

# Prediction of Oral Absorption of Nanoparticles from Biorelevant Matrices Using a Combination of Physiologically Relevant *In Vitro* and *Ex Vivo* Models

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## Abstract

The application of nanoparticles (NPs) in food products is ever increasing. For consumer safety it is essential to know the human oral bioavailability of these NPs. In the present study, a combination of physiologically relevant *in vitro* and *ex vivo* models are presented, which combined are considered to predict human intestinal digestion and absorption of NPs more accurately than commonly applied animal or cellular studies.

First, a computer controlled dynamic *in vitro* gastrointestinal model (tiny-TIM) is used for simulation of the digestive processes upon gastro-intestinal passage of non-biodegradable aminated, carboxylated, or unmodified fluorescent polystyrene NPs (PS-NPs). Then, the pretreated (digested) PS-NPs were consequently evaluated for absorption using *ex vivo* porcine intestinal tissue.

In this study, we found that the digestion of non-biodegradable aminated, carboxylated, or unmodified fluorescent polystyrene NPs (PS-NPs) leads to different bioaccessible concentrations, with unmodified PS-NP concentrations being 3.5- and 4.5-fold lower than the aminated and carboxylated PS-NPs, respectively. In addition, we observed a reduced absorption of PS-NPs by *ex vivo* porcine intestinal tissue from the digestive matrix when compared to the absorption of their undigested counterparts in Krebs-Ringer Bicarbonate (KRB) buffer. The current results prove that oral absorption of PS-NPs in humans is to be expected *in vivo*. In the risk assessment of potential oral exposure to NPs in food products it is therefore key that the exposure and hazard assessment are performed with the NPs in physiologically relevant conditions, since this may influence the absorption and hazard properties of the NPs.

## Keywords

Nanoparticle, Bioavailability, Digestion, Absorption, *In vitro*, *Ex vivo*

## Introduction

Nanotechnology has enormous technological and economic potential, but insight into the possible human health risks posed by nanoparticles (NPs) is essential for sustainable development and safe use of innovative products based on these structures. At present, however, various information gaps exist on the ability to become systemically available (absorption), the intrinsic toxic properties

(hazard), the potential human exposure and the relationship between exposure and health effects (risk).

An increasing number of food products contain NPs, because they contribute to enhanced physicochemical properties, for instance for an improved taste, mouth feelings or absorption of nutrients [1]. These enhanced product properties result among others from the different behaviours in the food matrices, due to their small particle sizes and relative large surface area, in comparison to their micro sized counterparts. Different material properties also come forth when NPs are released from their initial (food) matrix, for instance into biological matrices during digestion [2]. Whether or not these different properties are beneficial for e.g. the bioavailability of essential nutrients, or harmful upon uncontrolled intake of foreign NPs should be studied already during the food product development phase.

If ingested NPs are absorbed (*i.e.*, they are taken up from the gut lumen into epithelial cells and transported into the blood stream), there is a potential for systemic exposure (*i.e.*, the NPs may become bioavailable), which may lead to local or systemic toxicity [3]. Ideally, the oral bioavailability of NPs should be determined directly in humans, but without knowledge on the possible toxicity, there are some major ethical constrains.

A common approach to assess the oral absorption of conventional chemicals is by means of *in vivo* animal studies [4]. This approach could also be relevant for the assessment of NPs, since it allows both the assessment of the oral bioavailability as well as possible systemic effects [5]. Although animal studies allow the assessment of oral bioavailability and possible toxicity of NPs under physiological *in vivo* conditions, the outcome of these studies requires extrapolation to the human situation. It should be noted that animal studies may not always be predictive of the human situation due to differences in diet, gastro-intestinal anatomy and physiology, *i.e.*, food transit times, digestive enzymes or gastrointestinal absorption mechanisms [5, 6]. These factors have been shown to affect the release of NPs from their matrix into the lumen of the gastrointestinal tract and subsequently could affect the intestinal absorption and bioavailability [2].

As an alternative to animal studies, different *in vitro* models are often applied to estimate the absorption and/or transport of compounds across the intestinal wall. Usually a human intestinal epithelial cell system, such as the Caco-2 cell line, is applied [5, 7]. However, single cell type models do not represent the whole complexity of the intestinal wall *in vivo*. Different co- or tri-cultures with mucus secreting goblet cells and/or M-cells have therefore been developed to mimic the *in vivo* situation, however the discontinuous mucus layer and relative presence of M cells *in vitro* do not match the situation *in vivo* [8-10].

Alternatively, *ex vivo* human intestinal tissue can be used to investigate intestinal permeability of NPs, since *ex vivo* human intestinal tissue is the best representation of the *in vivo* situation with regard to anatomy and physiology. Previous studies with *ex vivo* human intestinal tissue have shown that

for pharmaceutical compounds the apparent permeability measured *ex vivo* is a good predictor of the fraction absorbed *in vivo* [11-13]. This suggests that *ex vivo* human intestinal tissue could also be used to study the permeability of NPs to indicate whether oral absorption of NPs is to be expected *in vivo*. However, to date, only a limited number of studies have been performed that determined the permeability of NPs across *ex vivo* human intestinal tissue [14-16].

