Annex 3 rev: Gastrointestinal Absorption of Microparticles and Nanoparticles after Oral Administration

According to the European Commission Recommendation, a **nanomaterial** is "*a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm*." (www.ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm)

There is a generally accepted cut-off between powders and massives of 1 mm particle diameter (e.g. for aquatic toxicity classification under CLP ECHA Guidance section IV.5.5 rev. 5 2017 or in Annex 9 of the UN GHS A9.7.5.4.3 GHS rev. 7 2017). Thus, micron-size particles can be expected to have diameters spanning from > 0.1 µm to < 1 mm.

Due to the difference in surface-to-volume or surface-to-mass ratio between nanoparticles (NPs) and microparticles (MPs), their behavior in the gastrointestinal tract following oral administration may differ. There are however, some similarities in the absorption of both MPs and NPs. The report below looks at the kinetics of gastrointestinal tract (GIT) absorption of MPs and NPs, and the factors affecting absorption.

Humans have been exposed to MPs and NPs in food and the environment throughout evolution. In fact, humans have evolved to incorporate MPs and NPs in their physiological machinery. For example, hemoglobin is a natural nanoparticle in the body, with a diameter of only 55 Å or 5.5 nm. Physiological MPs are found throughout the body and play varying roles, such as acting as messengers of biological information in different cellular processes (Hoyer et al, 2010). There are numerous uses of MPs and NPs, from the food industry, medicine, cosmetic industry to engineering. There is no escaping MPs and NPs, which are found naturally and through anthropogenic activities in foods, air (dust), beauty products, and general household products. MPs and NPs have been investigated and used for delivery of drugs and vaccines; nanomedicine is a pioneering field in the clinical applications of NPs. Thus, an understanding of how the body absorbs and handles these particles is important for risk assessment purposes and applications in these industries. O'Hagan (1996) writes that "*it is crucial that the quantitative extent of uptake is defined in the target species and the level of variability is determined*." As will become evident below, this is important due to the multivariate factors that affect absorption of particles in the GI tract. Some of the chemical factors that affect absorption of particles are **composition,** and **ion-to-particle ratios in aqueous suspensions**, whilst the physical factors include **particle size, size distribution, surface charge, polarity**, and **attachment of biomolecules**. Only the most relevant factors that research has demonstrated to have significant impact on particle absorption will be considered below. In bold are the particle sizes, length of exposure/administration and the methods used to measure particle uptake. Worth noting here is the fact that the absorption and distribution kinetics of particles administered via routes other than oral, such as inhalation, is different. The fluids, mucus layer, biomolecules that bind particles, and general absorption mechanisms are different between the oral and inhalation routes.

Four main mechanisms have been described for the gastrointestinal uptake of particles, namely persorption, phagocytosis, endocytosis, and transcytosis/transparacellular. The table below, from O'Hagan (1996), summarizes the mechanisms of particle uptake.

Table 1. *Mechanisms proposed to be involved in the uptake of particles*

Site/mechanism	Particle size
Villus tips—persorption	5–150 μm
Intestinal macrophages—phagocytosis	1 μm
Enterocytes—endocytosis	< 200 nm
Peyer's patches—transparacellular	< 10 μm

Persorption is a passive process by which particles in the GIT paracellularly penetrate the epithelial cell layer into the subepithelial layer from where they enter circulation via the lymph vessels and mesenteric veins (Volkheimer, 1974). The shedding of enterocytes from the villous tip into the gut lumen creates '*holes*' in the epithelium, permitting the translocation of both large and small particles. Persorbed particles are transported from the intestines by the lymphatic and portal systems. Per Volkheimer (1974), orally or rectally administered particles in the range of **15 to 90 μm** or occasionally **150 μm**, such as starch granules, silicate crystals, and powdered rabbit hairs were found in the region of the lumen of lymph vessels of rats, rabbits, dogs, chicken and guinea pigs corresponding to parts of the intestine where the particles were located. These particles were sometimes found in body fluids, chyle, blood, bile and urinary bladder a few minutes after oral administration. Additionally, **iron particles** up to **52 μm** were found in the chyle of dogs fed metallic iron powder. The rate of persorption is increased by cigarette smoking, caffeine and deep physiological sleep. Using **transmission electron microscopy** (TEM), Hillyer and Albrecht (2001) reported that NPs in size range **4 to 58 nm** were persorped through intestinal epithelium '*holes*' created by dead and degrading enterocytes, unequivocally demonstrating that NPs can also undergo persorption. Wells et al (1988) demonstrated that **1.09 μm fluorescein-labeled or 0.89 μm rhodamine-labeled latex beads** separately implanted into intestinal segments of dogs and rats were absorbed into the draining mesenteric lymph node by intestinal **phagocytic cells** consistent with macrophages.

