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Nanoparticles in Food

- with a focus on the toxicity of titanium dioxide

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Abstract

The use of nanoparticles, in many different fields, is rapidly increasing. What substances, and to what extent they exist in food and supplements, and the prevalence of such products on the market, is of interest to the Swedish National Food Agency. Answering those questions, as well as providing information regarding their potential toxicity, was the intent of this thesis.

To understand the difficulties, and possibilities, in analyzing nanoparticles in food, a methods-section was included, covering the most common techniques, the theory behind them and when they may be used. It became abundantly clear that analyzing nanoparticles in food, and investigating their potential toxicity, is anything but simple, and requires a combination of many techniques.

Quite surprisingly, it seems the occurrence of nanoparticles in food, as today, are not added on purpose, since not one nanosubstance to be used in food has been approved by EU. Rather, studies have revealed they originate from a nanosized fraction of food additives, such as TiO_2 (E171), which has attracted particular attention in this report. This nanofraction may be as large as 40%, and the estimated total intake of TiO_2 (as stated by EFSA) is 1.28 mg/kg/person, resulting in quite an amount of nano TiO_2, provided the numbers are correct.

The toxicity of silver has also been reviewed, since the colloidal form, which includes nanoparticles, is quite common as a health supplement.

Toxicity studies are not unambiguous though, some indicate geno-and–cytotoxicity and others do not. Importantly, the toxicity studies on TiO_2 have not been done on E171, but on many other forms of TiO_2, whose relevance is difficult to predict. TiO_2 is, however, classified as a possible carcinogen by IARC. Several groups have taken an interest in a potential relation between particle intake and various chronic inflammatory diseases, also discussed in this report.

In conclusion, the size, modifications of E171 and its toxicity, requires more attention in order for food authorities to confidently ascertain healthy food for the public.

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Aim

Many report of an increasing, though silent, interest within the food industry to use nano-materials in food for various applications. However, neither has the potential health risks been adequately evaluated, nor is the knowledge of our exposure to nanomaterial satisfactory. Therefore, knowing how little is known, the purpose of this thesis was to give an idea of what food items and supplements there is on the market, containing or based on nanoparticles, how these may be analyzed and characterized and, last but not least, what is known of the toxicity of silver and titanium dioxide nanoparticles, two of the most frequently used nanomaterials in food and supplements. Finally, from what has been mentioned and discussed, particularly concerning TiO_2, suggestions as to how the Swedish National food Agency may continue their work in assuring safe food for the people, will be given.

Background

Nanotechnology is a rapidly emerging science, by many thought to revolutionize our society, with applications as varying as electronics, aeronautics, cosmetics, chemical industries, medicines, diagnostics, textiles and clothing, solar energy, sports equipment, self-cleaning windows, dirt-resistant textiles, anti-microbials, bioremediation of polluted soil and water and- of special interest at The National Food Agency- in food and supplements. This report has an emphasis on TiO_2 and will throughout the text discuss if there is more than meets the eye, literally.

Nanomaterial

The definition of a nanomaterial, as used by the European Commission: *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.*

The term “nano” derives from Greek and means “dwarf”, indeed small, on the metric scale nano refers to 10^{-9} m. But being small does not necessarily mean being bad, which seems to be a growing apprehension of the public. An atom is in ångstrom range (10^{-10}) and the bonds between atoms in a crystal typically measure a few angstroms (Luykx *et al*. 2008). Just as synthesized nanomaterial measure 1-100 nm, so do many naturally occurring proteins, lipid structures or other biomolecules. Fig. 1 illustrates the sizes of various molecules, naturally occurring as well as engineerd. The issue with engineerd nanomaterial though, because there is one, is that they may be synthesized with specific surface characteristics, and these in turn decide the behavior, and may increase the reactivity and toxicity, of the nanomaterial.

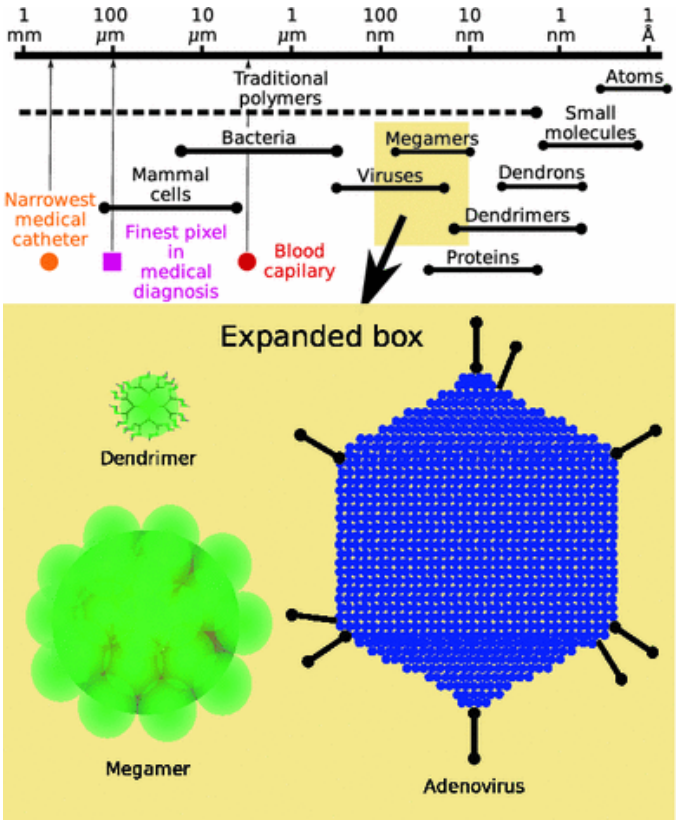


Fig. 1 illustrates the sizes of some naturally occurring structures such as small molecules, proteins and viruses, and engineerd ones, such as dendrimers and polymers. (Figure taken from Sebestik *et al*., 2010)

Nanomaterial can be categorized or classified in many different ways, one of them illustrated in Fig 2 below (categories from Borm *et al*. 2006).

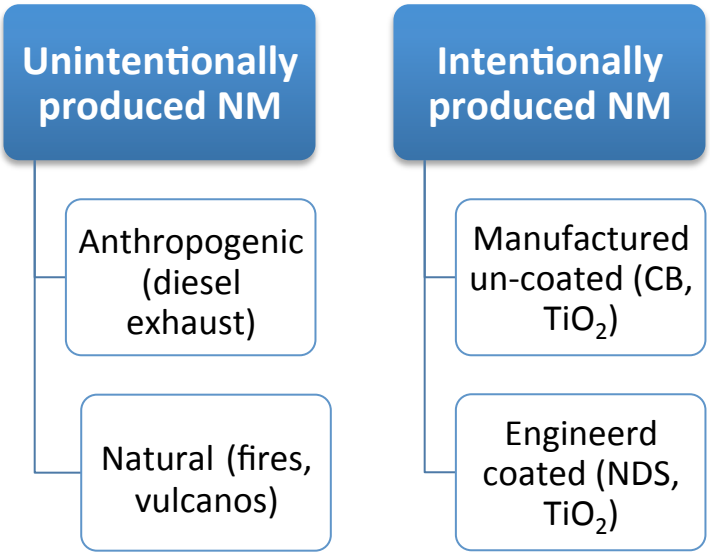


Fig. 2 Categories of nanomaterial often referred to. Abbr: NM; nanomaterial, CB; carbon black, TiO_2; titanium dioxide, NDS; nano delivery systems

It is important to remember that our exposure to diesel exhaust, for example, far exceeds the exposure of engineerd nanomaterial (SCHENIR, 2006) and the emitted unintentionally produced nanomaterial are not without adverse health effects (Oberdörster 2005). Identifying the health effects induced by engineerd nanomaterial, in for example industrial workers, and excluding those induced by nanoparticles (NP) of natural, or anthropogenic source, is not easy.

As mentioned, engineerd nanomaterial are structures with at least one dimension between 1-100 nm. These can be further categorized based upon their shape (Tiede *et al*. 2008).

- Nanofilms/nanocoatings: 1-100 nm in one dimension.
- Nanowires/nanotubes: 1-100 nm in two dimensions. These can be mm-long.
- Nanoparticles: 1-100 nm in three dimensions.
- Quantum dots: the smallest nanoparticles, measuring 1-10 nm (10-50 atoms)

Size, surface and reactivity

Size generally relates inversely to reactivity; smaller particles, higher reactivity. This is explained by the fact that smaller particles have a higher surface-to-mass ratio. As particles become smaller, the number of atoms on their surface increases exponentially (Mihranyan *et al*. 2012, FAO/WHO 2009, Borm *et al*. 2006). Also, as stated by Mihranyan, the characteristics of nanoparticles are, apart from the high surface-to-mass ratio, a large number of particles per weight unit. He explains that, while going from a particle size of 1μm to 10 nm, 100 "times smaller" results in a 100 times increase in *surface* per weight, the increase in *number of particles* per weight is not 100, but one million. Skocaj *et al*. (2011) further explains that in a particle of 30 nm approximately 20% of the atoms are on the surface, while in a particle of 10 nm approximately 35-40% of the atoms are on the surface. All these surface atoms result in increased reactivity. Hence, going from micrometer to nanometer not only results in more particles, they are also more reactive. Another definition of nanoparticles, as suggested by EFSA (European Food Safety Authority), apart from the one above, is a required surface area of at least 60 m^2/cm^3 or gram.

While understanding the reactivity of NPs is difficult, foreseeing the reactivity of NPs between 5-20 nm is next to impossible. These particles behave neither as the bulk material nor as its constituents. The change in physical characteristics and abilities are the reasons for using nano instead of bulk material, be it optical, electrical, magnetic, solubility or other (Chaudry *et al*. 2008, Mihranyan *et al*. 2012). Gold is an example of this. As a bulk material it has the colour of gold as we know it, but in nanosize it is blue. Also, while bulk material of gold is inert, particles of less than 10 nm burst into flames when in contact with oxygen (Maynard, 2012). The reason for putting TiO_2 in foods is to make it intensely white as TiO_2 around 200 nm gives that colour. Reducing the size of TiO_2, to around 20 nm, makes it an efficient UV-scavenger, but at the same time it does not respond to visible light and is transparent- two characteristics of nano TiO_2 exploited in sunscreens (SCENHIR, 2006, Borm *et al*. 2006.

The impact of the large surface area and the plentiness of nanoparticles per weight, as compared to the bulk material, have brought about new ways of thinking, and the old concept of mass concentration has been suggested being complemented with number concentration and surface area. One example to support this idea comes from an assay in which a difference between nanoparticles and microparticles was seen when measured as mass concentration, but when the mass concentration was changed to surface area, the difference was no longer there (Oberdörster, 2009). The same concentration of a material as measured in, say µg/ml, has immensely more particles in nanosize compared to microsize. Add to that the high surface reactivity of the nanoparticles, and that dose may become more potent.

But assessing an effect of a substance in terms of surface area or number concentration instead of mass concentration makes risk assessments complicated, as the concepts used are based on mass concentration (FAO/WHO, 2009).

Agglomeration/aggregation and consequences thereof

Pivotal for the destiny and impact of nanoparticles are their willingness to agglomerate or aggregate. The primary particles, single entities of crystals, might, depending on surface characteristics, form larger structures, so called secondary particles.

Agglomerates are formed between primary particles held loosely by van der Waals interactions. The surface area of an agglomerate corresponds approximately to the sum of the primary particles forming the agglomerate, as they are not tightly bound (Powell *et al*., 2010). (Fig 3 below illustrates an agglomerate and aggregate). This is important to remember as it has implications for the reactivity. One cannot assume lower reactivity of agglomerates simply because they are larger structures. It depends on what particles or structures are being investigated and in what context. It is said that the pores within an agglomerate can accommodate particles of 1-2 nm (depending on particle) and those immersed smaller particles would be exposed to both internal and external surfaces (Mihranyan *et al*., 2012). Just for comparison of sizes, the diameter of DNA is 2 nm and a C60 fullerene is 7Å (Xu *et al*., 2012). In assessments of the reactivity towards a large protein or an organell, however, the internal surface is irrelevant since that is not involved in the interaction. The agglomeration (and aggregation) of nanoparticles is one of the complicating factors in nanoresearch. Agglomerated particles may dissociate depending on factors such as pH, ion concentration, zeta potential of the particle and surrounding proteins (Gualtieri *et al*. 2011. Imagine a human ingesting nanoparticles of some sort. These will be exposed to certain proteins, a specific pH, ion concentration etc in the mouth. When it moves down to the stomach, where the pH is drastically lower, around 1-2 as opposed to 6.6 in the mouth (Fröhlich *et al*. 2012), the nanoparticles tend to agglomerate. This affects their uptake and reactivity in the stomach. When they travel down to the intestines, the pH is less acidic, allowing the particles to dissociate and adhere to new proteins, prevalent in that area (Gaultieri *et al*., 2012). These uncertainties make the understanding of nanoparticle reactivity

and fate after oral ingestion difficult, and perhaps contribute to the scarcity of oral studies on nanoparticle toxicity.