Currently, almost all *in vitro* and *ex vivo* permeability studies with NPs describe the absorption of pristine nanoparticles from cell-culture medium or buffered salt solution (e.g. Walczak et al. [8], Schimpel et al. [9], Lautenschlager et al. [14], des Rieux et al. [17]). However, it has been shown that NPs can be digested or otherwise structurally altered (including processes such as degradation and/or agglomeration) in the gut lumen *in vivo*, which might influence their absorption [19, 20]. To date, only a limited number of studies have included an *in vitro* digestion step of the NPs prior to determining their permeability across different cell systems (e.g. Lichtenstein et al. [21], Walczak et al. [22]). In these studies, the *in vitro* digestion matrix is diluted before being applied on the cell systems to prevent cytotoxicity already to the digestion matrix used. Consequently, this reduces the similarity to the *in vivo* situation. Therefore, a combination of physiologically more relevant digestion matrices and intestinal absorption models for humans is needed for a more accurate prediction of both the digestion and the oral absorption of NPs in humans.

To study *in vitro* digestion and degradation of chemicals, different models are available with varying levels of complexity, and as such varying similarity to the human situation. Commonly used static digestion models for pharmaceuticals and food products mimic the chemical and enzymatic conditions of the human GI tract in a flask or beaker glass, while being constantly mixed with an impellor, magnetic stirrer or shaker [5]. However, more sophisticated models, so-called dynamic models, also recreate the complex physical environments encountered in the GI tract, which has been shown to be an important factor in the disintegration of food particles *in vivo* [23, 24]. Therefore, these models have a high predictive value for the human situation [2, 25, 26]. Consequently, the characteristics of the digested and structurally altered NPs will be more representative for the human situation when applying the dynamic models.

In a previous study we have shown that the intestinal absorption of chemicals from undiluted digestion matrix can be studied by using *ex vivo* intestinal tissue segments from pigs [27]. Since pigs also have an omnivorous diet, their GI tract is very much comparable to that of humans [6, 28-31]. Since porcine tissue is generally more easily available than healthy human intestinal tissue it allows a more systematic approach to investigate (regional) intestinal absorption of NPs from a biorelevant matrix.

As a proof of concept, we here present a combined approach using a computer controlled dynamic *in vitro* gastrointestinal model (tiny-TIM), for the digestion of NPs, in conjunction with an *ex vivo* tissue model (InTESTine), in order to determine their intestinal absorption. This combined

approach allows the simulation of the transit through the stomach and small intestine and permeation through the small intestinal wall for any nanomaterial. Polystyrene NPs (PS-NPs) were selected as exemplary nanomaterial for this study because of their stability (being non-biodegradable) and availability of different surface modifications (aminated, carboxylated and unmodified).

## Materials and Methods

### Test products and chemicals

Aminated, carboxylated, or unmodified fluorescent PS-NPs (50 nm) with a red fluorophore core (excitation at 530 nm and emission at 590 nm) were obtained from Magsphere (Pasadena, California, United States). [ $^{14}\text{C}$ ] Caffeine (51.1 mCi/mmol) and [ $^3\text{H}$ ] mannitol (14.2 Ci/mmol), used as reference substances for absorption testing, were purchased from Perkin Elmer Inc. (Boston, Massachusetts, United States). All other chemicals were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands).

### *In vitro* digestion of NPs using tiny-TIM

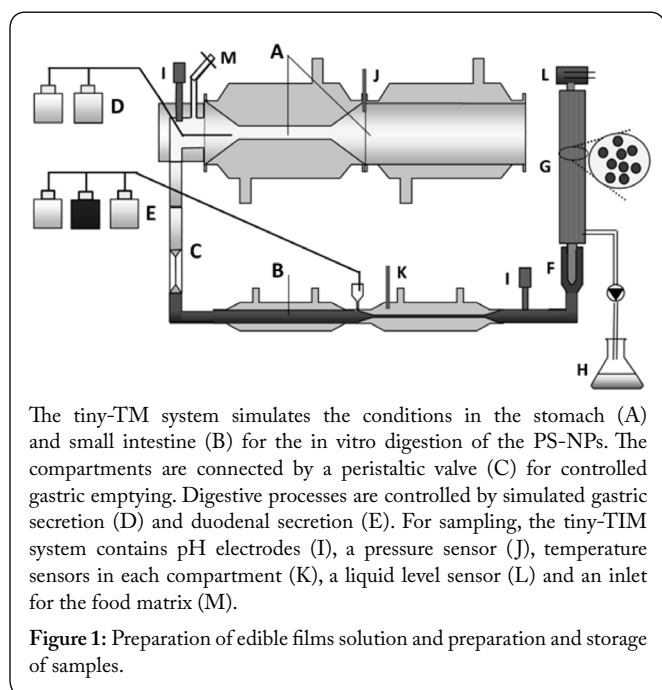
To expose the (non-biodegradable) PS-NPs to physiologically relevant gastrointestinal conditions, a computer controlled *in vitro* model was used (Figure 1). This model mimics the dynamically changing luminal conditions of the stomach and small intestine (tiny-TIM) of adults or infants and is described in detail by Havenaar et al. [32, 33]. In short the gastric and small intestinal compartments consist of flexible tubes inside a water filled glass jacket. Decreasing and increasing the volume of water (37 °C) in the jacket results in peristaltic movements and mixing of the luminal content with secretion fluids. Secretion fluids are pumped into each compartment, resulting in physiological relevant luminal concentrations. In the gastric compartment, gastric electrolyte