Further, although the regular function of the intestinal enterocytes is the uptake and transport of nutrients, they can also participate in the uptake of particles of **100 nm** average size by the process of **endocytosis**. As early as 1961, Sanders and Ashworth demonstrated the **pinocytic** transport (a type of endocytosis) in intact vesicles to the lymphatic system of **220 nm polystyrene latex particles** orally administered to rats. The predominant and most documented mode of transport of MPs and NPs is **transcytosis** through the M-cells of the Peyer's patches. A single intraoral delivery of **1.82 μm nonionic fluorescent polystyrene latex microspheres** to nonfasted

male Sprague-Dawley rats showed a higher uptake of the particles in the proximal Peyer's patch region (Hazzard et al, 1996), demonstrating the importance of the Peyer's patches in the uptake of particles. Uptake of whole particles were confirmed by maceration of tissues (bulk) or cryosectioning of tissues (morphological) followed by **epifluorescence.** A very early study by Sanders and Ashworth (1961) using **2200 Å (220 nm)** emulsions of **polystyrene latex particles** orally administered to rats demonstrated uptake into the intestinal and liver cells. **Electron microscopic** investigation of the absorption and transport pathway of the polystyrene latex particles showed uptake into the intestinal epithelium and transport in intact vesicles through the cytoplasm and ultimately into the lymphatics of the mucosa. This was one of the first studies to show the uptake and transport pathway of particles to organs following oral administration.

In addition to the above enumerated factors that affect particle absorption, confounding differences in **diet, pH, GI transit time, GI flora, composition and secretion rates of mucus, dose, species, age** and **gender** influence the GI absorption of MPs and NPs after oral administration (Frohlich & Roblegg, 2012). In 2007, Doyle-McCullough and colleagues demonstrated, following oral administration of **2 µm plain, non-ionic yellow-green fluorescent polystyrene latex microspheres** and estimation of particle numbers absorbed by **fluorescence microscopy**, that young adult rats of 7 weeks absorbed significantly more of total and percentage MPs than younger (3 weeks) or older (52 weeks) rats. Additionally, the authors demonstrated slightly higher MP uptake in female than male rats but only in the deeper regions and Peyer's patches. On a similar note, Seifert and colleagues (1996) investigated the bioavailability of **FITC-labelled 1 µm polystyrene** particles enterally administered to rats. The authors observed a higher uptake (775 particles) into lymph by 5-month-old animals than 9-month-old (518 particles) or 6-8-weeks-old animals (87 particles). The **effect of dose** on uptake was observed as increased number of particles in the lymph with increased dose of particles administered.

An important study by Sunderman and colleagues (1989) demonstrated that "*dietary constituents profoundly reduce the bioavailability of Ni^{2+} for alimentary absorption; approximately one-quarter of nickel ingested in drinking water after an over-night fast is absorbed from the human intestine and excreted in urine, compared with only 1% of nickel ingested in food."* This study was conducted in both male and female human subjects administered nickel sulphate (12-50 µg Ni/kg) added to drinking water or food after a 12-hr overnight fast, with Ni content in feces, blood and urine analyzed by **electrothermal atomic absorption spectrophotometry**. Compared with 0.7 % of Ni absorbed when ingested with food, there was ~40-fold increased absorption (27%) of Ni when ingested with drinking water. This effect of dietary constituents and fasted/nonfasted state on absorption of Ni after oral administration has been observed in another study by Solomons et al, 1982. The authors here observed that the absorption of 22.4 mg nickel sulfate hexahydrate is affected by beverage type (whole cowmilk, coffee, tea, orange juice, coca cola), dietary constituents (ascorbic acid, phytic acid), distinct foods (Guatemalan meal and North American breakfast), and chelating agents (NaFeEDTA). These studies demonstrate an **effect of dietary constituents** on particle uptake.

Hillery et al (1994) demonstrated that there is more uptake from lymphoid than non-lymphoid tissues of **60 nm polystyrene NPs** administered to female Sprague-Dawley rats by **5-days oral gavage** at 14 mg/kg. **Fluorescence microscopy** showed that there is 60% uptake in the Peyer's patches of the small intestine, compared to non-lymphoid tissues, demonstrating an effect of **GI tissue type** on particle absorption. Length of particle administration has been demonstrated to affect uptake. A higher uptake of **2 µm latex microspheres** at same dose was observed after prolonged feeding of same dosage for about **60 days** compared to **3 days** (Le Fevre et al, 1978). The surface charge of NPs, which strongly determines agglomeration and uptake in the GI, is affected by pH changes in the GIT, from the near neutral pH in the buccal cavity and small intestine to the strongly acidic pH in the stomach. Further, the GI transit time is not constant but varies between humans and animals, and healthy versus diseased states (Coupe et al, 1991). Variations in the thickness (Atuma et al, 2001; Matsuo et al, 1997) and pH (Schreiber et al, 2000) of the mucus along the length of the GI tract account for the differential absorption rates of different particles. The mucus layer is thick in the stomach and in the intestine, the thickness decreasing from the colon to the jejenum. Whilst the mucus in the buccal cavity and small intestines is nearly neutral, the gastric mucus is strongly acidic with a pH gradient existing along the length of the stomach. These and other multivariate factors, although not considered nor accounted for in most GI absorption studies, must be at the back of one's mind when interpreting these studies. Figure 1 below summarizes some factors influencing the GI absorption of particles. Table 1 below summarizes the oral absorption of selected particles, with some emphasis on metallic particles.