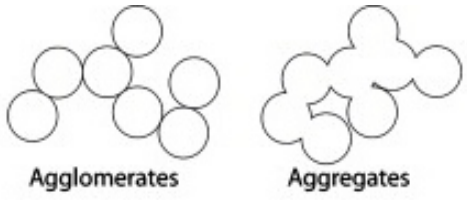


Fig 3 Schematic illustrating loosely connected primary particles in an agglomerate and more tightly bound particles of an aggregate. Aggregates can also form agglomerates. (*Fig cropped from Fasaki I, Ultrasound assisted preparation of stable water-based nanocrystalline TiO_2 suspensions for photocatalytic applications of inkjet-printed films, Appl Catalysis A: general, 411-412, 2012, 60-69)*

Aggregates, on the other hand, are tightly bound primary particles with a reduced surface area and, consequently, less reactivity (Powell *et al*., 2010). Aggregates do not as easily as agglomerates dissociate and re-associate. They may be taken up by cells, just as agglomerates or primary particles may be, although once there they are not equally reactive. But an aggregate of small nanoparticles could still be in nanoparticle size, ie. below 100 nm, and as such it may be more reactive than the corresponding microparticle. Note that aggregates also form agglomerates.

How easily nanoparticles form aggregates and agglomerates depend primarily on the zeta potential and size. Results from Andersson and collegues (2011) showed that the size of the primary particle has an impact on agglomeration, which was also shown by Gualtieri *et al*. (2011). Larger particles with higher zeta potential tended to agglomerate less, and were taken up by the cells to a larger extent than smaller particles that agglomerated more. The concept of zeta potential deserves some attention, as it is so fundamental.

Zeta potential

Fig 4 below illustrates the different potentials, including the zeta potential, that arise from particles in solution. A particle with a negative surface charge will attract positive charges of the solvent. The layer of bound positive charges is called the stern layer. Many negative charges binding many positive charges create a high potential. As the distance from the particle, into the solution grows, there will be a more balanced mix of positive and negative charges and the difference between charges of the solution and the surface of the particle, will be less clear, which results in lower potential. The zeta potential of a particle is found where the stern plane meets surrounding solvent. The surface charge and zeta potential usually have the same sign (Borm *et al*., 2006) but the are not the same.

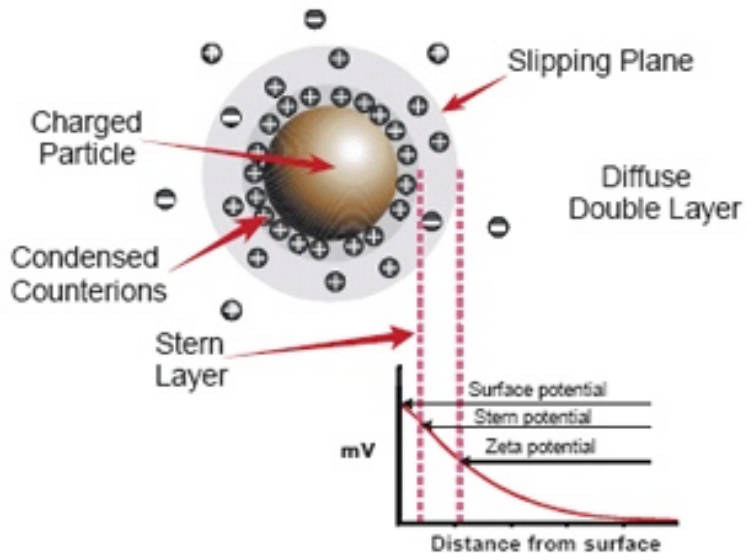


Fig 4 shows where surface potential, stern potential and zeta potential are found in a solution containing particles. The zeta potential is important as it decides how stabile particles in solution or an emulsion will be. It also has an impact on reactivity and toxicity. The figure shows a particle with a negative surface charge. Attached to this surface are positive charges of the solvent, resulting in a potential between the two layers. The potential is illustrated in the lower picture where one can see that at the surface, where charges of particle and solution meet, the potential is high (mV). The layer of positive charges surrounding the particle is called stern layer and the next phase is the diffuse layer. The potential in the diffuse layer decreases as the distance from the charged surface increases. In the interface between the stern layer and the surrounding solution, also called ”sliding plane”, is the zeta potential. (image from www.pharmaceuticals online.com)

In a solution of nanoparticles with high zeta potential, the particles will repel each other. As a consequence, the solution will be well dispersed and stabile. If the zeta potential is close to neutral, there will not be enough forces to repel each other and the particles may agglomerate or aggregate and precipitate (Tiede *et al*., 2009, Andersson *et al*., 2011). Knowing the zeta potential of a particle is crucial for an understanding of how it may react in solution, and as a result, what may happen to it in the body or environment. Normally, toxicity relates to dose in that higher dose is more toxic. But, because nanoparticles are more prone to aggregate and sediment at higher concentrations, a higher dose might actually turn out less toxic, while a lower dose, in which the particles stay more dispersed, might have higher effect (Tiede *et al*., 2009). This example touches again on how risk assessment of nanoparticles may turn traditional concepts of risk upside-down.

Nanoparticles and their “corona”

Nanoparticles adhere to proteins, lipids, nucleic acids or other molecules spontaneously in the body or biota forming a so-called “corona”, like a coating (Fig. 5). The material, size and surface characteristics of the nanoparticle affect to what biomolecules it will adhere (Cedervall *et al*., 2012), and the resultant corona, in turn, decides the destiny of the nanoparticle, in body or biota (Oberdörster, 2009, Gualtieri *et al*., 2011). Nanoparticles may bind to for example carboxyl-, hydroxyl-, amino-, or sulphydryl groups of biomolecules (EFSA journal, 2011).

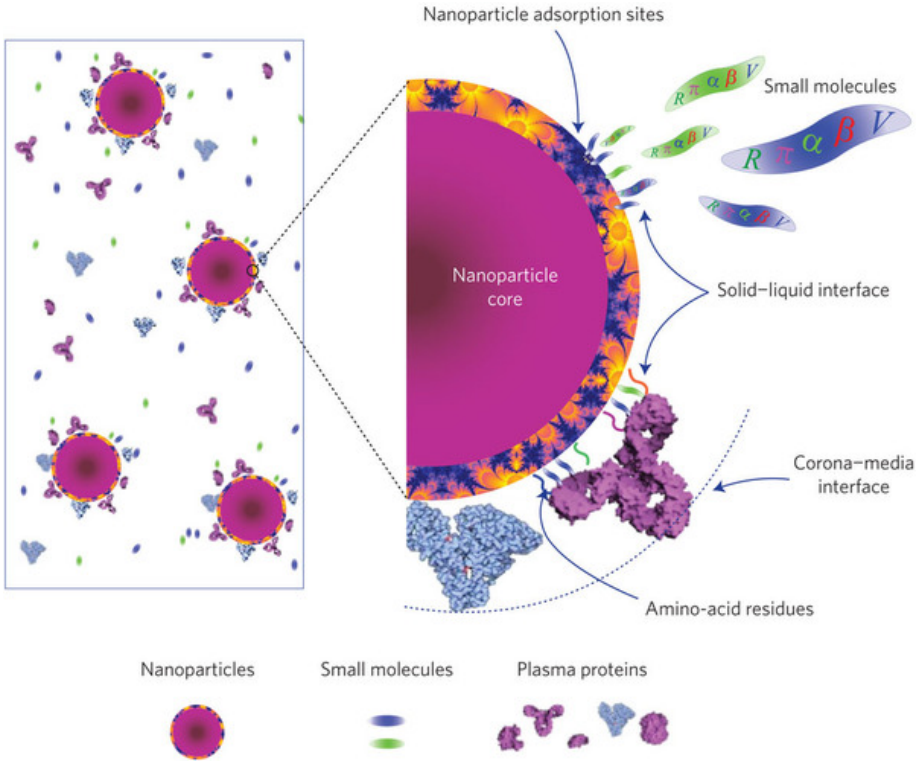


Fig 5 illustrates how nanoparticles can bind to various molecules, small or larger biomolecules. This binding can either determine the fate of the nanoparticle, or, if it interacts with an enzyme, depending on the site of interaction, the enzyme may lose its catalytic activity. (*Picture from Xia et al., An index for characterization o fnanomaterials in biological systems. Nat* Nanotechnol. 2010; 5:671-5)

When a nanoparticle binds to a protein, its tertiary structure or active site may be altered, rendering the protein dysfunctional (FAO/WHO 2009, Gualtieri *et al*., 2011). In food, nanoparticles also adhere to various molecules, which may affect their uptake.

An example often cited is the coating of nanoparticles with apolipoprotein E (ApoE) in the blood, giving them a ticket to the brain as Apo E binds to its receptor on endothelial cells of the blood brain barrier (BBB) (Oberdörster 2009, Mihranyan *et al*., 2012).

The corona also influences the agglomeration of nanoparticles. It has been shown in *in vitro* studies how adding BSA (bovine serum albumin) to the medium reduced agglomeration (Gualtieri *et al*., 2011).

Another interesting example of how the corona may affect uptake of NPs is illustrated by a suggested mechanism behind translocation of inhaled polystyrene nanoparticles to the circulation, involving the lipid lecithin (Oberdörster, 2005). The fluid lining the alvaeole wall in the lung contains lecithins, which function as surfactants. They may bind to both polystyrene nanoparticles and the lecithin-receptor in the epithelium, thereby providing a passage from lung to circulation. An assay showed that without the corona of lecithin, the polystyrene particles were not taken up.

The corona is far from static. It changes along with changes in pH, ion concentration, temperature, salt and protein contents, to mention a few. So, as the nanoparticle, coated by a protein, is transferred to a new region in the cell, or organ, where the microenvironment is different, the corona will change, giving the nanoparticles new characteristics (Cedervall *et al*., 2012, Mihranyan *et al*., 2012, Gualtieri *et al*. 2011). This contributes to the difficulty in characterizing nanoparticle uptake and toxicity.

Rutile and anatase

TiO_2 is a mineral that exists in three different crystal forms: rutile, anatase and the less common brookite. One unit of the respective crystal structure of anatase and rutile is shown in Fig. 6 below. The crystal form decides their uptake (Tiede *et* al. 2008, Allouni *et al*., 2012) and toxicity (Karlsson *et al*. 2009, Tiede, *et al*. 2009). Anatase, or a mix of anatase and rutile with anatase in majority, gives higher uptake and toxicity. In toxicity assays of TiO_2, P25, a TiO_2 powder of approximately 25 nm where 70-80% is anatase and 20-30% is rutile, is frequently used. However, it must not be forgotten that P25 has neither the same size nor structure as food grade E171, hence their uptake and toxicity likely differ (Weir *et al*., 2012).

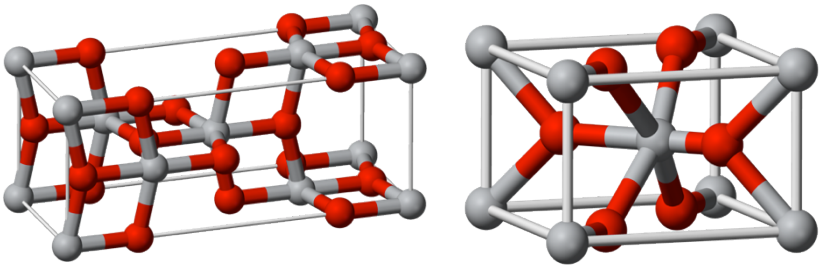


Fig. 6 Crystal structures of anatase (left) and rutile (right). (Figures from Wikipedia.org)

Possible occurrence in food and supplements

Institutes and analysts envisage a future in which we will be flooded by products with functions based on nano, in any form. This however, does not seem to be an imminent development in the food sector as authorities around the world (e.g. EFSA) have awakened and taken on a cautious approach, demanding that new products or substances be assessed with a focus on their nano-characteristics. That a substance has been approved for usage in foods as a bulk material, does not automatically make the nanoform of the same material accessible for product development. So far, not one food product with nanoparticles has been approved within the EU (personal communication Abramsson Zetterberg L, Svensson K). Or rather, no substance with deliberately added nanoparticles in a fraction of more than 50%, has been approved. Looking at the global occurrence, there are examples of foods with nanoparticles (e.g. bread, chocolates, water, oil), but they are still scarce (Project on Emerging Nanotechnologies).

For what purpose then would the industry want to add nanoparticles to our food? The possible applications seem endless, some even science fiction-like. From a FAO/WHO report (2009), and by looking at what is out on the market, the major industries that have found a use for nanomaterial are those producing functional foods and supplements. Functional foods are produced with the purpose of adding something health promoting to the food. A classical example would be yoghurts and sour milk enriched with certain bacteria for improved microbial balance in our gut. Now with the rising of nanomaterial, companies see a market where nano delivery systems (NDS) may be used to increase the bioavailability of certain vitamins and minerals. Vitamin C for example, which is hydrophilic, dissolves well in the blood but does not easily passes membranes, resulting in low bioavailability. Putting vitamin C in an NDS would increase its bioavailabilty as the NDS can be synthesized with specific surface characteristics for enhanced uptake (Luykx *et al*., 2008). The NDS may be coated to become amphiphilic, making them soluble in the blood as well as allowing them access to the cells. But, if these products would be approved, and become popular among consumers, the guiding authorities would have to keep up and change the recommendations of intake as these are based on bioavailability of a daily intake (FAO/WHO 2009, EFSA Journal, 2011). If not, people may unknowingly over consume vitamins that not seldom turn toxic at higher doses.