solution (artificial saliva: NaCl, KCl,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  at 0.5 mL/min) with alpha-amylase (Sigma Aldrich), gastric acid (1 mol/L at 0.5 mL/min), pepsin (Sigma Aldrich) and lipase (FA-P 15) were added. In the small intestinal compartment, bicarbonate (1 mol/L), bile (porcine bile extract, Sigma Aldrich, 4%, 0.5 mL/min) and pancreatic juice (Sigma Aldrich, 4x USP, 0.25 mL/min) was added at physiological levels and flow rates (Verwei et al. [33]). The gastric and small intestinal compartments are connected by peristaltic valves, mimicking the pyloric sphincter (allowing passage of particles 3–5 mm) and controlling the transit of solid and liquid luminal content from the gastric into the small intestinal compartment. Dialysing units (Sureflux<sup>TM</sup>, cut-off 7.5 kDa, Nipro Europe, Breda, the Netherlands) connected to the small intestinal compartment allowed the removal of digested materials to maintain the physiological composition of the chyme.

Experiments in tiny-TIM (n = 6) were performed using simulation of fed state conditions of healthy young adults as described before [34]. In short, the half time of gastric emptying was set to 50 mins, with a  $\beta$ -value of 1 (determining the shape of the gastric emptying curve), representative for the gastric behaviour of a light meal. The gastric intake consisted of 128 mL water and gastric electrolytes, 15 mL of citrate buffer to mimic the buffering capacity of a meal, 11 mg of alpha-amylase, 5 mL of gastric start residue and 2 mL of the respective PS-NP solution (2.5% w/v). The gastric pH followed a pre-set gastric pH curve from pH 6.0 to 3.5, 2.5 and 2.0, after 30, 60 and 90 mins, respectively. The gastric pH is computer controlled, in this way HCl is secreted when the actually measured gastric pH is higher as the set point and water is secreted upon a lower actually measured gastric pH. The small intestinal pH was kept at 7.0 during the experiment, representing an average small intestinal pH. Gastric luminal samples were withdrawn at t = 10, 60 and 90 mins and small intestinal luminal samples (*i.e.*, the biorelevant matrix) were withdrawn at t = 60, 120 and 180 mins for determining the bioaccessible concentrations and size distributions. For each PS-NP, small intestinal luminal samples from t = 120 mins were used for the permeability studies.

### Permeability studies with porcine intestinal tissue

Permeability studies (n = 4) with porcine intestinal tissue were performed as described in Westerhout et al. [27]. In short, intestinal tissue of four domestic pigs (*Sus scrofa domestica*, both male and female, bodyweight between 15 and 25 kg, one pig per day) was excised, flushed with ice-cold Krebs-Ringer Bicarbonate buffer (containing 10 mM glucose, 25 mM HEPES, 15 mM sodium bicarbonate, 2.5 mM calcium chloride, pH 7.4, and saturated with oxygen using a 95%/5%  $\text{O}_2/\text{CO}_2$  mixture by gassing for 120 mins, further indicated as KRB), stored in ice-cold KRB, transported to the lab, and immediately used for *ex vivo* preparation. Small pieces of intestinal tissue were opened longitudinally, continuously submerged under ice-cold oxygenated KRB buffer, and the serosa and muscularis propria were carefully stripped from the mucosa using a forceps. With a hollow punch, segments with a diameter of 20 mm were obtained. The basolateral sides of the epithelial segments were then, without stretching the



tissue, mounted on nylon mesh supports (NITEX 06-390/47, 390  $\mu\text{m}$  pores, obtained from Sefar B.V., Lochem, and the Netherlands). Subsequently, the supported segments were mounted in the InTESTine™ system with the mucosal sides facing upwards, resulting in a tissue exposure area of 0.785  $\text{cm}^2$ . Epithelial tissue segments were placed in basolateral compartments containing 7.5 mL of ice-cold KRB. Next, 1 mL of ice-cold KRB buffer was added to the apical compartment. The complete system was then pre-incubated for 60 mins in a humidified incubator at 37 °C with 90%  $\text{O}_2$ , 5%  $\text{CO}_2$  for equilibration of the tissue.