Table 1. Summary of GI absorption results for selected particles

Particle type, size, surface modifications	Dosage Duration	Species	Tissues examined	Absorption results	References
Fluorescent polystyrene, nonionic & carboxylated; 100, 500 nm, 1 & 3 µm	2.5% w/v 1.25 mg/kg/day, 10 days oral	Sprague Dawley rats (females)	Heart, lungs, kidneys, villi, spleen, Peyer's patches	Size-dependent uptake; higher uptake of 100 nm nonionic than carboxylated; no uptake of 3 µm	Böchmann et al, 2000
Fluorescent polystyrene, tomato lectin or N-acetylchitotetraose conjugated;500 nm	12.5 mg/kg 5-day oral gavage	Wistar rats (females) fasted	Colon, liver, spleen, lungs, kidneys, S.I., heart, stomach	23% systemic uptake of tomato lectin-conjugated vs <0.5% uptake of N-acetylchitotetraose-blocked	Abbracchio et al, 1982
Polystyrene, 50, 100, 300, 500 nm, 1 & 3 µm	1.25 mg/kg 10-day oral gavage	Sprague Dawley (females) fasted	Liver, spleen, blood, bone marrow	34% (50 nm), 23% (100 nm), 7% (1 µm) uptake. 3 µm ND in most tissues	Schreiber et al, 2000
TiO_2, anatase/capsular, 160 & 380 nm	Single oral	Human volunteers	Blood	Size-dependent direct bimodal uptake at 2 hr. with peak at 6 hr. post-administration	Jani et al, 1992
f-TiO_2, 260 nm	100 mg single oral	Human volunteers	Blood	Direct bimodal uptake into blood	Eldridge et al, 1990
TiO_2, 15, 100 nm, <5 µm	5 mg/kg bw, single oral	Human volunteers	Urine	<0.1% uptake of given dose	Wang et al, 2007

TiO_2, 500 nm	2.5 % w/v 12.5 mg/kg, 10 days	Wistar rats	Liver, spleen, lungs, peritoneal tissues	11.9% uptake of given dose	Cho et al, 2013
f-TiO2, with (w/v) 1% albumin or 1% glucose, 117 nm	500 mg/kg, single oral	Sprague Dawley rats	Lungs, liver, spleen, kidneys	0.7 % uptake with 1% albumin, 0.30% with 1% glucose, 0.01% with DW	Sadat et al, 2016
TiO_2, 7-220 nm	34.1-59.9 mg/kg bw, 5-day oral	Wistar rats	Liver, spleen	<0.09 µg Ti/g tissue absorbed, size-dependent uptake	Jani et al, 1994
TiO_2, 25, 80, 155 nm	5 g/kg bw, single oral	ICR mice, fasted	Lungs, liver, kidneys, spleen	<0.01% uptake of given dose.	Park et al, 2010
TiO_2, 26 nm ZnO, 89 nm	10 ml/kg bw, 7 days/wk for 13 wks	Sprague Dawley rats	Liver, spleen, urine, brain, kidneys, feces	Uptake Zn >Ti. At same dose, uptake of ~3 µg/g Zn & ~0.4 µg/g Ti in blood	Jani et al, 1989
Au, metallic colloidal; 4, 10, 28, 58 nm	$2x10^5$ ppb, 7-day ad libitum	BALB/c mice	Blood, brain, liver, lungs, heart, S.I., kidneys, etc	Size-dependent uptake, uptake in lungs of 32.42 ppb (4 nm), 8.55 ppb (10 nm), 0.80 ppb (28 nm).	Volkheimer et al, 1974
Au, carboxylated or aminated 2.8 nm; 1.4, 5, 18, 80, 200 nm	1-27 µg/kg intra-oesophageal instillation	Wistar-Kyoto rats	Blood, RES, kidneys, S.I., L.I., lungs, heart, brain, urine	Size-dependent uptake; 0.37% of aminated vs 0.14% of carboxylated. <1% given dose absorbed.	Le Fevre et al, 1978
AgNPs, 22, 42, 71, 323 nm	1 mg/kg 14-day oral	ICR mice	Liver, lungs, brain, testis, kidneys	Size-dependent uptake. No uptake of 323 nm. NOAEL = 100 $\mu g/m^3$ for liver, lungs.	Pele et al, 2015
$AgNO_3$; AgNPs uncoated 17.7 nm, PVP-coated 12.1 nm	9 mg/kg bw $AgNO_3$; 90 mg/kg bw AgNPs oral gavage	Sprague Dawley rats (males)	Liver, lungs, kidneys, S.I., L.I., spleen, stomach	<1% given dose absorbed. ~15 µg Ag/kg blood $AgNO_3$ & ~2 µg Ag/kg blood AgNPs.	Hussain et al, 1997
$AgNO_3$, AgNPs 3.8 nm	2 or 20 mg/kg single oral	Sprague Dawley rats (males)	Liver, lungs, kidneys	Dose-dependent higher uptake of Ag+ from $AgNO_3$ than AgNPs	Schäffler et al, 1997
AgAc; PVP-coated AgNPs 14 nm	90 mg/kg bw 28-day oral	Wistar rats (females)	Liver, lungs, kidneys, brain, blood, etc	Nano-sized granules in tissues of AgAc & AgNPs-exposed rats	van der Zande et al, 2012