Nanoencapsulation. One example is the use of nanoliposomes for nano encapsulation or as emulsifiers, to make a low fat product seem high fat in texture (Garrec *et al*., 2012). The idea is the same as for microencapsulation used regularly in foods, only more efficient as nano, they say. Another application of nano encapsulation, in line with nano delivery systems for vitamins, is the encapsulation of additives and colorants in food. Encapsulated, the additives would be more soluble, thus requiring less of the substance. It seems a bit odd, though, if that idea has arisen as a response to the general public who nowadays seeks products with less additives. Reducing the amounts of additives by putting them in nano capsules might not be exactly what the picky public had in mind.

Masking a bad taste is another example of how nanomaterial may be used. One example is the Australian bread, Tip-Top Up bread, which for health purposes contains Omega-3 fatty acids from fish, only nano encapsulated to hide the taste (note; though often mentioned in the literature, their home page does not mention nano so it is not confirmed).

Agriculture. As a means to limit global starvation, groups are trying to develop crop insensitive to extreme weather conditions such as drought, using nanomaterial (FAO/WHO, 2009). Other agricultural related applications are pesticides that are sensitive to specific weather conditions and released only then, thereby limiting its use. Another example on this theme is adding nanoparticles that function as catalysts to the pesticides, with increased efficiency and reduced pesticide volumes as a result (Nanoforum report, 2006).

Bioremediation. Nanoparticles of zerovalent iron have been shown to be efficient in sanitation of chlorinated organic compounds and metals, such as arsenic, in ground water. However, as the long term environmental effects of this application are not clear, authorities in Europe have been cautious in its implementation, more so than corresponding authorities in the US (Mueller *et al*., 2012).

Antimicrobials. Silver has antimicrobial ability and is used for that purpose. Colloidal silver is taken as a supplement, though, at least in Sweden, it is not allowed to be sold as a supplement, only as a water treatment product. The particle size of silver in colloidals typically ranges from 10-1000 nm (Faunce and Watal, 2010). Though silver has been used as an antimicrobial for a long time, with its biocidal ability attributed to the ionic form, it is under debate whether the antimicrobial activity is mediated by particulate silver or by released silver ions (Faunce and Watal, 2010, Li *et al*., 2012, Asghari *et al*., 2012). In a watersolution of metallic silver there is a release of silver ions, according to the formula provided below (Tolaymat *et al*., 2010).

$$O_{2(aq.)} + 4\ H_3\ O^+ + 4\ Ag_{(s)} \rightarrow 4\ Ag^+_{(aq.)} + 6\ H_2O$$

Tolaymat *et al.* (2010) emphasize that the antimicrobial ability, and toxicity, might be mediated through both forms of silver. They also consider that the antimicrobial effect seen in bulk material is likely enhanced in nanosilver, together with increased toxicity.

The production of silver as an antimicrobial increased 500 times between 2000-2004 (Lau, 2011) and the resulting high levels released into the environment has gained attention. Of concern are both toxic effects on aquatic organisms (Asghari *et al*., 2012) but also, since the highest levels are found in surface water and around wastewater plants (Lau, 2011), it may kill the nitrifying bacteria used in the handling of our wastewater (Faunce and Watal, 2010).

Food Contact Materials. There is a lot of research going on in the field of food contact materials (FCM). There would be a lot to gain for producers and retailers if the FCMs were designed in such a way that they enhanced shelf life, for example by improving the barrier properties. Or, a nanofilm in the package could change colour when the environment in the food changes, as a result of microorganism contamination, change in pH or other reason.

Even futuristic products such as a nano wine whose taste can be altered from a Merlot-like to, for example a Cabernet Sauvignon-like or Shiraz-like by microwaving it at a specific effect for a certain number of seconds, has been developed. The wine contains nano capsules that in the microwave are opened with the subsequent release of different flavours. Different capsules respond differently to time and effect. As funny and mind blowing as that application may be, a huge –and lasting- market would be surprising.

Returning to real life, only one FCM has been approved within EU, Ti_4N_3, (personal communication Svensson K) and as eager, and admittedly creative, as the food industry may be in finding new possibilities and solutions using nano, the first nanomaterial in food has yet to be approved by EU.

Nano Delivery Systems

Nano delivery systems (NDS) are evolving rapidly with many exciting and useful applications. They may be dendrimers, liposomes or micelles whose function is to deliver something, either to the food as such, or to the person ingesting it. It has been mentioned how

these may be exploited for increased uptake of for example vitamins. They may also be used for a controlled release of preservatives, antioxidants, additives or other substances in the food (Luykx *et al*., 2008). For example, the release of antioxidants may be regulated to occur only when the food is oxidized and there is a need for it.

Dendrimers and drugs

As beneficial as dendrimers might prove for increased uptake of vitamins in supplements, in clinical sciences there are great expectations on NDS to revolutionize the administration of certain medicines, particularly in cancer therapy. They may increase the bioavailability of a poorly soluble drug, or protect the drug from degradation in the stomach, for example by using archaeosomes. These are liposomes made from lipids from archae bacteria that contain an ester bond, instead of the ether bond used by other organism. The ester bond is a lot more stabile in extreme pH or temperature, so they may be used for an adequate oral delivery of substances, that otherwise would be degraded in the acidic stomach (Luykx *et al*., 2008). Note that liposomes and micelles have since long been used as microstructures, the novelty is to make them in nano size. Fig. 7 illustrates how dendrimers may carry drugs. Either, the drug is attached to the surface by covalent bonds (as in A), or encapsulated within the dendrimer (B) (Cheng *et al*. 2008). Through covalent binding, as in A, the release can be controlled enzymatically.

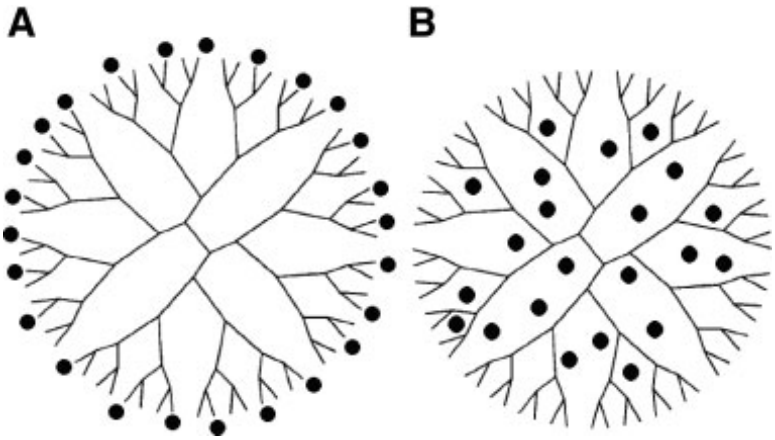
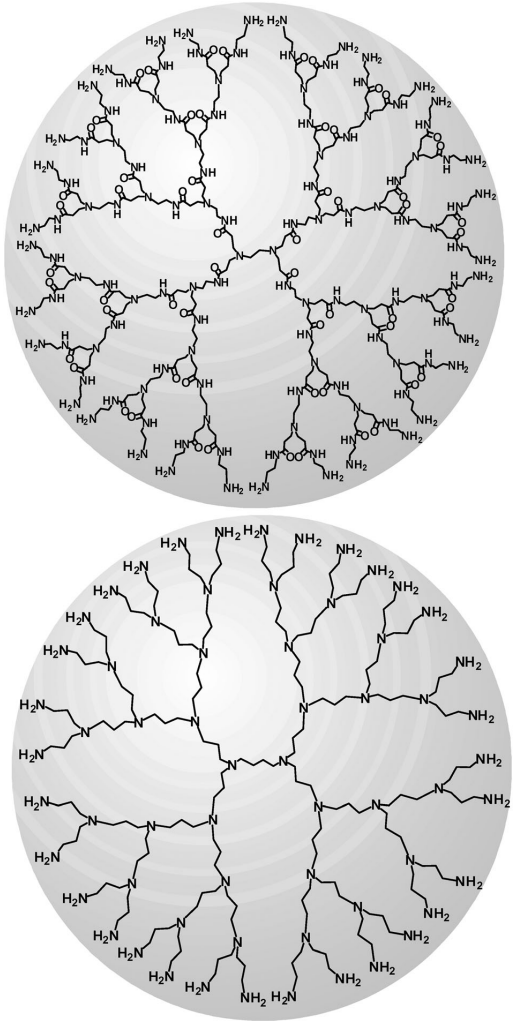


Fig. 7 illustrates how dendrimers may carry drugs. Either bound by covalent bonds on their surface, as in A, or encapsulated within, (B). The covalent attachment allows enzymatically controlled release of the drug. (Figure from Cheng *et al*. 2008)

Not only the drug to be delivered can be attached to the surface. Molecules that direct the dendrimer to a specific target can also be attached to the NDS. There is a lot of research on how to use dendrimers for targeted cancer therapy. They may for example be coated with antibodies or “epidermal growth factor“ (Cheng *et al*. 2008) that will direct them to a specific tissue. That way, the systemic administration of chemotherapy, known to cause a lot of suffering, can be avoided. A common problem in cancer therapy is the development of resistance. Much to blame for this are the P-glyco proteins (P-gp) that pump out the drug.

However, dendrimers with specific surface molecules may be used to overcome this problem by evading the P-gp (Cheng et al. 2008, Ziemba *et al*. 2011, Sebestik *et al*. 2011. One such example is the coating of PAMAM (poly(amidoamine)) dendrimers with the beta blocker propranolol which has been shown to reduce the efflux (basic structure of cationic PAMAM without propranolol is shown in Fig. 8). Hence, using dendrimers coated with, for example propranolol, for oral delivery of drugs would not only increase the bioavailability in terms of increased solubility and uptake, but also through reduced efflux (Cheng *et al*. 2008). This is not only beneficial for targeted cancer therapy, but for oral delivery of drugs in general. Substances that hold great promise as drugs often have to be discarded because of low bioavailability, but with dendrimers as carriers, this obstacle can in some cases be overcome.



But, as enthusiastic and hopeful as researchers may be, dendrimers (or liposomes) are not always without toxicity. Ziemba *et al*. (2011) investigated the toxicity of poly(propyleneimine) dendrimers (PPI) (structure of cationic PPI also shown in Fig. 8) and saw clearly that the toxicity depended on the amine groups. These positive groups bound to negatively charged membranes. When the amines were 100% masked by maltotriose, the toxicity vanished. There have been expectations to use positive dendrimers in gene targeting, as they may bind the negative DNA, but the results of Ziemba are discouraging. However, they did observe that masking them by 25% reduced the toxicity, but still left them with an ability to bind to DNA. Many have shown hemolysis as a result of positive dendrimers binding to and disrupting plasma membranes (Chen et al., 2008, Ziemba *et al*. 2011, Sebestik *et al*. 2011). This however, does not only apply to dendrimers. Gold NPs with positive coating have also been shown to be more toxic and haemolytic compared to negative. In the case of TiO_2, a hydrophobic coating has been shown least pro-inflammatory (Borm *et al*., 2006).

Fig 8 (taken from Cheng *et al*., 2011) shows the basic structure of cationic PAMAM and PPI dendrimers. Note that these are both shown in their cationic form, with amines at the surface. They may however, be anionic with carboxyl groups on the surface, or any other surface groups, giving them the desired characteristics.

Nanoparticles, metals and fish

There are environmental concerns regarding the high amounts of nanoparticles that are released from cosmetics, lotions, toothpaste, industry and other sources. Many groups (Johnston *et al*., 2010, Tan *et al*., 2012, Cedervall *et al*., 2012, Pakrashi *et al*., 2012) discuss the environmental aspects of released nanoparticles and how/if they, for example, may be transferred along the food chain. It has been shown that TiO_2 NP in the water increases the uptake of Cd and As in fish (Tan *et al*., 2012) and it has been suggested that they may travel up the food chain (Cedervall *et al*., 2012), where we stand as top consumers. Other results (Johnston *et al*., 2010), however, indicated that nanoparticles of metal oxides (TiO_2, CeO_2, ZnO) do not pose a threat to the environment, as they, due to aggregation, had a low bioavailability. But, as the authors pointed out themselves, they used uncoated particles that aggregated. As already discussed, nanoparticles added to food (E171) and cosmetics, are coated for increased solubility.

Hu and co-workers (2012) in an evaluation of desorption of metals from TiO_2 NP, saw that at pH 2, more than 98% of adsorbed metals, binding to TiO_2 NP were released. Since our stomach has pH 1-2, perhaps, ingested Cd or As bound to nanoparticles that have been taken up in fish, would be released in our stomach. Our intake of cadmium is already considered being on the verge of acceptable and need not be increased with the help of nanoparticles.