After pre-incubation, the mucosal KRB buffer was used to remove the mucus build-up and was aspirated off. Afterwards, either 1 mL of KRB containing different concentrations of PS-NPs (125  $\mu\text{g}/\text{mL}$ , 250  $\mu\text{g}/\text{mL}$  or 500  $\mu\text{g}/\text{mL}$ ) or 1 mL of biorelevant matrix (containing the PS-NPs) was added to the apical compartment in triplicate ( $t = 0$ ), and the basolateral KRB buffer was replaced by 7.5 mL fresh, pre-warmed KRB buffer (37 °C). The InTESTine™ system was then incubated on a rocker platform at 60 rpm at 37 °C in an incubator that provides approximately 90%  $\text{O}_2$ , 5%  $\text{CO}_2$  and a high humidity. PS-NP absorption was measured by collecting samples from the apical (100  $\mu\text{L}$ ) and basolateral side (2 mL) after 45 and 105 min. At the first time point the basolateral KRB buffer was replaced by 7.5 mL pre-warmed KRB buffer (37 °C), whereas the apical sample remained untouched. Samples were analyzed as described below and recovery of the test compound was calculated. In each permeability study, parallel incubations were included to monitor the permeability of caffeine and mannitol, and leakage of fluorescein isothiocyanate-dextran, MW 4000 (FD4), a membrane integrity marker. Studies were only approved when the ratio of  $P_{\text{app}}$  caffeine/ $P_{\text{app}}$  mannitol was higher than 3 and the FD4 leakage was less than 0.5%.

### Analytical methods

Samples from both the apical and basolateral compartments containing the PS-NPs with a fluorescent core or FD4 were analyzed using a FLUO star OPTIMA fluorescence spectrometer (BMG Labtech, Ortenberg, Germany) at excitation wavelengths 530 nm or 490 nm and emission wavelengths of 645 or 520 nm for the PS-NPs and FD4, respectively. PS-NP calibration curves consisted of concentrations ranging from 0.1 to 250  $\mu\text{g}/\text{mL}$ .

Radioactive labeled compounds were measured on a Tri-Carb 3100TR Liquid Scintillation counter (LSC, Perkin Elmer, Boston, Massachusetts, United States) after adding scintillation liquid (Ultima Gold, Perkin Elmer Inc., Boston, Massachusetts, United States) to apical and basolateral samples.

The average size and the size distribution between 0.4 and 6000 nm of the PS-NPs in KRB and in the biorelevant matrix were analyzed with a Zetasizer-Nano ZS Instrument (Malvern, Etten-Leur, and the Netherlands). Dynamic Light Scattering (DLS) used to determine the size distribution of the particles is based on the quantification of dynamic fluctuations of light scattering intensity caused by Brownian motion of the particles. This technique yields a hydrodynamic

diameter that is calculated via the Stokes-Einstein equation from the aforementioned measurements. The result of the measurements is the average hydrodynamic diameter of the particles, the peak value is the hydrodynamic diameter distribution and the polydispersity index (PDI) that describes the width of the particle size distribution. The PDI scale ranges from 0 to 1, with 0 being monodisperse and 1 being polydisperse. Each assigned size and PDI was the mean of 9 runs. All measurements were carried out in triplicate with a temperature equilibration time of 1 min at 20 °C.

### Calculations

The apparent permeability values ( $P_{\text{app}}$  values,  $\text{cm}/\text{s}$ ) representing the apical (mucosal) to basolateral (serosal) permeability of the PS-NPs were calculated according to equation 1.

$$P_{\text{app}} = \frac{dA/dt}{SA * CA_{pi,0}} \quad (1)$$

Here,  $dA/dt$  indicates the appearance rate of the test compound at the basolateral side over time,  $SA$  is the surface area of the exposure area and  $CA_{pi,0}$  is the initial donor concentration of the PS-NPs.

## Results

### Digestion of PS-NPs in tiny-TIM

The impact of the different digestive processes on the size and availability of the different PS-NPs was studied in the tiny-TIM system, which simulates the physiological processes of digestion in the stomach and small intestine for healthy young human adults over time. The presence of electrolytes, bile, pancreatic juice and enzymes in the tiny-TIM system causes an increase in overall particle size in both the gastric compartment and small intestine compartment (Figure 2). The PS-NPs in the stock solution (dose material) had a mean size of 50 - 60 nm with a size range of 20 - 120 nm as determined with DLS (Figure 2A). Once administered to the gastric compartment, the average size increased to 1500 - 3000 nm (range 600 - 6000 nm) due to interaction with the gastric matrix (Figure 2B). In the small intestine compartment, the average nanoparticle size decreased to 150 - 850 nm, but the particle size range increased to 50 - >6000 nm (Figure 2C).