ND = Not detected
S.I = Small intestine
L.I = Large intestine

Particle size, as demonstrated by numerous studies, is the single most important factor affecting absorption of MPs and NPs following oral administration. In a study on the effect of particle size on GI absorption, Desai et al (1996) used **biodegradable polylactic polyglycolic acid copolymer particle sizes** of **100 nm, 500 nm, 1 micron** and **10 microns**. Uptake of the particles was investigated by **histology** following *in situ* infusion of the particle suspensions in clamped duodenal and ileal segments of the small intestine. The authors observed that the *in situ* intestinal uptake of the 100 nm particles was 15-250X higher than uptake of the larger particles. The location of particles in the intestine and type of tissue in that location also influenced

absorption; absorption in the Peyer's patch tissue was 2-200 times higher than absorption from non-patch tissues from the same region of the intestine, in agreement with the Hillery et al (1994)

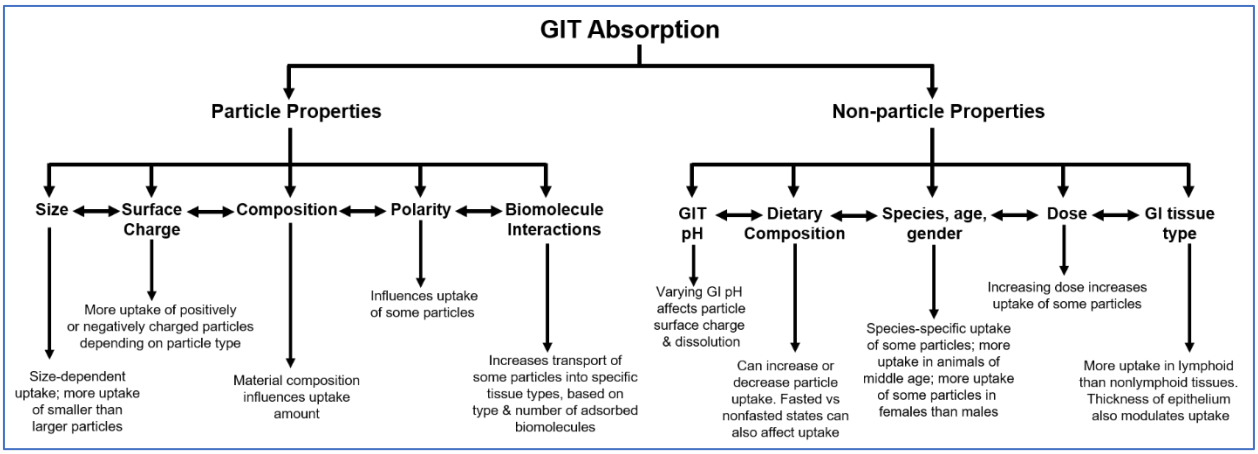


Figure 1. Summary of some factors influencing GI absorption of particles.
The interdependence of these factors in affecting uptake is indicated by sideways arrows.

study referenced above. This confirms that absorption occurs predominantly in the M-cells of the Peyer's patches. The experimental design and non-ionized property of the particles indicates the uptake of whole polyglycolic acid copolymer MPs. This *in situ* report of size-dependent particle absorption in the gastrointestinal tract has been confirmed by numerous *in vivo* studies. Jani et al (1990) investigated the absorption of **50 nm** to **3 µm monodispersed non-ionized polystyrene microspheres** given by oral gavage at 1.25 mg/kg for **10 days** to female Sprague Dawley rats. **Gel permeation chromatography** demonstrated size-dependent variation in the absorption of intact polystyrene microspheres. The absorption rates decreased from 34% for the 50 nm particles to 26% for the 100 nm particles; 7% of the absorbed 50 nm particles and 4% of the absorbed 100 nm particles were found in the liver, blood, spleen and bone marrow. Minimal-to-no absorption were observed for the larger sized particles.

According to Le Fevre and colleagues (1980), **5.7 µm synthetic spherical polystyrene MPs** but not **15.8 µm MPs** were absorbed into Peyer's patches, mesenteric lymph nodes and lungs of mice after chronic oral gavage for **60 days**. Here, it appears "*that most particles are sequestered in Peyer's patch macrophages. Particles that escape sequestration are transported by lymph rather than by portal blood*." The work of Jenkins and colleagues (1994) buttressed this statement by showing that there is more efficient and rapid (5 min) uptake of **0.15 µm and 1.0 µm fluorescent polystyrene microparticles** into mesenteric lymph of male Wistar rats, with absorption peaking 65 min after particle administration. In support of the higher absorption of smaller-sized MPs over larger-sized MPs, Ebel (1990) noted that **2.65 µm fluorescent, monodisperse, polystyrene latex spheres** were absorbed more than **9.13 µm latex spheres** in mice. Young female BALB/c mice were given **0.2 ml single** oral gavage of particle suspensions; fed mice were given ad libitum access to food and in fasted mice, particle administration was preceded by 12-hr fasting. Ebel (1990) observed a maximum absorption <0.01% of the 2.65 µm particles 48 hours after administration. These studies demonstrate direct uptake of the particulate forms of these

microparticles, with more uptake of the smaller-sized MPs than the larger-sized MPs. Please note that almost all the studies referenced thus far were conducted with polystyrene MPs. An upper limit of **≤10 µm**, depending on species, has been suggested for the uptake of particles into the Peyer's patches (Jani et al, 1992; Eldridge et al, 1990). In fact, particles between **5-10 µm** have been suggested to be more sequestered in the Peyer's patches whilst particles **<5 µm** escape sequestration and are transported in the lymph (Eldridge et al, 1990), suggesting that there is increased absorption and transport into secondary organs of particles <5 µm than particles >5 µm.