Exposure of (nano) TiO_2 from intake of food

Titanium dioxide has been allowed as a food additive (E171) for decades and no acceptable daily intake (ADI) has been set by EFSA (EFSA opinion, 2004) based on what was stated by JECFA in 1969 who, in their comments wrote, "*Titanium dioxide is a* ***very insoluble compound****. The studies in several species, including man, show neither significant absorption nor tissue storage following ingestion of titanium dioxide.* ***Studies on soluble titanium compound have therefore not been reviewed.*** *Establishment of an acceptable daily intake for man is considered unnecessary".* This thesis will return to that statement.

While on the subject of references for allowing TiO_2 (E171) as a food additive, the NCI (National Cancer Institute) carcinogenesis bioassay comes off as particularly intriguing and worth mentioning (NCI, 1979). Though there are more examples for the sceptic reader. The TiO_2 used in their assay, provided by the American Cyanamid Company, was bulk material sifted for removal of larger particles, before put in the food. The size was not further specified. Since one of the main business areas for the American Cyanamid Company is manufacture of pigmentary TiO_2, as 200 nm, it is surprising that such material was not provided (or ordered) to be used in the carcinogenesis assay. More, while one of the reviewer of the assay considered the statistics, showing dose relation (p- value 0.013) for C-cell adenoma or carcinoma of thyroid in rat, with a p-value of 0.043 between high dose and control group, cause for concern, that opinion was not shared by others who required a p-value below 0.025 for significance. It was hence negotiated out of the conclusion, and instead a recommendation to consider a retest of TiO_2 was included.

This gets particularly interesting, or troubling if you will, when put in the context of the IARC evaluation which classified TiO_2 as a possible carcinogen, category 2B, based on inhalational animal studies (IARC monograph, 2010). It should be mentioned also that TiO_2, or E171, is allowed to be but in food at "*quantum satis*" in Europe, which means as much of the substance that is needed for the desired effect, but not more.

What has recently been shown, is that not a negligible amount of TiO_2 in food may well be in nano size. One group (Weir *et al*., 2012) found the nano sized titanium dioxide in one E171 batch to be approximately 36 % (fig. 9) and another group, who examined chewing gums from six different brands, found the mean nano sized TiO_2 to be somewhat over 40% (Chen *et al*., 2012). This is in agreement with what was stated by Skocaj *et al*. (2011) that the "*submicron-sized powders always contain a certain proportion of nano-sized particles"*. The term submicron usually refers to sizes between 0.1-1µm.

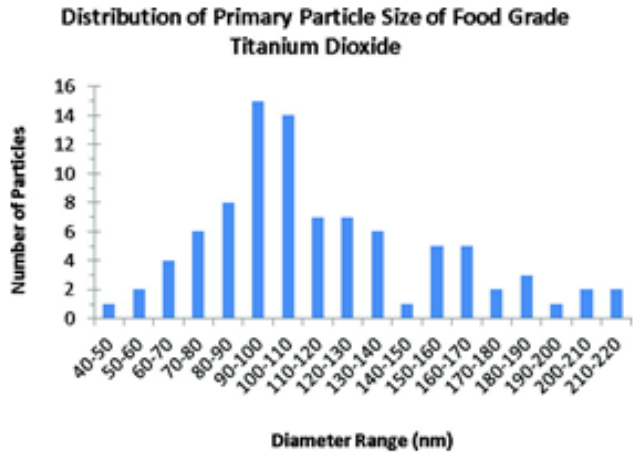


Fig. 9 illustrates the size distribution of one batch of E171. It can be seen that most of the particles are below 200 nm in diameter and 36% are below 100 nm. Mean diameter is 110nm. (Fig taken from Weir *et al*., 2012)

It may be interesting to note that FAO/WHO in one of their own reports (2009) stated the same fact about the occurrence of nano-sized particles, albeit referring to the bulk material. This, however, is evidently not enough to question the use of E171 in food items, or to inquire as to the extent of TiO_2 nanoparticles in E171. On the other hand, even if 40 % seems a lot and is surprising to some, it still would not be classified as a nanomaterial, as the definition requires that at least 50% is in nanosize. Adding to the concern does the fact that the anatase form of TiO_2 in nanoform is said to be 100 times more toxic than the rutile form (Weir *et al*., 2012) and in the characterization of the crystal structure of the nanosized TiO_2 from chewing gums, Chen *et al*. (2012) found it to be in the anatase form, as confirmed by both XRD and high-resolution TEM (transmission electron microscopy). That E171 is often in the anatase form in food, is corroborated by Powell *et al*. (2010), who detected anatase TiO_2 of approximately 100-200 nm in human intestines.

In the approval of the rutile form of TiO_2 as E171, EFSA included exposure data on TiO_2 intake, as presented by the petitioner. The estimated intake was 1.28 mg/kg-bw/day including medicines and supplements, in which case a person of 70 kg would consume roughly 90 mg TiO_2 daily. This was claimed to be a gross overestimation. If realistic, and if the quantization of the nano content in E171 of 36-40% is correct, and applicable to E171 in general, this 70 kg person would consume around 35 mg nano TiO_2 per day. Either our consumption of TiO_2 has increased greatly over the last decades, as suggested by some, or our consumption patterns vary a lot between countries, since the Ministry of Agriculture Fisheries and Food, in UK in 1993, estimated their intake of TiO_2 to be 5.4 mg/day (corresponding to 0.077 mg/kg-bw/day), not including medicines and supplements. Also, Lomer *et al*. (2002) reported a daily intake of 5.9 mg/person of TiO_2 in the UK.

According to data from Powell *et al.,* (2007) based on intake data from Lomer of 5.9 mg/pers/day, the proportion of TiO_2 particles ingested through food, pharmaceuticals and toothpaste are 47%, 48% and 5%, respectively. Silicates and aluminosilicates are also frequently used as additives with an estimated intake of 34 mg/pers/day (Lomer *et a*l., 2002), but there the data are reversed, with food, pharmaceuticals and toothpaste corresponding to 14%, 44% and 42%, respectively.

Several groups (Chen *et al*., 2012, Weir *et al*., 2012, Powell *et al*., 2000) have taken an interest in the probable high intake of nano-and micro sized TiO_2 and the potential consequences for both human health and environment. While Chen and Weir analyzed the nano-TiO_2 in food items, Powell, with an interest in chronic inflammatory diseases, analyzed TiO_2 content as such, in food. It is apparent from their results that intake may vary greatly between individuals as there are some food groups with an extreme TiO_2 content, unmatched by other food items. In general, chewing gums and confectionaries and chocolates with a hard shell contain the highest levels of TiO_2. Below is a list of a few items and their TiO_2 content (Table 1).

Table 1: Food items and their TiO_2 content (Chen *et al*. 2012, Weir *et al*., 2012, Powell *et al*. 2000). The center section contains the amount of TiO_2 per gram food. In the right section the TiO_2 content has been adapted to serving size.

Food item	**TiO_2 content (mg/g food)**	**TiO_2 content (per serving)**
Powdered donut	2	100 (50g)
Salad dressing	7.5	225 (30g)
M&Ms	1.25	45
Chewing gum	1.51-3.88	2.4-7.5 (average 5) per gum
Marshmallows	2	10 (5g)
Creamed horseradish	2.84	56.8 (20g)
Teamate	7.82	35.19
Cake icing (on tube)	1.83	55 (30g)
Low fat Caesar dressing	0.93	27.9

It must be mentioned that some of these values are from the year 2000 and recipes may have changed since. For example, in the contents analysis by Powell *et al*., (2000) "Smarties" from Nestlé was included. However, E171 is not on the label here in Sweden and e-mail correspondance with Nestlé confirmed that, in Europe, TiO_2 has been replaced with natural colorants. The recipe in the Middle East remains the same though.

It can be seen from the list that the amount vary a lot and included are the items on the top of the list. Still, it shows how choosing a life style with lots of certain candies and a frequent use of certain processed ingredients, and a high intake of pharmaceuticals, could result in a TiO_2 intake many fold higher than estimated. Add to that, the possibility that 36-40% may be nanosized, with increased uptake and reactivity, surely a fraction of the consumers would have a quick glance at the contents label, if they knew that is.

Needless to say, children due to their lower weight, end up with a higher exposure per kg body weight. Also, they are generally not renowned candy-haters and not all countries have the tradition of "Saturday-candy" as in Sweden. The intake of E171 in children under the age of ten years is estimated being twice that of adults (Weir *et al*., 2012).

In agreement with the importance of a full characterization of nanoparticles that will be added to food, EFSA has suggested that "*the characterization of engineerd nanomaterial should ideally be determined in five stages, ie. as manufactured (pristine), as delivered for use in food/feed products, as present in the food/feed matrix, as used in toxicity testing, and as present in biological fluids and tissues"* (EFSA Journal, 2011). Thus, from this it follows that toxicity testing of various forms of TiO_2, other than E171 with its specific size and modifications, cannot be considered adequate or, in some cases, even relevant.

Toxicity

Toxicokinetics of TiO_2

As not many oral studies on TiO_2 have been performed, data concerning its toxicokinetics after oral administration is sparse. Hopefully the data presented here will suffice to give an idea of its administration, distribution, metabolism and excretion (ADME). Data from *in vitro* studies are included as they may be a complement in the understanding of the fate and reactivity of nanoparticles in organisms.

In a **human** study by Böckmann and collegues (2000), six male volunteers ingested TiO_2 (powder or tablet) either of 160 or 380 nm diameter. Administration of 23 or 46 mg resulted in a blood level of 50 and 100 ug/L, respectively. Background levels varied between 6-18 ug/L. Uptake of 160 nm particles was higher than that of 380 nm particles. In spite of the low uptake, it might not be insignificant when ingested daily in foods. This will be put in a

context later in the section of "Particle intake, microvilli and IBD". (Note: only abstract for this study was obtained, but it has also been referred to in the IARC evaluation of TiO_2).

Oberdörster (2009) discussed how nanoparticles in general are found in the liver after **intravenous administration** (i.v.) and in the bone marrow after **intratracheal administration**. He reasons that the protein corona surrounding the particle, as well as the proximity to a draining lymph vessel, decide where the particles are to be found in the body, much the same idea as mentioned previously (in the "Background" section). This is supported in an ***in vivo*** assay by Umbreit and collegues (2011), who analysed the tissue distribution of TiO_2 in mice after i.v or sub-cutaneous injection. What they could see from gross inspection was that liver, lung and spleen were brighter in colour than controls, due to accumulation in those organs. The mice were inspected at weeks 2, 4, 12 and 26. Over that time they detected microgranulomas (clusters of macrophages and lymphocytes with agglomerated TiO_2) in the liver. In the subcutaneously injected mice, particles were found at the sight of injection long after the time of injection, and the area was subsequently infiltrated by macrophages and fibroblasts. In the gut, the M-cells lining the Peyer's patches, are considered being responsible for most of the particle uptake after oral intake (Powell *et al*., 2010, Lomer *et al*., 2002, Butler *et al*., 2007). The particles then accumulate in macrophages in Peyer's patches, which, in the microscope, can be detected as pigmented cells.

With the help of TEM, the ***in vivo*** study by Wang *et al*. (2012) detected particles (60-200 nm) that adhered to villi and were absorbed in the stomach and small intestine, although only a small fraction, and did not reach the circulation. TiO_2 in food is roughly 100-200 nm and is suggested being taken up in the intestine, as has been seen (Powell *et al*., 2010, Lomer *et al*., 2002) but not to reach the circulation. But, as shown, (Chen *et al*., 2012, Weir *et al*., 2012) there is evidence to support that a substantial fraction of the food grade titanium dioxide (E171) actually is below 100 nm. Those particles would most certainly also be absorbed, even to a larger extent, and perhaps reach circulation. It is suggested by Chaudry *et al*. (2007) that nanoparticles are taken up 15-250 times more efficiently in the GI tract than microparticles. A study by Jani (1990), frequently referred to in the literature, investigated particle uptake in the GI tract of nano sized polystyrene particles and revealed that 7% of the 50 nm particles were taken up by Peyer's patches and from there transported to liver, spleen, blood and bone marrow.

The effect of agglomeration on TiO_2 fate was examined by Creutezenberg *et al.* (2012) in an **instillation assay** of Wistar rats, each given 0.3 mg TiO2. In their experiments they compared agglomeration of two different titanium nanoparticles, TiO_2 P25 and TiO_2 T805 both with a primary particle size of 21 nm. What differed though, was that P25 was hydrophilic and T805 hydrophobic, which, as was shown, influenced their kinetics in the lung. P25, being hydrophilic, tended to form agglomerates quicker than T805, probably due to the hydrophobic environment in the lining fluid of the lung, as speculated by the authors. Along with their agglomeration it could be seen that the particles were internalized in the alveolar macrophages. The T805 also formed agglomerates, but slower. The authors detected that the agglomerated particles were taken up and cleared by alveolar macrophages and

because of that they concluded that agglomerates are unlikely to reach circulation and other organs.