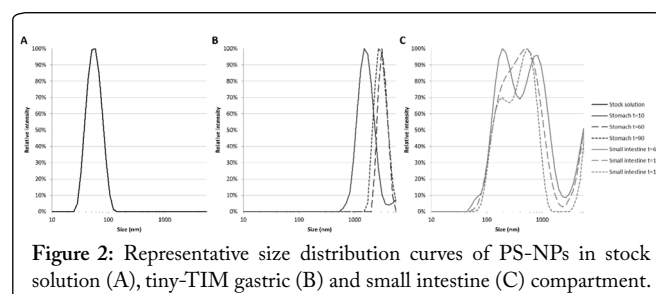


Figure 2: Representative size distribution curves of PS-NPs in stock solution (A), tiny-TIM gastric (B) and small intestine (C) compartment.

We determined the concentration of available PS-NPs in the small intestine compartment at  $t = 60$  and  $t = 120$  min. PS-NP concentrations in the small intestine at  $t = 120$

were between 1.5 and 2-fold higher than at  $t = 60$  min. Peak concentrations of  $55.5 \pm 1.8 \mu\text{g/mL}$  for the unmodified PS-NP,  $194.3 \pm 16.7 \mu\text{g/mL}$  for the aminated PS-NP and  $247.2 \pm 14.3 \mu\text{g/mL}$  for the carboxylated PS-NP in the small intestine compartment of the tiny-TIM system at  $t = 120$  min were measured.

### Permeability of PS-NPs in presence of biorelevant matrices

The permeability of aminated, carboxylated, or unmodified fluorescent PS-NPs in KRB and biorelevant matrix was measured across porcine jejunal tissue mounted in the InTESTine™ system. When the PS-NPs were applied to the tissue in KRB buffer, the average particle size on the apical side of the tissue was 50 - 60 nm as determined with DLS, with a size range from 20 - 150 nm (results not shown). In contrast, the average particle size in TIM matrix was at least 3-fold larger at 150 - 850 nm (Figure 2C). This difference is partially caused by the different matrix, since the biorelevant matrix without PS-NPs also contains particles in the size range of 100 - 3000 nm (results not shown). Interestingly, the size of the PS-NPs that were found in the basolateral compartment were comparable with an average particle size of 160 - 220 nm for the PS-NPs from KRB (Figure 3A) and 190 - 260 nm for the PS-NPs from TIM matrix (Figure 3B).

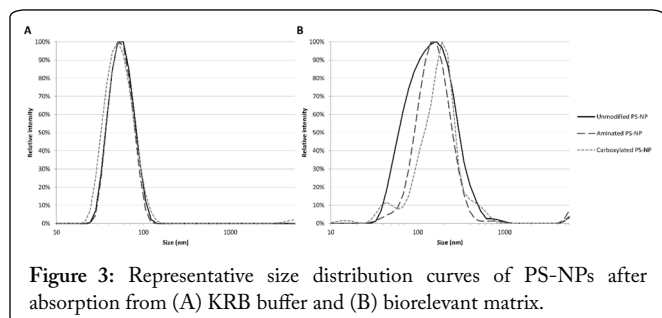


Figure 3: Representative size distribution curves of PS-NPs after absorption from (A) KRB buffer and (B) biorelevant matrix.

Exposure of porcine jejunal tissue segments to increasing concentrations of PS-NPs in KRB buffer resulted in a dose related decrease in the relative fraction absorbed (Table 1). The unmodified PS-NPs were found to be absorbed to the highest extent, ranging between 0.25 and 1.09%, followed

Table 1: Absorption parameters for the different NPs in KRB buffer.

Nanoparticle	Apical concentration in KRB ( $\mu\text{g/mL}$ )	Amount absorbed ( $\mu\text{g}$ )	Fraction absorbed (%)	$P_{\text{app}}$ value ( $\ast 10^{-6}$ cm/s)
Unmodified PS	127.8-127.9	1.32-1.45	1.03-1.14	3.66-4.02
	213.4-249.5	0.95-1.45	0.38-0.68	0.96-2.41
	470.6-473.2	1.03-1.29	0.22-0.27	0.78-0.97
Aminated PS	102.2-102.5	0.72-0.79	0.71-0.77	2.50-2.72
	206.0-244.5	0.65-0.72	0.29-0.32	1.04-1.12
	n.d.	n.d.	n.d.	n.d.
Carboxylated PS	102.0-103.2	0.38-0.50	0.37-0.48%	1.31-1.71
	197.9-254.4	0.19-0.38	0.08-0.19	0.27-0.67
	503.8-508.9	0.27-0.31	0.05-0.07	0.18-0.21

n.d.: not determined

by the aminated PS-NPs (up to 0.74%) and carboxylated PS-NPs (up to 0.43%). Even though the fractions absorbed were dependent on the apical concentration applied, the total amounts absorbed for each PS-NP were comparable for each of the doses tested. Apparent permeability ( $P_{\text{app}}$ ) values ranged between  $0.87$  and  $3.84 \ast 10^{-6}$  cm/s for the unmodified PS-NPs, between  $1.1$  and  $2.6 \ast 10^{-6}$  cm/s for the aminated PS-NPs and between  $0.20$  and  $1.51 \ast 10^{-6}$  cm/s for the carboxylated PS-NPs.