On the other hand, oral administration of micron-sized **anatase or capsular titanium dioxide** (TiO_2) particles to men aged 24-66 years showed direct absorption of particulate TiO_2 into the blood, as demonstrated by **inductively coupled plasma atomic emission spectroscopy (ICP-AES)** (Böckmann et al, 2000). In agreement with previous reports of the size-dependent uptake of particles, the **0.16 µm TiO_2** particles showed more absorption than the larger-sized **0.38 µm TiO_2**. These reports demonstrate that the size-dependent uptake of particles is species-independent; this size-dependent uptake has been demonstrated in rats, mice, and humans. In human subjects administered a **single oral dose** of **260 nm pharmaceutical/food grade anatase TiO_2**, **dark field microscopy** and **ICP-MS** confirmed that some of the TiO_2 was absorbed into the bloodstream in the particulate form (Pele et al, 2015). However, the authors did not quantify how much of the absorbed TiO_2 is contributed by the ionic or particulate forms. Jani and colleagues (1989) also investigated the uptake of **non-ionic and carboxylated (negatively charged) fluorescent polystyrene microspheres (100 nm, 500 nm, 1 µm, 3 µm)** orally administered to female Sprague Dawley rats for **10 days**, followed by **fluorescence microscopy** imaging of tissue sections. There was clear uptake and translocation of the non-ionic microspheres, with more uptake of the 100 nm and 500 nm microspheres and less efficient uptake of the 1 µm microspheres. Uptake of the carboxylated microspheres was less clear, even for the smaller-sized 100 nm particles. In contrast to the observation here on uptake of carboxylated microspheres, particle surface charge has been demonstrated to affect gastrointestinal absorption (see below). No uptake of the 3 µm particles was observed. Higher absorption of the smaller-sized particles was confirmed by orally administered ^{125}I-radiolabelled 100 nm and 1 µm microspheres. These studies buttress the size-dependent uptake of MPs and also demonstrate the uptake of whole particles.

Size-dependent particle uptake is significant not only for MPs but also in the uptake of NPs. According to Hillyer and Albrecht (2001), oral administration of **4, 10, 28 and 58 nm metallic colloidal gold (Au) NPs** (AuNPs) resulted in a size-dependent absorption; the smaller particles were more easily taken up than the larger particles. Using **Instrumental Neutron Activation Analysis (INAA)** to measure the amount of AuNPs absorbed into tissues and organs, Hillyer and Albrecht observed that the AuNPs were absorbed into blood, brain, lung, heart, kidney, spleen, liver, stomach and small intestine, with the 58 nm NPs not detected in the blood, lungs, heart and spleen. As previously stated above under absorption mechanisms, TEM of the 4 nm and 10 nm Au particles demonstrated persorptive uptake through degrading enterocytes. The electron micrographs also showed uptake of individual particles in the tissues, with no "*large particle-to-*

particle aggregates" due to the "*spontaneous conjugation of the particles to endogenous molecular species in the mouse saliva immediately upon contact, as indicated by salt stability tests*." The effect of such "*endogenous molecular species*" or biomolecules on particle absorption is succinctly considered below. In the Hillyer and Albrecht study, absorption is strictly of the particulate AuNPs since Au is chemically inert without any chemical modifications. On a similar note, a **14-day**, repeated oral administration of **silver (Ag)** particles to mice demonstrated that the smaller-sized NPs (**22 nm, 42 nm, 71 nm**) were absorbed and distributed to the liver, kidney, brain, lung, and testis but the larger-sized particles (**323 nm**) were not (Park et al, 2010). Here, the authors did not investigate if the absorbed Ag particles was due to absorption of particulate or ionic forms. It is interesting to note here that 323 nm Ag particles were not absorbed into the organs investigated, especially when considered that microparticles of different materials larger than this are absorbed, suggesting a nanomaterial (NM) or micromaterial effect on the size-dependent uptake of particles. Thus when comparing uptake across MPs and NPs of different materials, it is generally correct but too simplistic to assume more uptake of the NPs than the MPs.