This is in accordance with results from Weir *et al*. (2012) and Andersson *et al*. (2011) who both observed a relation between size and agglomeration. In the ***in vitro*** study by Andersson, this was also related to uptake in lung epithelial cells. Smaller particles formed larger agglomerates and were less taken up in the epithelial cells, whereas larger molecules agglomerated less and were more extensively taken up. Thus, from those reports it seems particles that form larger agglomerates or aggregates become phagocytosed and cleared by macrophages, whereas particles less prone to agglomerate, may instead be taken up by other cells, such as epithelial cells. The results regarding nuclear uptake of TiO_2 are also conflicting. Andersson *et al*. did see nuclear uptake, though only to a minor extent, while Dodd *et al*. (2009), on the other hand claim there is no uptake in the nucleus.

Weir and co-workers (2012) also clarified that size and surface characteristics are quintessential for the reactivity of nanoparticles, as illustrated in a comparison between E171 and P25, the latter frequently used in toxicity assays. While P25, approximately 25 nm, rapidly aggregated into particles of 2.5 μm after sonication, E171, with a mean diameter of 110 nm, stayed more soluble in solution. While size is indeed important, the fact that E171 is allowed to be, and is, modified in order to "improve the technological properties", is also crucial (Borm *et al*., 2006). As E171 need to be soluble in food preparations it is coated to that end, but what implications do that coating have for its distribution, uptake and reactivity in humans? This is important to keep in mind when discussing ADME of TiO_2 because, in the literature search for this report, only two *in vitro* toxicity assays on either food grade TiO_2 (Butler *et al*., 2007) or E171 (Chen *et al*., 2012) were found. Knowing how uncertain extrapolations from *in vitro,* or even *in vivo,* to humans are, not even investigating the very same substance, should make those extrapolations extra fragile. Still, if the observations on agglomeration and uptake are correct, it can be speculated that since larger, more soluble particles, are more readily taken up than smaller particles that agglomerate, it may not be unrealistic to assume that E171, being soluble and larger, is more readily taken up in the body than, for example, P25.

Allouni *et al*., (2012), in an ***in vitro*** assay of fibroblasts, detected a higher uptake of anatase than rutile TiO_2. Tiede *et al*. (2008) also reported higher uptake of anatase than rutile.

Skocaj *et al*. (2011) in their review of the safety of TiO_2, refer to ***in vivo*** and ***in vitro*** studies in which nanoparticles of 20 and 50 nm were not endocytosed and cleared by phagocytes, as were larger particles of either aggregates or in micro size. In one ***in vivo*** rat assay referred to, nanoparticles of TiO_2 administered by inhalation, were later detected in epithelial cells, endothelial cells and fibroblasts.

Koeneman and collegues (2010) wanted to assess whether nanoparticles of TiO_2 passed the intestinal epithelium and in what way. To that end they used the Caco-2 cell line, which is frequently used as a model of human intestinal cells. Their results indicated that the TiO_2, sized between 80 and 350 nm, crossed the epithelial lining through transcytosis (Fig. 10),

without damaging the cells. Transcytosis is a mechanism by which epithelial cells transport nutrients from GI-lumen to the circulation. They detected a higher cross-over to the baso-lateral side at 10 µg/ml (14, 4%) as compared to the higher dose of 1000 µg/ml (2, 2%). Perhaps it can be speculated that this difference was due to aggregation of particles at higher concentrations, thereby denying them entrance.

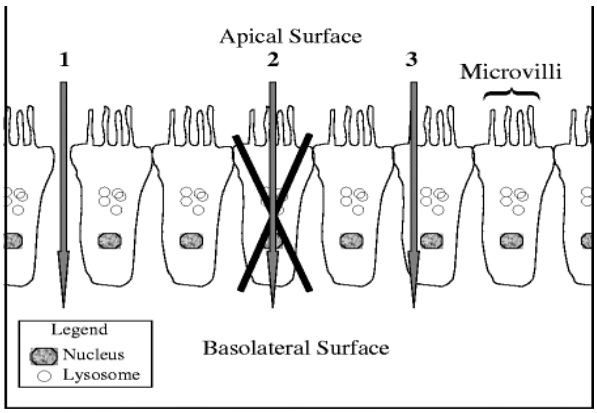


Fig 10: Illustration of possible passages of TiO_2 NP across the epithelium, (cropped figure from Koeneman *et al*. 2010). 1) Particles could be transferred between epithelial cells if the barrier is compromised, or 2) a possible passage when an epithelial cell has died, and 3) illustrates transcytosis where the particles are transferred through the cells from the apical surface in lumen to the basolateral surface. The data showed neither disrupted junctions nor dead cells, suggesting transcytosis as the pathway for TiO_2 NP.

Table 2. Genotoxicity, cytotoxicity, neurotoxicity and immunotoxicity studies of TiO_2

System	Assay	Conc. µg/ml	Result	Size nm	Ref.
In vitro, human lung cell line A549	Comet (geno)	40, 80	Pos, at 80 *µ*g/ml	63	Karlsson (2009)
In vitro, human cell line A549	Viability' mitochondria	40, 80	Neg	63	Karlsson (2009)
In vitro, human skin fibroblast	γH2AX (geno)	10, 25, 50, 100, 250, 500, 1000	Pos at 10 and above, not dose rel.	22	Setyawati (2012)
In vitro, human fibroblast	Proliferation (cyto)	10, 25, 50, 100, 250, 500, 1000	Pos, at 250 and 500 *µ*g/ml	22	Setyawati (2012)
In vitro, PBL	MN (geno)	20, 50, 100	Pos, dose rel. Sign at 50, 100	25	Kang (2008)
In vitro, PBL	Comet	20, 50, 100	Pos, dose/time rel.	25	Kang (2008)
In vitro, PBL	Viability and ROS (cyto)	20, 50, 100	Pos, dose/time rel. decrease in viability	25	Kang (2008)

System	Assay	Conc. µg/ml	Result	Size nm	Ref.
In vitro, RAW 264.7, phagoc	Oxidative stress, pro-infl	0.5 µg/**L**	ROS formation only under abiotic cond.	20-30	Xia (2006)
In vitro, human lung cancer cell line A549	MN	1, 10, 50	Pos, at 10 and 50	<25	Srivastava (2012)
In vitro, human cell line A549	MTT (cyto)	1, 5, 10, 50, 100	Pos, at 10, 50, 100	<25	Srivastava (2012)
In vitro, human cell line A549	LDH (cyto)	1, 5, 10, 50, 100	Pos, at 50, 100 after 6h, at 10, 50, 100 after 24h	<25	Srivastava (2012)
In vitro, salmonella	Ames (geno)	0-4950 ug/plate	Neg	10	Woodruff (2012)
In vitro, TK6[)]	Comet	0, 50, 100, 150, 200 ug/ml	Neg	10	Woodruff (2012)
In vitro, fathead minnow, neutrofil function assay	NET	0.1, 1, 10, 100, 1000	Pos, increased respiratory burst at all conc.	66	Jovanovic (2011)
In vivo, fathead minnow (Pimephales promeloas)	FET, 7 d (cyto)	0.01, 0.1, 1, 10, 100, 1000	Neg	<25	Jovanovic (2011)
In vivo, fathead minnow (Pimephales promeloas)	Neutrofil function assay on adult fish	10 µg/g	Pos, suppressed neutrophil function	86	Jovanovic (2011)
In vitro, Caco-2, human cell line	Intracellular Ca Microvilli changes	1, 10, 100, 1000 µg/ml	Pos at 10 µg/ml and all above, reduced microvilli, increased intracellular Ca	80-350	Koeneman (2010)
In vitro, fibroblasts	Microvilli changes	0.05, 0.5, 5 mg/L	Pos, reduced microvilli at 0.05, collapsed at 0.5 and 5		Allouni (2012)

System	Assay	Conc. µg/ml	Result	Size nm	Ref.
In vivo, CD-1 female mice	Nasal instillation	500 µg every other day for 15 days.	Pos, changes in neuron morphology, enzyme activity and ROS	80 and 115	Wang (2008)
In vitro, mouse Leydig cells	Proliferation and viability	1, 10, 100, 1000 µg/ml	Pos, dose rel. reduction of viability Time-dep decrease in prolif.	25-70	Komatsu (2008)
In vitro, WIL2-NS, human lymphoblastoid cells	MN	26, 65, 130 µg/ml	Pos, dose rel.	7	Wang (2006)
In vitro, WIL2-NS	MTT	26, 65, 130 µg/ml	Pos, dose rel.	7	Wang (2006)
In vitro, WIL2-NS	Comet	26, 65, 130 µg/ml	Pos at 65	7	Wang (2006)
In vitro, WIL2-NS	HPRT	26, 65, 130 µg/ml	Pos. dose rel.	7	Wang (2006)
In vivo, Sprague-Dawley rats	30 day oral study of young and adult rats	10, 50, 200 mg/kg/day	Pos, at 50 and 200	75	Wang (2012)
In vitro, A549 lung epithelial cell	Cytokine and ROS production	5, 50, 100, 200 µg/ml	IL-8, MCP-1, superoxide prod. (various conc dep on particle size)	5, 9, 14, 21, 60	Andersson (2011)
In vivo, Male CBAxB6 mice	Comet Liver	40, 200, 1000 mg/kg-bw/d	Genotoxic (not dose rel) in bone marrow at 40 (both 33, 160 nm) liver (33 nm) at 200), increased mitotic index.	33, 160	Sycheva (2011)
In vivo, Male CBAxB6 mice	MN	40, 200, 1000 mg/kg-bw/d	Pos at 1000 (160 nm)	33, 160	Sycheva (2011)

System	Assay	Conc. µg/ml	Result	Size nm	Ref.
In vivo, F344/CrlBR rat, B3C3F1/CrlBR mouse and LVG BR hamster	13 weeks, subchronic inhalation- lung burden, cytology	10, 50, 250 mg/m^3,	Pos in rat at 50 and 250, LDH and epithelial hypertrophy and hyperplasia	Pigm TiO_2	Bermudez (2002)
In vivo, mice, C57Bl/6Jp^{un}/p^{un}	MN	50, 100, 250, 500 mg/kg/d for 5 days	Pos at 500	160	Trouiller (2009)
In vivo, mice, C57Bl/6Jp^{un}/p^{un}	γ-H2AX (geno)	50, 100, 250, 500 mg/kg/d for 5 days	Pos, dose rel.	160	Trouiller (2009)
In vivo, mice, C57Bl/6Jp^{un}/p^{un}	Comet	50, 100, 250, 500 mg/kg/d for 5 days	Pos at 500	160	Trouiller (2009)
In vivo, mice, C57Bl/6Jp^{un}/p^{un}	Inflammation	50, 100, 250, 500 mg/kg/d for 5 days	Pro-infl: TNF-α, IFN-γ, IL-8 at 500	160	Trouiller (2009)
In vivo, mice, C57Bl/6Jp^{un}/p^{un}	DNA-deletion *in utero*	500 mg/kg/d	Pos, 27% increase in DNA deletions in embryo	160	Trouiller (2009)

Abbreviations: MN; micronucleus assay, MTT; methyl tetrazolium assay, LDH; lactat dehydrogenase, PBL; peripheral blood lymphocytes, CHO; chinese hamster ovary, RTG; rainbow trout gill, GFSK; gold fish skin cell line, FET; fish embryo toxicity assay, γ-H2AX (measurement of phosphorylated histone 2A, indication of DSB), TNF; tumour necrosis factor, IFN; interferon, IL; interleukin, pigm; pigmentary, MCP-1; monocyte chemotactic protein-1, NET; neutrophil extracellular traps

Discussion of TiO_2-toxicity

Traditionally, TiO_2 has been considered inert (JECFA 1969, Skocaj *et al*., 2011) with no toxicity and therefore used as negative control in many assays (Ema *et al*., 2010). Actually, in the evaluation of TiO_2 as a possible carcinogen, IARC pointed out that in some of the toxicity assays on TiO_2, TiO_2 was also used as a negative control. Any result implying lack of toxicity, deriving from such an experimental setup, cannot possibly be reassuring. As can be seen from Table 2 there is reason to doubt a general lack of toxicity. A clinical example to illustrate the reactivity of TiO_2 comes from titanium implants. It is used as such because of its inertness. But TiO_2 on the surface of implants has been released in patients, particularly after physical abrasion against the bone or another implant. Released TiO_2 could then migrate to other parts of the body, adhere to proteins and stimulate an immune response with subsequent

inflammation (Allouni *et al*., 2012, Lomer *et al*., 2002). This inherent ability of TiO_2, and other nanoparticles, to adhere to proteins and possibly function as adjuvants in the onset of an immune response, will be discussed again below. The results on display in Table 2 above show toxicity in various biological systems from *in vitro* human cell assays to *in vivo* studies on mouse, rat, hamster and fish. The toxic effects discussed below include cyto-, geno-, immuno-, reproductive and neurotoxicity. But, as can be seen from Table 2, the assays are far from conclusive, with several examples illustrating a lack of response in various cells. This discrepancy of the results only confirms that the evaluation of nanoparticle toxicity is anything but straightforward. It should be emphasized that the majority of the assays are *in vitro*, from which conclusions regarding toxicity in man, are difficult to draw.