When the PS-NPs were present in the biorelevant matrix, we found very limited absorption with basolateral concentrations close to, or even lower than, the estimated limit of detection ( $0.02 \mu\text{g/mL}$ ). Consequently, the calculated overall fraction absorbed is less than 0.3% for all PS-NPs, with  $P_{\text{app}}$  values of less than  $1 \ast 10^{-6}$  cm/s (Table 2). Even though we were not able to quantify the fraction absorbed, the DLS measurements support the observation that absorption of PS-NPs from biorelevant matrix does occur. In comparison to the fraction absorbed from KRB buffer, the fraction absorbed is up to 3-fold lower when PS-NPs are present in biorelevant matrix. However, when looking at the actual amount absorbed, the difference is between at least 2-fold for the carboxylated PS-NPs and at least 9-fold for the unmodified PS-NPs, indicating a restricted absorption from biorelevant matrix.

Table 2: Absorption parameters for the different NPs in biorelevant matrix.

Nanoparticle	Concentration in biorelevant matrix ( $\mu\text{g/mL}$ )	Amount absorbed ( $\mu\text{g}$ )	Fraction absorbed (%)	$P_{\text{app}}$ value ( $\ast 10^{-6}$ cm/s)
Unmodified PS	53.7-57.7	$\leq 0.15$	$< 0.3$	$< 1$
Aminated PS	181.3-218.7	$\leq 0.15$	$< 0.1$	$< 0.3$
Carboxylated PS	233.2-266.5	$\leq 0.15$	$< 0.1$	$< 0.3$

## Discussion

In the present study we successfully applied a combined approach to assess the oral absorption of nanoparticles from biorelevant matrices using a dynamic *in vitro* human digestion model in combination with *ex vivo* porcine intestinal tissue.

With an ever increasing use of NPs in consumer products, it is essential to establish the potential risks of such materials for humans [35, 36] as early as possible. Since the actual risk of NPs is determined by the relationship between exposure and hazard, both the exposure and hazard assessment are important. With NPs that can enter the body via the oral, dermal and inhalation routes, it is already quite a challenge to estimate the exposure of NPs specifically from food products. Because of the different exposure routes and the problems associated with quantification of NPs, it is even more difficult to achieve a good estimate of a potential systemic exposure to these NPs. In case of potential oral exposure to NPs in food products, the first step is often to determine their behavior in digestive fluids and subsequent absorption potential of the NPs [37].

For the estimation of oral absorption using *in vitro* models, the transport of compounds across an epithelial monolayer of Caco-2 cells is often applied. However, the absorption of NPs is assumed to occur primarily via transcytosis in the M-cells of the Peyer's Patches [37]. In addition, clathrin- and/or caveolin-mediated endocytosis of NPs is also possible, but these processes are under-represented in Caco-2 cells [38]. Different co- or tri-cultures with mucus secreting goblet cells and/or M-cells have been used to get more representative results of these studies for the *in vivo* situation, but it remains difficult to accurately mimic the physiological complexity of the gastrointestinal tract *in vivo* with regard to anatomy and physiological processes [2, 8-10]. Alternatively, animal studies can be applied to determine the oral absorption of NPs in physiological environments and during physiological process. However, animal studies may not always be predictive for the human situation with regard to oral bioavailability [39], due to differences in anatomy and physiology [5, 6].

This implies the need for more predictive models for the human situation, while taking into account the essentials of the 3R principle in replacing, reducing, and refining animal testing [40, 41]. To that extent, the use of *ex vivo* human intestinal tissue segments has been suggested [8]. However, due to the limited availability of sufficient healthy human intestinal tissue to investigate the absorption of different types of compounds under different conditions in a more high throughout fashion, we have previously suggested the use of *ex vivo* intestinal tissue segments of pigs [27]. The limited number of available permeability studies of NPs with *ex vivo* intestinal tissue segments (either from humans or animals) all have one thing in common: none of these studies have studied the absorption of nanoparticles from a physiologically relevant matrix but rather used saline or a buffer such as KRB buffer. Consequently, even though the tissue segments are more representative of the *in vivo* situation than the different cell systems, the lack of a biorelevant matrix limits the translational options. It has been shown before that the different digestive processes to which the NPs are exposed *in vivo* will alter their characteristics and thereby potentially also their absorption [2, 8].

In our efforts to mimic the human *in vivo* digestive processes as closely as possible we suggest that more sophisticated models than the commonly applied stirred flask are required. To that extent, we have previously developed a dynamic computer-controlled *in vitro* gastrointestinal model of the stomach and small intestine (TIM-1; Minekus et al. [24]). This system consists of four compartments representing the stomach, duodenum, jejunum and ileum and offers insight into the solubility, release and availability for absorption of a compound within the GI tract. Recent efforts aimed at reducing the complexity of the system while maintaining its predictive power for the human situation and increasing the throughput [32-34, 42]. This has resulted in the tiny-TIM system as applied in this study. In contrast to the TIM-1 system, the tiny-TIM system only has one intestinal compartment and no ileal effluent, but luminal conditions are comparable between both systems.