In a study on the effects of NMs on absorption, Cho et al (2013) observed limited, concentration-independent absorption into blood of **26 nm TiO_2 NP** but a concentration-dependent increased absorption of **89 nm ZnO NP** into blood following oral administration for **7 days/week** for **13 weeks** to rats. Here, the type of NMs more than the size of NPs influenced GI absorption, given that larger-sized (89 nm) ZnO particles were absorbed more than smaller-sized (26 nm) TiO_2 particles. **ICP-MS** measurement of TiO_2 and ZnO in tissues showed no significant distribution of TiO_2 in liver, spleen, kidneys or brain but significant dose-dependent distribution of ZnO in the same tissues. The authors did not investigate if the absorption and tissue distribution was mostly due to ionic or particulate ZnO or TiO_2. However, the higher dissolution of ZnO compared to minimal dissolution of TiO_2 in acidic gastric fluid suggests that more ZnO particles than TiO_2 NPs dissolves in the stomach, releasing more Zn^{2+} ions that are absorbed into systemic circulation and may subsequently form Zn granules or particles. The absorption of ionized elements and subsequent formation of granules or particles *in vivo* has been demonstrated for soluble and nanoparticulate Ag (see van der Zande et al, 2012 below). How much of the absorbed ZnO particles and TiO_2 NPs is contributed by the ionic or particulate forms remains to be adequately addressed.

Studies of the effect of particle size on gastrointestinal absorption of orally administered TiO_2 MPs and NPs have yielded some contradictory results. In a report by Wang et al (2007), there was absorption (<0.01% of injected dose) into the liver, kidneys, lungs, and spleen of **25 nm, 80 nm** and **155 nm TiO_2 particle suspensions** given **once orally** to fasted adult female mice at a high dose of 5 g/kg bw; **only the liver absorptions of 25 nm and 80 nm NPs reached statistical significance**. It is worth noting here that there was more absorption of the larger sized 80 nm Ti into the liver than the smaller-sized 25 nm particles (3970 ng Ti/g liver versus 106 ng Ti/g liver respectively), contrasting with previous reports showing a size-dependent absorption of particles. But the fact that **this observation was made only for the liver suggests preferential**

accumulation or sequestration of the 80 nm particles in the liver. Here, Wang and colleagues measured elemental Ti levels in the tissues and blood with **ICP-MS**. Jones et al (2015) studied the uptake of 5 mg/kg bw of **15 nm, 100 nm** and **<5 µm TiO_2** particles dispersed in water and orally administered as a single dose to human volunteers (lower dose and different species from Wang et al study). The authors observed that <0.1 % of the administered dose was absorbed, with no difference in absorption between nano-sized and micron-sized TiO_2 particles. The latter observation is contradictory to previous observations referenced above. The smaller-sized TiO_2 particles were shown to agglomerate more in simulated gastric fluid than the larger-sized particles. If agglomeration affects uptake, that was not observed in this study.

Additionally, a study of tissue distribution after a **5-day** oral administration to Wistar rats of 34.1-59.9 mg/kg bw TiO_2 particles with size ranging from **7 – 220 nm** demonstrated that limited absorption occurred in the GI tract, indicated by the observation of low TiO_2 levels (<0.10 µg Ti/g tissue) in the mesenteric lymph nodes, spleen and liver (Geraets et al, 2014). Here, the authors determined the Ti levels in blood and tissues using **High-Resolution Inductively Coupled Plasma Mass Spectroscopy (HR-ICP-MS)**. Although limited absorption occurred, it was also noted that elimination was limited, resulting in some accumulation of these particles in secondary organs. In contrast, Jani et al (1994) reported that **500 nm TiO_2** administered orally as a 2.5% w/v suspension (12.5 mg/kg/day) to rats for **10 days** showed moderate absorption into the liver, spleen, lung, peritoneal tissues and the major tissues of the gut associated lymphoid tissue. The colon showed the highest total TiO_2 absorption (1125 µg) whilst the kidney showed the lowest absorption (2 µg). The total percent uptake of the dose administered was 11.9 % or 5.0 % of the dose in µg/g of all tissues analyzed. The authors used a combination of **histology, SEM**, and **ICP-AES**, to confirm that uptake of TiO_2 particles occurred but could not distinguish between uptake of whole TiO_2 MPs or ionic Ti. Less than 0.1 % of administered TiO_2 dose was absorbed in all these studies except the Jani et al (1994) study. The duration of administration and dose of TiO_2 differed in these studies; the duration ranged from a single dose to 10 days repeated dosing and the dose ranged from 12.5 mg/kg to 5 g/kg bw. However, these differences did not appear to correlate with the uptake results.

From the above studies, it is apparent that particle size, although important, is not the sole determinant of absorption levels of MPs and NPs. The surface charge of particles also affect gastrointestinal absorption. In a Schleh et al (2012) study, **AuNPs** with core diameter ranging from **1.4-200 nm** were administered via **intra-esophageal instillation** to non-fasted female Wistar-Kyoto rats. The 2.8 nm particles were coated with carboxyl groups for a negative surface charge or with amine groups for a positive surface charge to investigate the effect of surface charge on NP absorption. The absorbed NPs in tissues and circulation was determined by **gamma-spectroscopy** measurement of ^{198}Au radioactivity of the samples. There was low absorption of total NPs in the 24-hr. period. The smallest particles were absorbed more into the circulation than the largest particles, in agreement with numerous studies on size-dependent uptake of particles. Therefore, significantly more of the 1.4 nm NPs were found in the blood, kidneys, reticulo-endothelial system, and urine but interestingly, more of the 18 nm NPs than the 1.4 nm

NPs were found in the heart and brain. This observation, just as was made by Wang and colleagues in 2007 for the *80 nm* TiO_2 NPs in the liver, may be due to preferential accumulation of the 18 nm AuNPs in the heart and brain.