Interesting results from Karlsson and co-workers (2009) suggested that the nanoform of TiO_2 is less toxic than corresponding microform when it comes to DNA damage, as showed by the Comet assay. They also stated that, both nano and micro particles are taken up by cells, but the size determines in what form (ie with/without vesicle) which in turn affects its toxicity. Strangely enough, they did not detect any cytotoxicity or mitochondrial assault from neither form of TiO_2, which disagrees with other findings. For example, Srivastava *et al*. (2012) (see Table 2) detected mitochondrial damage using the same cell line. They even detected toxicity at 10 μg/ml. The particle sizes differed between the two assays, but not radically, <25 vs 63 nm. Many other groups agree that one of the primary mechanisms behind TiO_2 toxicity is through oxidative stress. The fact that the microform was suggested being more genotoxic than the nanoform, and is taken up by cells equally well, is worrying in the context of titanium dioxide being a food additive.

All assays referenced to Kang (2008) (micronucleus, comet, ROS and viability) showed time and dose related toxicity. Interestingly, addition of NAC (N-acetyl cystein) to the system significantly reduced the oxidative stress and DNA strand breaks, as shown in the comet assay. Since NAC is a precursor of the tripeptide glutathione (GSH), an abundant and essential antioxidant, this indicates that the DNA damage resulted from oxidative stress. The notion that the DNA damage induced by TiO_2 is a secondary effect due to oxidative stress, is supported by others (Setyawati *et al*., 2012, Srivastava *et al*., 2012, Trouiller *et al*., 2009).

Clearly demonstrating the highly discrepant results on the toxicity of TiO_2, Xia *et al.* (2006) compared the ROS producing ability and pro-inflammatory effects of various nanoparticles. While their results demonstrated that TiO_2 NP in aqueous solution had the highest ability to induce ROS, in cell culture media it did not. Nor did TiO_2 NP induce pro-inflammatory mediators in the *in vitro* assay on RAW 264.7 cells. They also investigated any binding to, and disruption of, mitochondria as well as the intracellular calcium levels, and did not observe any effects induced by TiO_2 NP. They also investigated amine-coated polystyrene (NH_2-PS) spheres whose effects were significant for all the parameters investigated. In aqueous media, the TiO_2 NP and NH_2-PS had a zeta potential of -16.4 and +40.3, respectively, whereas in culture media they were -12.4 and -11.1, respectively (see Fig 4, page 9 for a reminder of zeta potential). Hence, it would be difficult to attribute any differences in reactivity in culture media, to their respective zeta potentials.

Srivastava and collegues (2012) performed a number of tests, apart from those shown in Table 2, to further evaluate oxidative stress caused by TiO_2 NP and any consequential change in transcription. The reduction of GSH and increase in lipid peroxidation are indicators of oxidative stress. Up regulation of p53 and p21 and down-regulation of anti-apoptotic Bcl-2, at transcriptional and translational level, also imply a mode of stress induced by TiO_2 NP. Beside cytotoxicity, they also investigated any genotoxicity, using the micronucleus assay, with a positive result at 10 and 50 µg/ml.

Not supporting the results from Karlsson *et al*. (2009) and Kang *et al.* (2008) Woodruff and co-workers (2012) did not observe genotoxicity in the comet assay or Ames test. The lack of genotoxicity in Ames test is not surprising given that their TEM-images (transmission electron microscopy) revealed that the particles did not even enter the bacteria. Noteworthy was, however, the lack of genotoxicity in the comet assay, without significance even at 200 µg/ml. In an attempt to put forward reasons for this disparity, the cell types used is one suggestion. Different cell types often respond differently to substances, and if the genotoxicity is dependent on the antioxidative abilities of the cells (as indicated by loss of toxicity when NAC was added), perhaps the TK6 cells (human lymphoblastoid cells) have a more powerful defense against this kind of insult. Another obvious difference, though not obviously the explanation, is particle size. The group of Woodruff (2012) used particles of 10 nm, compared to 25 and 63 nm particles used by the other groups. It is recognized that nanoparticle toxicity is linked to particle size, but normally, decreased size renders particles more reactive, and larger ones less so. Hence, abiding by that gross generalization, size is probably not the explanation in this case. Also, particles of 10 nm and 25 nm are probably considered being in the same size range.

As mentioned by Jovanovic and co-workers (2011), the concentration of TiO_2 able to stimulate respiratory burst by neutrophils (0.1 µg/ml) is four times higher than estimated environmental concentration (0.025 µg/ml). In Table 2 it can be seen that, while TiO_2 NP increased respiratory burst *in vitro*, the *in vivo* experiment indicates a suppression of neutrophil function. The authors discuss that the major target organ of TiO_2 NP accumulation in fish is the kidney, which also happens to be a lymphopoietic organ. From this, they were tempted to speculate that the neutrophils would be long term exposed throughout their maturation, leading to suppression. Another interesting aspect they discuss is the fact that after TiO_2 uptake, neutrophils continuously produce ROS through respiratory burst, which leads them to release of so-called NETs (neutrophil extracellular traps). The release of NETs is considered a way of the neutrophil to get rid of the TiO_2, after which it dies. This means that the particles are not cleared, but remain in the system.

If this should be put in a human context, the kidney is not a lymphopoietic organ, but the spleen is. It has been shown in several studies that the spleen is a target for nanoparticle accumulation in rodents (KemI rapport 2007, Borm et al., 2006) maybe so in humans as well. If that were the case, a low but prolonged exposure, leading to accumulation in the spleen, could perhaps be a means for immunosuppression, or activation.

Koeneman *et al*. (2010) registered a raise in intracellular calcium level, already at 10 µg/ml. The intracellular calcium concentration is meticulously regulated, as it is a tool to govern many processes in the cell. It acts as a second messenger in cell signaling, and a seriously elevated calcium level pushes the cell into apoptosis or necrosis. The authors also noted morphological changes of the microvilli, which could have implications for nutrient uptake. These results are interesting from several aspects. As the TiO_2 in their assay spanned from 80-350 nm, they encompassed the size used as food additive (E171), which is usually around 200 nm (FAO/WHO 2009, Lomer *et al*., 2002). Adding to that, it has been discussed by Lomer and collegues, that elevated levels of TiO_2 in our diet may account for the increased incidence of Crohn's disease, a trend seen in developed countries. They, in their investigations, noted uptake of TiO_2 into Peyer's patches in the GI tract, through M-cells in the mucosa. There, the particles could accumulate and, if not induce acute toxicity, give a chronic inflammatory response, just as in Crohn's disease. Apart from that, they, along with others (Fröhlich *et al*., 2012) believe that the nanoparticles taken up, due to their charged surface, could function as a Trojan horse, facilitating the uptake of other, unwanted molecules, which adhere to the reactive surface of the nanoparticles. Those undesired particles could be LPS (lipopolysaccharide) or toxins from bacteria, which may elicit an immune response. The occurrence of calcium increases the binding of TiO_2 to LPS, which increases the inflammatory response (Powell et al., 2007).

Mice nasally instilled with TiO_2 of either 80 nm rutile or 115 nm anatase clearly displayed neurotoxic effects (Wang *et al*., 2008). The changes induced from the exposure varied from morphological changes and loss of neurons in *hippocampus*; altered enzyme activity of catalase (CAT), superoxide dismutase (SOD) and acetylcholine esterase (AchE); lipid and protein oxidation- clearly indicating oxidative stress, and increased level of the neurotransmitter glutamate. The effects inflicted by anatase were greater than those from rutile, again demonstrating the higher toxicity of anatase. This study is not the only one showing the ability of nanoparticles to enter the brain via the olfactory neuron (Oberdörster, 2005).

The *in vitro* study on mouse Leydig cells (cell line TM3) by Komatsu and collegues (2008) investigated the effects on proliferation and viability. They observed, using TEM, that TiO_2 NP were taken up and formed agglomerates in the cytoplasm, but did not enter the nucleus. Nor did the particles adhere to the mitochondria, which is otherwise common nanoparticle behaviour (Oberdörster, 2005, Andersson *et al*., 2011). Note that while they detected a decrease in both viability and proliferation, this study did not present any sign of oxidative stress induced by TiO_2, which is also often intimately related to nanoparticle toxicity (Setyawati *et al*., 2012, Xia *et al.*, 2006, Srivastava *et al*., 2012). They speculate if the inhibition of viability and proliferation of Leydig cells, could be a mechanism for the, by others (Ono *et al*. 2007), observed impairment of male mouse reproduction following TiO_2 exposure, as the Leydig cells are the testosterone producing cells. Corroborating this finding, Ema *et al.* (2010) listed studies on reproductive toxicity which, along with detection of TiO_2 in Leydig and Sertoli cells, noted decreased epididymal sperm motility, reduced number of

Sertoli cells and histopathological changes in testis in pups from pregnant ICR: mice exposed subcutaneously to 100 μg per day on four different occasions.

Wang *et al*. (2006) wanted to assess cyto-and genotoxicity of TiO_2 NP in human lymphoblastoid cells, WIL2-NS. To that end they used an array of assays including MTT (methyl tetrazolium assay), LDH (lactate dehydrogenase), HPRT (hypoxanthine-guanine phosphoribosyltranferase), CBMN (cytokinesis block micronucleus), comet and apoptosis assay. They concluded not only that nano TiO_2 is both genotoxic and cytotoxic, but so in a dose-dependent way. Reduction in cell viability, as measured in the MTT assay, was also time-dependent.

In the toxicity assessment of TiO_2 in food, Chen *et al*. (2012) evaluated the cytotoxicity and ROS production in GES-1 and Caco-2 cells, of purified E171 from chewing gums. They did not see reduced viability or increased toxicity, as shown in the LDH assay, but they did see an increase in ROS production. This does not correlate with the assays discussed above that have shown cytotoxicity. But, as discussed by Setyawati and co-workers (2012) and Powell *et al*. (2010) toxicity is highly dependent on the cell type under investigation, and the response of GES-1 or Caco-2 cells may be different from that of epithelial cells, fibroblasts or lymphoblastoid cells.

Sycheva *et al*. (2011) investigated the geno- and cytotoxicity of 33 nm and 160 nm particles. Though they observed a significant increase in micronucleus formation, it was not dose related and they counted too few cells to be considered highly significant. However, they noted increased mitotic index (more than two-fold) in epithelial cells of the forestomach and colon in mice administered both 33 and 160 nm particles, indicating increased replication as a result of near-pigmentary-sized TiO_2 exposure. The testis also showed highly significant values for mitotic index, though only in the 33 nm group. This latter result, however, contradicts the results by Komatsu *et al*. described previously, which noted a decrease in proliferation of Leydig cells after TiO_2 exposure.

Based on the estimated higher exposure of dietary TiO_2 in children compared to adults, the group of Wang (2012), wanted to compare the effects of TiO_2 nanoparticles in young and adult Sprague-Dawley rats. Their finding indicated that young and adult rats responded differently to the particles. Young rats displayed hepatic edema and biochemical abnormalities concerning increased serum total bilirubin, increased alanine aminotransferase (ALT) and decreased aspartate aminotransferase (AST), all indicative of hepatic dysfunction. They also detected increased blood glucose and LDL-C (low density lipoprotein cholesterol). They saw no toxic effects on the kidney. The adult rats showed increased number of inflammatory cells in the liver, but no other toxicity. This assay points to the liver as the main target for TiO_2 toxicity.

Perhaps more intriguing was their finding that the particles diminished the uptake through the intestine, which can be detrimental in terms of reduction of nutrient uptake. What makes this finding even more interesting is the fact that, as mentioned above, Koeneman *et al*. (2010) observed how the morphology of the microvilli on the intestinal cells changed and were

reduced in numbers, after addition of TiO_2, which made them speculate if that could result in a reduced nutrient uptake. The finding that microvilli may be reduced after exposure to TiO_2 has been corroborated in an assay by Allouni *et al*. (2012), which, at 0.05 mg/L, detected reduction of microvilli, along with other morphological changes of fibroblasts, and a complete loss of microvilli at 0.5 mg/L. It must be emphasized though, as explained by Powell *et al*. (2010), that one cannot apply results obtained from non-gut cells to the situation in the gut. The reduction of microvilli seen by Allouni and co-workers on fibroblasts already at 50 μg/L, were, in the assay by Koenemann on intestinal Caco-2 cells, seen at a much higher concentration (10 μg/ml). However, regardless of the applicability on intestinal cells, the fact that fibroblasts are affected to that extent, at such low concentration, is interesting.