In a previous study, it was shown that undiluted luminal

TIM-1 samples can be applied to *ex vivo* intestinal tissue from pigs to determine the fraction absorbed of pharmaceutical compounds under physiological conditions [27]. In contrast, undiluted luminal TIM-1 samples were found to be cytotoxic for both Caco-2 cells, as well as a co-culture of Caco-2 and mucus secreting goblet cells (HT29-MTX). It was also shown that the intestinal tissue mounted in the InTESTine™ system is viable with an intact and functional barrier (including metabolism and active transport processes) within the time period tested (120 min). In this study, the same approach was applied to establish the absorption of nanoparticles. We selected PS-NPs with a fluorescent core for this study since they are non-biodegradable and thereby allow quantification of the absorption of intact NPs and not just the fluorescent label. In addition, by selecting PS-NPs with different surface modifications (aminated, carboxylated and unmodified), we were able to determine their relative impact on the absorption of the NPs. Ideally, these studies should also be performed with other NPs for which oral exposure is more likely, such as biopolymeric NPs used in edible coatings and films [1], or silver NPs or silicon dioxide NPs from migration from food contact materials [43]. However, determining the digestion and absorption of these type of NPs presents additional analytical challenges and needs to be addressed in future research.

As a proof of concept, the absorption of the different PS-NPs from KRB buffer was first studied. When different apical concentrations (between 125 and 500 µg/mL) were applied, comparable concentrations in the basolateral compartment were measured. This indicates that the absorption of nanoparticles under these specific conditions appears to be a saturable process. However, since some of the basolateral concentrations were close to the limit of quantification (0.05 µg/mL), we did not test any lower apical concentrations. We were therefore not able to establish the apical concentration at which the absorption is not saturated.

In our next step, we applied a dose of 50 mg PS-NPs to the gastric compartment of the tiny-TIM system and applied the luminal samples of the intestine compartment at t = 120 to the apical compartment of the InTESTine™ system. Interestingly, the different surface modifications had a significant impact on the bioaccessible (i.e., available for absorption) concentrations in the biorelevant matrix, which were lowest for the unmodified PS-NPs and highest for the carboxylated PS-NPs. Initial experiments with a lower dose of 25 mg resulted in a similar ratio of bioaccessible concentrations (results not shown), indicating that the type of surface modification determines the level of interaction with the biorelevant matrix. Unfortunately, the fraction absorbed from the biorelevant matrix was too low for accurate quantification but we did establish that the absorption of PS-NPs takes place, albeit to a lesser extent than when using KRB buffer. The increase in average particle size in biorelevant matrix to 150 - 850 nm could possibly explain why the absorption is reduced when compared to KRB buffer, since larger particles are generally absorbed less well [19, 20]. In addition, the presence of the biorelevant matrix may trigger mucus secretion of the tissue segments and thus provide an extra barrier for absorption.

The fractions of PS-NPs absorbed from KRB, as observed in this study, are relatively high (0.06 - 1.09% in 1h) when compared to the results obtained by Walczak et al. [18], who studied the absorption of the same PS-NPs from cell culture medium across Caco-2 cells, a co-culture of Caco-2 and HT29-MTX cells and a tri-culture with Caco-2 cells, HT29-MTX cells and M-cells (0 - 7.8% in 24 h). This difference could be caused by the difference in average particle size used in the dose solutions. In our study, the average particle size of the PS-NPs in dose solutions as determined with DLS was 50-60 nm (ranged from 20 - 150 nm), while the average particle size of the Walczak study was between approximately 100 and 200 nm, and larger particles are generally less well absorbed [18]. Walczak and colleagues also studied the effect of *in vitro* digestion on the absorption of the same PS-NPs across Caco-2 and HT29-MTX cells [22]. In contrast to our own study, which showed that upon *in vitro* digestion a decrease of absorption of PS-NPs is observed, the Walczak study showed an increased absorption after *in vitro* digestion. This difference in results may be explained due to the fact that the digestion matrix in the Walczak study had to be diluted to prevent cytotoxicity to the Caco-2 and HT29-MTX co-culture. This dilution may influence the characteristics of the PS-NPs, which in turn may influence the absorption. In addition, it is known that the Caco-2 and HT29-MTX co-culture do not mix well in culture, resulting in a discontinuous mucus layer [8]. Without proper protection the diluted digestion matrix may still be cytotoxic to the Caco-2 cells to a certain degree, which in turn may have led to increased absorption of the PS-NPs.

