment is stopped with the sampling. For a better understanding, the sampling procedure is displayed in Figure 2.

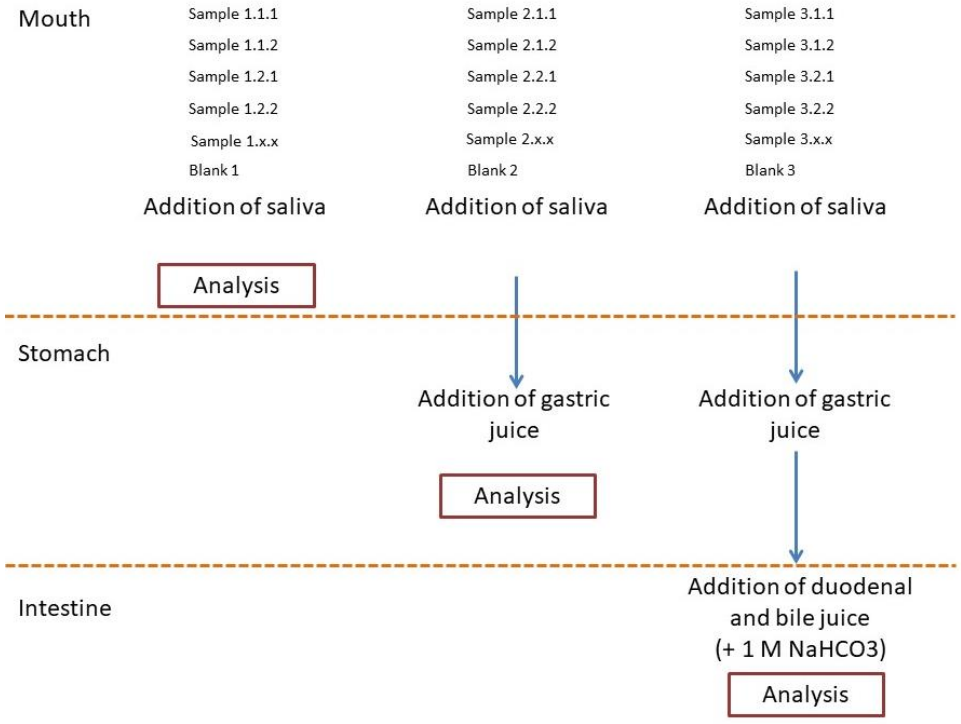

Figure 2: Sampling setup.

e) Controls

Blank measurements with UPW instead of CNC samples have to be conducted by replacing the CNC suspension by the same amount of UPW. Hereby, the same procedure is used as described for experiments with CNC samples.

f) Data analysis

The described method for an *in vitro* digestion study with CNC, generates samples, which can be subsequently analyzed by TEM or AF4 (or other analytical methods).

g) Testing errors

This *in vitro* digestion study presents a simplification of the real process. The above-described procedure was performed with CNC and MilliQ water as food matrix substitutes. This was considered a worst-case-scenario as more complex food components may cause agglomeration or other effects of CNC particles and may therefore change their behavior and mobility.

For example, the mechanical size reduction by the teeth is neglected. Inhomogeneities of the food matrix could cause differences compared to the true behavior of CNC during digestion in the human body.

Complex food matrices, which may often include fats, might complicate the *in vitro* digestion and may induce protein and/or CNC agglomeration. Moreover, complex food matrices might also cause challenges for the applied analytical technique and may require additional sample preparation and digestion steps.

Different matrices from simple to complex should, therefore, be used in the implementation.

7. SCOPE/AREA OF APPLICATION

This method of an *in vitro* digestion study can be performed with any engineered nanomaterial and/or food-matrix blends. Further, Versantvoort et al (2005) used this method for determining the bioavailability of mycotoxins and Peters et al. (2012) used SAS (synthetic amorphous silica) and n-SiO_2 as food additives for a safety assessment of silica nanoparticles in food.

8. APPENDIX

1 Formulation of “SALIVA inorganic components”

Materials	Used amount
KCl, Carl Roth	299 mg
KSCN, Th. Geyer	66.7 mg
NaH_2PO_4 x H_2O, Carl Roth	340.3 mg
Na_2SO_4, Carl Roth	190 mg
NaCl, JT Baker	100 mg
$NaHCO_3$, Merck KGaA	564,7 mg

The prepared solution should be clear without precipitate.

2 Formulation of “Gastric Juice inorganic components”

Materials	Used amount
NaCl, JT Baker	917 mg
NaH_2PO_4 x H_2O, Carl Roth	102 mg
KCl, Carl Roth	275 mg
$CaCl_2$ x $2H_2O$, Carl Roth	133,8 mg
NH_4Cl, JT Baker	102 mg

The prepared solution should be slightly turbid.

3 Formulation of “Duodenal Juice inorganic components”

Materials	Used amount
NaCl, JT Baker	2337.3 mg
$NaHCO_3$, Merck KGaA	1129.3 mg
KH_2PO_4, Th. Geyer	26.7 mg
KCl, Carl Roth	188 mg
$MgCl_2$ x $6H_2O$, Merck KGaA	17 mg

The prepared solution should be turbid.

4 Formulation of “Bile Juice inorganic components”

Materials	Used amount
NaCl,JT Baker	1753 mg
$NaHCO_3$, Merck KGaA	1128.3 mg
KCl, Carl Roth	125,3 mg

The prepared solution should be turbid.

9. HEALTH, SAFETY AND ENVIRONMENTAL CONSIDERATIONS

Standard safety aspects and local laboratory rules have to be considered. Personal protective equipment (lab coat, gloves, etc.) has to be worn.