The results from Wang *et al*. (2012), showing reduced uptake in the intestine, and those from Allouni (2012), showing reduced microvilli, both indicate Koeneman *et al*. (2010) may not be far from the truth in the speculation that chronic exposure to TiO_2 NP may result in reduced nutrient uptake. Wang and co-workers also investigated if TiO_2 could elicit an allergic response. They reasoned that since inhaled particles may be aggravating for an asthmatic, and application of particles on the skin may do the same to people with atopic dermatitis, perhaps oral ingestion of particles may increase an allergic response or inflammation in the gut, particularly applicable to patients suffering from Crohn's disease. They did indeed see an increase in mast cell number in the stomach, though not in the small intestine. The hypothesis that increased dietary TiO_2 is contributing to the increased incidence in Crohn's disease, as suggested by Lomer *et al*. (2002) and Powell *et al*. (2007) applies primarily to the small intestine as particles of titanium, alumina and silica have been visualized in macrophages in Peyer's patches of the small intestine. But, TiO_2 NP can pass the epithelial barrier and be taken up elsewhere along the GI tract as well.

In further support of the theory of a relation between TiO_2, inflammation and Crohn's disease are the results from Trouiller and colleagues (2009) who, in an *in vivo* study on mice orally exposed to TiO_2 NP through the drinking water, showed a significant increase in the pro-inflammatory cytokines TNF-α (tumour necrosis factor), IFN-γ (interferon) and IL-8 (interleukin). No increase in the anti-inflammatory cytokines TGF-β, interleukin-4 and interleukin-10 was detected. They not only consider a link between particle ingestion, inflammation and Crohn's disease, but their results also showed genotoxicity manifested as DNA strand breaks (DSB) in bone marrow shown with the γ-H2AX-assay, deletions in embryo exposed through the mother (eyespot assay), DNA strand breaks in peripheral blood (comet assay), clastogenic effects in bone marrow (micronucleus assay) and oxidative DNA damage (8-hydroxy-2'-deoxyguanosine, (8-OHdG)). Their results will be discussed further in the section of "Particle intake, IBD and genotoxicity" below.

As the particle uptake and inflammatory response in the gut has been discussed thus far, it might be interesting with a comparison of those events, to the response in rat, mouse and hamster observed after inhalation of pigmentary TiO_2, as evaluated by Bermudez and co-workers (2002). The primary outcome of their study was the species differences, which clearly showed rat to be most susceptible. Rat, and mouse, of the high-dose groups still had 75% of inhaled particles in the lung at 52 weeks post inhalation. All three species showed

increased infiltration of neutrophils after inhalation, but while the neutrophils of the hamster had returned to normal at 26 weeks post-exposure, an elevation remained in rat and mouse 52 weeks post-exposure, indicating a chronic inflammation. Lesions in the alveolar lumen were also detected, at mid and high doses, together with aggregations of overloaded alveolar macrophages. In the direct vicinity of the aggregations of particle-loaded macrophages, hypertrophy and hyperplasia (increase in cell size and cell number, respectively) of type II epithelial cells, was observed. These lesions lasted throughout the 52 weeks recovery period. Also, in the highest dose group, metaplasia of alveolar cells along with fibrosis was detected and the authors relate these events to the chronic inflammation, in turn mediated by the high, and lasting, neutrophil count. Umbreit *et al*. (2011) investigated tissue distribution after intravenous or subcutaneous injection and their results pointed in the same direction, with infiltration of fibroblasts and macrophages in the injected area and activation of interstitial mononuclear cells. However, in the lung, the particles were detected along the epithelial wall, and not in alveolar macrophages as seen by Bermudez *et al*.

The above mentioned responses to inhaled or injected TiO_2, much resemble those reported from orally administered TiO_2, and indicate that responses need not differ greatly between different routes of administration. If that is agreed upon, can it be ruled out then, that some of the mechanisms behind the carcinogenesis of TiO_2, responsible for placing it in category 2B by IARC, are also applicable to orally ingested TiO_2? Particularly since the carcinogenesis of TiO_2 is considered a secondary effect, resulting from oxidative stress, and not due to an organ specific mechanism.

TiO_2, inflammatory bowel disease and genotoxicity

More than a decade ago researchers started contemplating a relation between intake of various particles via food, such as TiO_2 and AlSi, and the incidence of inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and Ulcerative colitis (UC), as both particle intake and inflammatory diseases have increased over the last half of a century (Lomer *et al*., 2002, Butler *et al*. 2007). Indeed, the literature holds many fascinating examples, suggestions and pieces of evidence to their relation, some of which will be mentioned here. Of course, they may all be merely coincidences.

Mentioned many times by now is the ability of nanoparticles to induce ROS formation and inflammation. These go hand in hand as ROS may induce damage and attract inflammatory cells, but inflammatory cells, in turn, also release ROS or RNS (reactive nitric species) for example in the respiratory burst, as a means for protection against pathogens (Butler *et al*., 2007, Trouiller *et al*., 2009). An acute inflammation must be resolved by anti-inflammatory mediators, or it will become chronic. Chronic inflammation is related to many diseases, cancer being one (Westbrook, 2009).

Crohn's disease and Ulcerative colitis are states of chronic inflammation. In the case of CD it may arise throughout all parts of the GI tract, whereas UC refers to the colon. Peyer's patches are lymphoid aggregates in the small intestine, which are lined with M-cells. These take up

foreign substances from the gut lumen and transport them to antigen presenting cells (such as macrophages and dendritic cells) and T-cells within the Peyer's patches (Lomer *et al*., 2002) (see Fig 11).

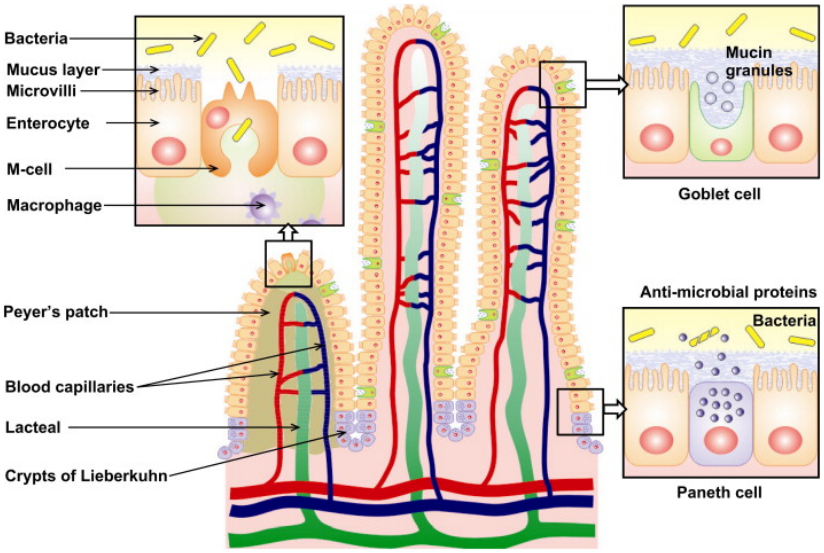


Fig. 11 An illustration of the small intestine showing a Peyer's patch (green) with M-cells that take up the pathogen and a macrophage in the lymphoid structure that phagocytoses the pathogen and presents it to T-cells (not shown). Blood capillaries are also show. Nanoparticles ingested in food has been suggested to reach the circulation through uptake in Peyer's patches, then cross the endothelial cells and enter the circulation. Once there they can reach distant organs such as liver, spleen and lung (Trouiller, 2009) (*Fig taken from "A review of the prospects for polymeric nanoparticle platforms in oral insulin delivery" (Chen, 2011))*

These cells may then elicit an immune response against the invaders, with infiltration of inflammatory cells, such as neutrophils, and secretion of pro-inflammatory cytokines. Therefore, it is not surprising that Peyer's patches, being frequently exposed to ingested particles or pathogens, can mediate diseases such as Crohn's disease. Interestingly, Lomer and collegues reported that many people, diseased or not, have pigmented cells in the Peyer's patches. An investigation of the pigments showed them to be silica, alumina and titanium, all of which are common in food additives. Further characterization of the titanium revealed that it was the anatase form of TiO_2. Moreover, macrophages loaded with these pigments are commonly seen in patients with Crohn's disease, and they are particularly common in the diseased area of the intestine.

It was mentioned above that Umbreit *et al*. (2011) observed microgranulomas in the liver of mice exposed to TiO_2. Granulomas are common in certain diseases as a response to non-degradable substances, and they are frequently seen in patients with Crohn's disease (Butler *et al*., 2007).

Inflammation and genotoxicity

In the light of the inflammatory response observed by many, it has been reasoned that frequently replicating cells, such as epithelial cells in the lining of the gastro intestinal tract (GI), or blood cells from the hematopoiesis in the bone marrow, are targeted by pro-inflammatory mediators, which may either further stimulate replication or inhibit apoptosis (Westbrook *et al*., 2009). The combination of genotoxicity and pro-inflammatory mediators seen after administration of TiO_2 NP (Trouiller *et al.,* 2009), and enhanced proliferation, is unfortunate as they provide the basis for carcinogenesis. It has been illustrated in a study (Westbrook *et al*., 2009) how inflammation in the intestine, acute or chronic, chemically induced by DSS (dextran sulfate sodium) or genetically induced in interleukin-10 knock-out mice, respectively, resulted in systemic genotoxicity. This was shown by *in vivo* MN assay, comet assay, oxidative DNA damage assay and γ-H2AX on peripheral blood. These results support the acknowledged connection between IBD and colon cancer, as well as the general apprehension of a relation between inflammation and cancer.

Trouiller and collegues (2009) hypothesize that the systemic genotoxicity induced by TiO_2 NP or inflammation, may arise from circulating activated inflammatory cells such as macrophages. These may then, through oxidative burst, release reactive species that cause DNA damage in peripheral blood cells, bone marrow cells or embryo, as shown in their study. This observed relation between TiO_2 NP, inflammation and genotoxicity ought perhaps warrant some sort of restriction regarding E171 as an additive.

Particle intake, microvilli and IBD

Another interesting feature of cells exposed to TiO_2 NP is the destroyed microvilli, as observed by both Allouni *et al*. (2012) and Koeneman *et al*., (2010), mentioned above. Quite fascinating, in a colonoscopy performed on patients with UC, it could be seen that the epithelial cells were damaged with reduced microvilli (Fratila *et al*., 2010). It is interesting to relate these findings to the human oral study by Böckmann (2000) which revealed that ingestion of 23 mg of TiO_2 microparticles resulted in a blood concentration of 50 µg/L (and 46 mg resulted in 100 µg/L) Recall that the dose in the Allouni *in vitro* assay, that reduced the microvilli, was the very same, 50 µg/L. Normally, *in vitro* doses are quite high, one of the factors making extrapolation from *in vitro* to *in vivo* difficult. Also recall that the, by EFSA, estimated exposure of E171 is approximately 1.28 mg/kg bw/day, and the calculation suggesting a 70 kg person ingests around 32 mg nanosized TiO_2. In the colonoscopy of UC patients it was also observed that the structure of mitochondria and Golgi was altered. It has been described that nanoparticles tend to bind to organelles (Dodd *et al*., 2009), with mitochondria frequently targeted. If so, could that result in distorted structure?

Speculations on particles, obesity and related diseases

Another exciting aspect of Crohn's disease and particle intake is the significantly higher intake of sugar in CD patients, compared to healthy individuals (Lomer *et al*., 2002). Remember that E171 primarily is found in processed foods and confectionaries. Data on

particle intake among diseased and healthy have not been published, but the mere fact that patients with CD eat more sugar is interesting, particularly in the light of macrophages in the intestines of CD patients being loaded with TiO_2. This is fascinating on many levels as it tempts to speculations on links between obesity and the many illnesses generally related thereto. Powell, Lomer and Butler have taken an interest in particle intake and CD. Consider if, for example, cancer, chronic inflammation, cardiovascular diseases or allergy, all overrepresented in obese people, are not always solely due to obesity *per se*, but aggravated by a higher particle intake. As discussed by Powell *et al*. (2007) the uptake of potentially immunostimulatory molecules, such as LPS, or proteins in the gut, together with TiO_2, synergistically increases the immune response. Can this be attributed to food proteins as well, explaining the increase in food allergies in Western countries?

That TiO_2 results in inflammation and that inflammation can result in cancer has been discussed thus far. Among the results on effects of TiO_2 NP there are also data on cytokine profiles and T-cell activation. Of particular relevance in a discussion on allergy is the T-helper type 2- (Th2)-mediated response, or rather, the balance between Th1-and Th2. Butler *et al*. (2007) evaluated the effect of micro particles (such as food grade TiO_2) *in vitro* on macrophages and their phagocytic ability. Their results indicated that TiO_2 acted as an adjuvant, increasing the production of the cytokines TNF-α, IL-8 and IL-10, as compared to the production stimulated by LPS alone. The adjuvant activity was particularly strong in the presence of calcium, which binds to both TiO_2 and LPS, linking them together (Powell *et al*., 2010). IL-10 is a Th2 cytokine, which generally suppresses responses of Th1 (Kuby, 4th ed.) and as such shifts the balance towards the allergy inducing Th2 subset. What they also detected was a decreased phagocytic ability of macrophages, leading them to speculations if that, similar to the hypothesis for inhaled TiO_2 in the lung, (Bermudez *et al*. 2002) could lead to "particle overload" in the intestines.