In order to determine the appropriate *in vitro* model to study the intestinal absorption of NPs, Schimpel and colleagues studied the absorption of PS-NPs across Caco-2 cells, a Caco-2 and HT29-MTX co-culture and a Caco-2, HT29-MTX and M-cell tri-culture, which was then compared to the absorption of neutral PS-NPs (50 and 200 nm) across *ex vivo* intestinal tissue from pigs [9]. Interestingly, they reported that basolateral concentrations of PS-NPs were comparable between the study using *ex vivo* porcine tissue and Caco-2 cells. In contrast, basolateral concentrations were significantly lower when using the Caco-2 and HT29-MTX co-culture, whereas the highest basolateral concentrations were observed with the Caco-2, HT29-MTX and M-cell tri-culture [9]. Unfortunately, the apical concentration and basolateral volumes were not reported for the study with *ex vivo* porcine intestinal tissue, so it is not possible to derive the actual fraction absorbed from the reported concentrations. Therefore, the comparison of basolateral concentrations between porcine intestinal tissue and the different cell systems is not fully justified.

All in all, there is currently no single *in vitro* or *in vivo* method available that allows accurate prediction of human oral bioavailability for a wide variety of compounds, including NPs. Often, permeability studies are combined with metabolism studies to allow prediction of human oral bioavailability. As described above, there are numerous different *in vitro* and *in vivo* methods to determine intestinal permeability, which can be a good predictor of the fraction absorbed in humans for a specific set of compounds [11-13, 44-46]. In addition, there are

different intestinal metabolism methods (e.g. van de Kerkhof et al. [47]) and liver metabolism methods (e.g. Zhang et al. [48]) available that allow estimation of pre-systemic metabolism. It has been shown before that a combination of different *in vitro* and *in vivo* methods allows a better prediction of human oral bioavailability [49, 50]. This combined approach appears to be promising for common chemicals, such as pharmaceuticals, since pre-systemic metabolism is known to potentially alter the molecular structure, thereby reducing the bioavailability of the parent compound. However, the potential impact of food matrices on the release, absorption and metabolism of these chemicals is often neglected [51, 52].

In case of NPs, pre-systemic metabolism may leave the particles intact, while their surface properties are altered. Our results indicate that digestive processes could influence the size and surface characteristics of the NPs, which in turn could influence their oral bioavailability. This has major implications for the risk assessment, since the right size and surface characteristics of the NPs should be considered in the appropriate toxicity tests. We therefore suggest that a combination of physiologically more relevant intestinal digestion and absorption models is required for an improved prediction of both the digestion and the absorption of NPs in humans. This approach can also be applied to other chemicals or formulations for which an effect with the food matrix is to be expected. However, this approach also has its limitations: 1) the biorelevant matrix limits the analytical possibilities with regard to characterizing and quantifying the NPs; 2) the relatively short time for absorption (60 min) requires a sensitive analytical method to detect low concentrations of NPs; 3) extrapolation of study results to the human situation has not yet been established. For risk assessment purposes, we would like to suggest to expand the current setup by applying the digested and absorbed NPs, rather than pristine NPs, to the appropriate toxicity tests.

In order to be able to extrapolate these results for the safety assessment of oral exposure to NPs, additional research is required. First, we need to establish if the *in vitro* digestion of NPs in the tiny-TIM or TIM-1 system results in the same NP forms and characteristics as *in vivo*. Then, we need to establish for which types of NPs *ex vivo* porcine intestinal tissue is a good surrogate to human intestinal tissue to study intestinal absorption. And finally, we need to incorporate first-pass metabolism into the equation, since all elements combined will determine the oral bioavailability of NPs and ultimately the systemic exposure to NPs. As a first step in that direction, we have started studying the absorption of different chemicals across both *ex vivo* human and porcine tissue and found similar results with regard to the calculated  $P_{app}$  values (unpublished results). This is promising with regard to being able to use generally more available *ex vivo* porcine intestinal tissue instead of human tissue for more fundamental research questions, including regional absorption across healthy and diseased tissue in the presence of different food matrices.

## Conclusion

In this study, we have successfully applied the tiny-TIM

system in combination with *ex vivo* porcine intestinal tissue to study the effect of *in vitro* digestion on the absorption of PS-NPs from undiluted biorelevant samples. We showed that aminated and carboxylated PS-NPs have higher bioaccessible concentrations after digestion when compared to unmodified PS-NPs. In addition, the digestion resulted in a reduced absorption when compared to the absorption of the same particles from KRB buffer. Unmodified PS-NPs were absorbed to a higher extent than aminated PS-NPs, with the lowest level of absorption for the carboxylated PS-NPs. Current results do not allow estimation of human oral bioavailability, but nevertheless proves that oral absorption of PS-NPs across the intestinal wall is to be expected *in vivo*. This indicates that the risk assessment should consider systemic toxicity as a realistic possibility. In the risk assessment of potential oral exposure to NPs in food products, it is key that the exposure and hazard assessment are performed with the physiologically relevant size and surface characteristics of the nanoparticle under investigation, since each specific nanoparticle may have specific absorption and related hazard properties.

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