As is noted below on the effects of proteins on particle transport, the ***differential binding of serum proteins to particles may ferry the particles to different cells or organs, leading to differential accumulation***. Negligible amounts of the 200 nm NPs were found in the blood, kidneys, urine, and reticulo-endothelial system; none of the 200 nm NPs were found in the heart and brain. No significant effect of dose on the absorption and distribution was observed, determined by applying 20X the original dose of the 1.4 nm NPs. The highest absorption across intestinal membranes was found for the negatively charged 2.8 nm AuNPs than the positively charged NPs. Over 2X more of the negative AuNPs than the positive AuNPs was found in the circulation. This agrees with observed higher phagocytosis of crystalline NiS and CoS with net negative surface charges compared to low phagocytosis of amorphous NiS and CoS with net positive surface charge in different cell lines (Abbracchio et al, 1981). The observations here with AuNPs, NiS and CoS may be metal-specific because some *in vitro* and *in vivo* studies report more uptake of positively charged nonmetallic particles (des Rieux et al, 2005; Foged et al, 2005; Sadat et al, 2016; Jani et al, 1989). The negative charge of cell membranes endorses higher uptake of positively charged particles due to the attractive forces between the negative surface of phospholipids and positive charge of particles. Schleh and colleagues attributed the higher uptake of negatively charged AuNPs over positively charged AuNPs to adsorbed proteins, which could reverse the surface charge of the particles. Thus, the positively charged particles may become negatively charged due to adsorbed proteins, and vice versa. The adsorption of proteins on the surface of metals may be different from nonmetals. An earlier study demonstrated that specific amino acids alter the cell uptake of ionic nickel (Abbracchio et al, 1982). It is noteworthy, though, that the positively charged 2.8 nm AuNPs were detected in the brain but not the negatively charged 2.8 nm NPs by Schleh and colleagues (2012).

On the issue of particle-biomolecule interactions, Jo and colleagues (2016) demonstrated an effect of albumin and glucose on the absorption of TiO_2 NPs. **Food grade (f-TiO_2) TiO_2** of average particle size **117 nm** was administered as a 500 mg/kg **single oral dose** to male Sprague Dawley rats in the presence or absence of 1% glucose, 1% albumin or distilled water. There was a significantly higher absorption of f-TiO_2 in the presence of 1% albumin (0.78 %) and 1% glucose (0.30 %) than in distilled water (0.01 %), showing that endogenous biomolecules can interact with and influence the absorption of particles. The higher absorption in the presence of 1% albumin over 1% glucose may be due to the stronger adsorption of proteins to the surface of NPs. However, the authors did not demonstrate direct, physical adsorption of the biomolecules on the surface of f-TiO_2. There was more uptake of f-TiO_2 than **general grade TiO_2 (g-TiO_2)** into kidney, liver, lungs and spleen of rats given 500 mg/kg TiO_2 NPs in 5% albumin. **Gel permeation chromatography** of tissues of female Wistar rats fed **fluorescent 500 nm polystyrene particles** orally for **5 days** showed a 23% uptake of the particles conjugated with tomato lectin and <0.5% uptake of particles blocked with N-acetylchitotetraose (Hussain et al, 1997), clearly

demonstrating that **particle-molecule interactions influence absorption rates**. NP-protein interactions, so called protein corona, leads to the accumulation of NPs in organs and cells that would otherwise not accumulate such tiny particles. These proteins function as ferry boats to transport the attached NPs by interactions with other membrane, cytosolic or nuclei proteins. Proteins also bind MPs but their large size prevents efficient and/or sufficient protein-mediated transport. Here too, particle size, as well as particle surface properties, influence the interactions with proteins. In a 24 hour, *in vitro* incubation of **5, 15** and **80 nm** diameter **monodisperse, negatively charged AuNPs** with mouse serum in PBS, there was a size-dependent qualitative and quantitative adsorption of proteins to the AuNPs (Schäffler et al, 2013). The 5 nm AuNPs had the most adsorbed proteins (40), followed by the 15 nm AuNPs (35) and the 80 nm AuNPs (19). The adsorbed proteins were identified using MALDI-TOF-MS-Proteomics analyzer after separating from the non-adsorbed proteins by centrifugation and gel electrophoresis. The size-dependent effect on adsorption of proteins suggests this may contribute to the size-dependent uptake of particles.

A few studies have demonstrated the absorption and tissue distribution of ionic particles. In a **28-day** daily repeated oral administration of **soluble $AgNO_3$, <20 nm (17.7 nm) uncoated or <15 nm (12.1 nm) polyvinylpyrrolidone (PVP) coated AgNPs** to male Sprague-Dawley rats, a low absorption of Ag was observed, especially following treatment with AgNPs, as indicated by the >99% AgNPs excreted in the feces and the low amounts in blood and tissues (van der Zande et al, 2012). However, there was 7-10X higher uptake of Ag+ ion into the blood from the soluble $AgNO_3$ than from the two AgNPs at all time points. There was time-dependent release of Ag+ from the <15 nm PVP-coated AgNPs, showing that uptake of AgNPs may be mediated by released Ag+ ions. Rapid clearance of Ag from the blood occurred one day post-exposure. Significantly more of the absorbed Ag in tissues like liver, spleen, kidneys, and lungs came from the $AgNO_3$ (Ag+) treated group. To determine whether Ag in the tissues are present in the soluble or particulate form, the authors applied **single particle inductive coupled plasma mass spectroscopy (SP-ICP-MS)**. The AgNP treated groups and the soluble $AgNO_3$ treated groups demonstrated the presence of nano-sized particles in lungs, liver, spleen and gastrointestinal contents. The presence of nano-sized particles in the group treated with soluble $AgNO_3$ is interesting, strongly suggesting the formation of nano-sized granules or precipitates *in vivo* from soluble Ag+ ions, possibly by binding to sulphur or chloride present in cellular structures to form Ag salts. The threshold NP measurable size for SP-ICP-MS is 20 nm, suggesting that NPs smaller than 20 nm were probably not detected.

In a related study, a **single oral dose** of **soluble $AgNO_3$** or **~3.8 nm AgNPs** administered to rats resulted in higher blood levels and higher organ distribution levels of Ag when soluble $AgNO_3$ was administered relative to when AgNP was administered, demonstrating higher absorption of the Ag+ ions than the AgNPs (Park 2013). Ag concentration in tissues, blood, urine and feces was measured using **ICP-MS**. Similarly, after **28-day repeated oral** exposure of female Wistar rats to **soluble AgAc** or **14 nm AgNPs coated with PVP**, TEM revealed the presence of nano-sized granules in the ileum and lysosomes of macrophages in both groups exposed to AgNPs or AgAc

(Loeschner et al, 2011). The granules diameter was close to the diameter of the AgNPs. **Energy dispersive x-ray spectroscopy** showed that the lysosomal granules from both AgNP and AgAc exposed groups composed of Ag, S and Se. This confirms the findings of van de Zande et al (2012) that Ag+ is absorbed from AgNP and soluble Ag, forming nano-sized particles *in vivo* following absorption. The tissue distribution pattern was similar between the soluble Ag and AgNPs. A theory of Ag absorption suggests "*that the deposition of particulate silver following the oral administration of nanoparticulate solutions is not due to the whole particle's being deposited, but rather due to the ionic silver contained in the suspension or ionic silver that is released from the particle surface. The reason being the similar composition of the deposited particles regardless of whether ionic or nanoparticulate silver was administered*" (Hadrup and Lam, 2014). Absorption of ionic, nanoparticulate and colloidal Ag following oral administration ranged between 0.4-18% in animals and humans (Hadrup and Lam, 2014).

Finally, the **polarity** of particles or ionic elements also influence absorption. A seminal report by Ishimatsu et al (1995) demonstrated that 24 hours after oral administration of micrometer-sized nickel compounds of varying solubilities, the amount of nickel in the secondary organs was higher in the rats that received the water-soluble nickel compounds nickel nitrate, nickel chloride, and nickel sulphate than in the rats that received the less-soluble nickel oxide (black and green), nickel subsulphide and metallic nickel. This finding may partly be reflected in the **differential bioaccessibility of the Ni compounds in simulated biological fluids**. Tilaki and colleagues (2007) demonstrated that the average size of AuNPs in water is 6 nm, with the size increasing to 18 nm and showing strong aggregation in ethanol. This suggests that the liquid environment of the GI tract can impact the size and aggregation properties of particles, and ultimately their absorption.

From the above studies, the following general conclusions can be made:

- Dose-dependent absorption of some particles observed.
- There is species-independent uptake of smaller particles than larger particles.
- More particle uptake in young adults than young or old animals, although both young and old do not represent worst cases.
- Dietary composition affects particle uptake, possibly by mediating pH variations in the different zones of the gastrointestinal tract.
- Thus, fasted and nonfasted states can alter uptake results.
- Prolonged particle exposure or administration increases uptake of particles.
- More particle uptake in the lymphoid than nonlymphoid tissues of the GI tract.
- Higher uptake of smaller particles than large particles demonstrated.
- Depending on particle properties, absorption occurs a few minutes to days after administration, and the particles may persist in body for several days.
- More sequestration and less absorption of the larger-sized particles.
- Direct uptake of particulate and charged particles occur.

- Release of ionic elements following dissolution in GIT fluids increases absorption of elements and contributes to total absorption of particles.
- Uptake of ionic elements released from particles can reform precipitates and granules of nano-size *in vivo*.
- Type of material affects uptake of particles, such as <0.1 % uptake of micron-size TiO_2, ~0.1 – 5.0 % uptake of AuNPs, 0.4 – 18 % uptake of AgNPs, and >10% uptake of ZnO NPs.
- There is more GIT uptake of some negatively charged metallic particles/compounds, such as NiS, CoS and AuNPs.
- Interaction of particles with biomolecules modulates uptake.

In conclusion, absorption of ionic elements and particles occur in the gastrointestinal tract, with transport and distribution into secondary organs through lymph. Factors attributable to particle and non-particle properties affect the absorption and distribution of ionic elements and particles.

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