Nogueira *et al*. (2012) investigated cytokine production in mice after exposure of nanoparticles and microparticles of TiO_2. Their results were not very clear as to the ability of TiO_2 to induce either Th1 or Th2, as cytokines of both subsets were secreted (IL-4, IL-12, IL-23, TNF- α, IFN-γ and TGF-β), (though in their analysis they considered it a Th1 response). However, they noted a significant increase in Th2 cells in the small intestine. Perhaps that could have implications for food allergies? This idea, of a relation between particle intake and allergy, is partly based on the same criterion as that for Crohn's disease, namely an increase in the Western world over the last decades, and whose cause remains unexplained. The hygiene hypothesis, by some thought to explain the increase in allergy in developed countries, suggests that our habits of excessive cleaning would shift the balance between Th1 and Th2. Customs not shared in developing countries. But what if particles we ingest tilt the balance of Th1/Th2, as also speculated by Butler *et al*. (2007) in the context of Crohn's disease. Or, the ability of particles to bind to proteins in the intestines and function as adjuvants, as shown by Powell *et al*. (2010), could that boost the response against food proteins or result in new epitopes (small fragments recognised by antibodies or T-cell receptor) that stimulate the immune system, much the same way as was seen for TiO_2 released from surgical implants.

It has also been shown in several studies that nanoparticles may affect the coagulation process in the blood and lead to thrombosis (Seaton *et al*., 2010, Jovanovic *et al*., 2012). Many pieces of evidence have linked inhaled nanoparticles with atherothrombosis. Particles are taken up in the lung, not cleared by macrophages, enter the circulation where they may affect endothelial cells and blood platelets. For example, inhaled 30 nm gold particles have been detected in blood platelets where they affected their aggregation (Oberdörster, 2005). For another interesting detail, recall that in the study by Wang and co-workers (2012) they observed increased blood glucose level and increased LDL-C in rats after oral intake of TiO_2 NP. Arteriosclerosis is common in obese people, and is promoted by the so-called "bad cholesterol" LDL. Though not a believer in the diet "low carb high fat" (LCHF), it cannot be denyed that followers quite often sidestep the classical dogma of high animal-fat intake-bad blood cholesterol levels. They may end up with fantastic cholesterol values and feeling great, in spite of eating loads of fat (though for some it is a hazardous diet). What they do, though, is they do not eat processed junk food or candies of any kind, that way evading TiO_2 and other additives.

Admittedly, the speculations just presented may be very farfetched, probably even ridiculous. But it is interesting to note that obese people are known to suffer more from chronic inflammation, cardiovascular diseases, allergies and certain cancers, than non-obese. Those diseases may all have other explanations, but perhaps the contribution from nanoparticles is enough to make a difference in susceptible individuals. Again, their food habits generally include a substantial amount of processed foods and high intake of confectionaries, which contain E171 and other particulate food additives. Also, what if the unknowingly omitted TiO_2, and other additives such as AlSi, have been a confounding factor in epidemiological studies aiming to elucidate relations between food intake and health or disease.

Toxicokinetics of AgNP

In a 28-day oral study on Sprague-Dawley rats, Van der Zande and co-workers (2012) wanted to assess toxicity, distribution and elimination of silver nanoparticles (Ag NP). In their assay they included a group of rats that was given ionic silver ($AgNO_3$) in order to determine if any toxicity was attributed to the nanoform, or to silver as such.

There was a difference between the nanoparticle and ionic forms regarding their uptake, with ionic $AgNO_3$ more readily taken up. The blood level of silver was significantly higher in the $AgNO_3$ group. It must be mentioned here that in a suspension of Ag NP, a fraction is in ionic form; in the assay discussed here, approximately 7%. In an attempt to take this into account, the authors normalized the silver content on the soluble form in both groups (7% in the Ag NP and 100% in the $AgNO_3$) and this resulted in no significant difference in blood levels between the groups. They thus speculate that the silver detected in the blood in the Ag NP group was in ionic form, released by the nanoparticles.

The two forms showed similar patterns of distribution with gastrointestinal tract, liver and spleen primarily being targeted and testis, kidney, brain and lung less so. Just as in the blood, the organs contained higher levels of ionic than particulate silver. The detection of silver in the brain is corroborated by others. In the present study they could not clearly distinguish if the silver had passed the blood brain barrier (BBB) or ended up in the endothelial cells of the barrier. This however, was clarified by another group who, by using TEM-EDX (TEM-energy dispersive X-ray spectrometry), reported on silver in neuronal cells after subcutaneous administration (Tang, 2009, only abstract attained). The very same group also detected nano silver inside cells of some of the target organs, eg. liver and kidney. If silver has the ability to pass the BBB and accumulate in the brain, this could have serious health consequences. A study in which mice were exposed to Ag NP, either by systemic, intracerebral or intranasal route showed that sensory, motor and cognitive functions did alter, and dose dependently so (Sharma, 2012). Unfortunately, that study did not administer ionic silver to specify if it was the nanoform that was responsible. The same group has examined the effects of silver in nanoform administered intravenously and intraperitoneally on the blood-brain barrier and their results clearly indicated the particles had an ability to disrupt the barrier and cause brain edema in rats (Sharma, 2010). BBB disruption was also seen by Tang and co-workers (2009) as well as by Trickler *et al*. (2010) in an *in vitro* assay (more under discussion of Ag toxicity).

Elimination of silver from blood was rapid in all groups. One day after the last exposure it was significantly reduced and one week after exposure it was below detection.

However, they found that silver was eliminated very slowly from both testis and brain, with considerable amounts remaining 2 months after exposure. They also refer to another study in which it was shown that silver remained in the brain for 13 months. These are disturbing results. If silver, in nano form or ionic form, is taken up in the brain and remain there for months, this could of course have serious health implications. Silver is known to bind to sulfur groups, which could distort protein configurations and ruin their catalytic activity. The affinity to sulfur could also explain the relatively high distribution in liver, where many enzymes and antioxidants contain sulfur groups.

What more, van der Zande *et al*. (2012) detected silver nanoparticles in the rats that had been given ionic silver, indicating nanosilver may be formed from ionic silver. This illustrates once more the complexity of nanoparticles, how they vary and change depending on an array of parameters. Needless to say, given all the factors that influence the outcome of nanoparticle research, and adding to that, the recently suggested possibility that the particles change form once in the animal, one cannot be completely certain of form or size of the particle under investigation.

Table 3. Geno-, cyto-, immuno-, neuro- and reproductive toxicity of Ag:

System	Assay	Conc./Dose	Result	Size nm	Ref.
In vitro, BRL 3A rat liver cells	MTT	2.5, 5.0, 10.0, 25.0, 50.0 μg/ml	Sign. reduction at 5-50, dose rel.	15, 100	Hussain (2005)
In vitro, BRL 3A rat liver cells	LDH	2.5, 5.0, 10.0, 25.0, 50.0 μg/ml	Pos, at 10-50, dose rel.	15, 100	Hussain (2005)
In vivo, Sprague-Dawley rat	28-d oral toxicity	90 mg/kg bw	Neg.	<20	Van der Zande (2012)
In vitro, rBMEC	BBB inflammation and permeability	6.25-50 μg/ml	Pos at 25 and 50 Cytotoxicity, decreased viability, morph changes	25, 40, 80	Trickler (2010)
In vivo, F344 rats	90 d, oral toxicity	30, 125, 500 mg/kg bw	Liver toxicity pos at 125 and 500 mg/kg	56	Kim (2010)
In vivo, Sprague-Dawley rat	BBB disruption	30 mg/kg bw intravenously	Pos, BBB disruption	50-60	Sharma (2010)
In vitro, C18-4 cell line from type A spermatogonia	Mitoch. function, LDH leakage,	5, 10 μg/ml	Pos LDH assay at 5 (at 10 precipitation)	15	Braydich-Stolle (2005)
In vivo, *Daphnia magna*	Acute toxicity of AgNP and Ag^+	0.001-0.32 mg/L	Pos, at 0.0015, 0.0017, 0.003 resp. both NP and ionic	5-25, colloidal and ionic	Asghari (2012)
In vitro, human lymphoblastoid TK6	MN	10, 15, 20, 25, 30 μg/ml	Pos at 25 and 30, dose rel.	5	Li (2012)

Abbreviations: rBMEC; rat brain microvessel endothelial cells, LDH; lactate dehydrogenase, EC_{50}; effective concentration, BBB; blood brain barrier

Discussion of Ag-toxicity:

Hussain and co-workers (2005) performed several assays on Ag NP, which clearly indicated cytotoxicity, both cases shown in Table 3 being dose related. Moreover, the toxicity at 25 and

50 µg/ml in the LDH assay was significantly higher for particles of 100 nm as compared to those of 15 nm. Results from the other assays of the same reference, examining ROS production and GSH status, clearly indicated that nanoparticles of Ag are potent inducers of oxidative stress.

The 28-day oral study carried out by van der Zande (2012) did not, however, show any significant immunotoxicity or hepatotoxicity of nanoparticle silver.

The subchronic oral toxicity study of silver nanoparticles by Kim *et al*. (2010) found, like others, liver to be the target organ. Elevated levels of cholesterol and alkaline phosphatase were significant, and histopathology showed bile-duct hyperplasia and increased foci. They also noted that the accumulation of silver was twice as high in the kidney of female rats as compared to that of male rats, at all three dose groups.

To assess the effect silver nanoparticles might have on the permeability of BBB, Trickler *et al*. (2010) used rat brain microvessel endothelial cells (rBMEC) to measure the transport of fluorescein over the monolayer after exposure to Ag NP. They noted a time-dependent increase in permeability, which was associated with an increase of the pro-inflammatory mediators IL-1B, TNF and PGE_2. This effect was clear for the smaller particles (25 and 40 nm) but not for 80 nm-particles. Further, they detected morphological changes and reduced viability of the cells, with subsequent perforations in the monolayer. Their study implies an impact of Ag NP on the morphology and viability of rBMEC, which could have consequences on the permeability of BBB.

In an interesting *in vivo* study on rats by Sharma *et al*. (2010) BBB-disruption after intravenous administration of 50-60 nm Ag NP was seen. The same outcome was noted for Cu NP but not Al NP exposure of same sizes, ruling out the mere prescence of nanoparticles as the culprit. The BBB- disruption was detected using Evans blue and radioiodine tracer, both of which were clearly spread throughout the brain. An intraperitoneal administration of the same particles (at 50 mg/kg bw) did not give the same result, pointing to the importance of the route of administration and resultant high concentration of those NP for their effect on BBB.

In the evaluation of cytotoxicity of silver nanoparticles on cells derived from mouse spermatogonia, effects in terms of LDH leakage, mitochondrial function and necrosis/apoptosis were investigated (Braydich-Stolle *et al*., 2005). The authors used CdO, a verified cytotoxicant, as positive control. Surprisingly, the EC_{50} value they got in the LDH assay was the very same as that of CdO, 2.5 µg/ml. From phase-contrast microscopy they could see the impact silver had on cell morphology with the appearance of both necrotic and apoptotic cells. This study revealed the potency of silver nanoparticles as a cytotoxicant on germline stem cells and ought not be forgotten in a risk assessment of silver.

Asghari and co-workers (2012) wanted to address whether silver in the form of nanoparticles or in ionic form was most toxic to the crustacean *Daphnia magna*. They investigated the acute toxicity of two forms of ready-to-use colloids of nanoparticles, one suspension of nanoparticulate silver and ionic silver in the form of $AgNO_3$. Along with aggregation and

sedimentation of the silver suspension, it turned out least toxic. The two colloidal forms, however, remained well dispersed and did not sediment. The two colloidals and the ionic silver were equally toxic, and according to the GHS (globally harmonised system) for classification of toxic substances, they should be classified as "category acute 1" for *D. magna*. The authors also discuss the potential of trophic transfer (up the food chain) of silver as it may be ingested by crustaceans and other lower organisms, which are subsequently eaten by fish and higher animals.

Last, Li *et al*. (2012) in their *in vitro* MN assay on human lymphoblastoid TK6 cells got significance for genotoxicity. They did not, however, get a positive result in Ames test. There may be several reasons for that, as discussed by the authors. Many nanoparticles cannot pass the bacterial cell wall, which gives negative results in Ames test. Or, if it can penetrate the cell wall, being an antimicrobial it may thus be toxic to *S. typhimurium,* in which case the dose necessary to induce genotoxicity might never be reached before the bacteria die.

Nanoparticles of different sorts have been shown many times to induce ROS formation, oxidative stress and pro-inflammatory cytokines. These may together, as shown by Trickler *et al*. (2010), disrupt the morphology of the endothelial cells that make up the BBB, thereby robbing them of their protective ability. The toxicity of silver nanoparticles does not seem to be acute or severe, but their apparent ability to reach the brain, and other organs, accumulate there and induce a stress and an inflammatory response- not to mention providing access to the brain of other chemicals-is alarming. Therefore, one might want to think twice before taking silver regularly as a supplement to promote health, since one cannot rule out the possibility that the outcome will be the opposite.

Techniques and methods for characterization and determination of nanoparticles

Characterization of nanoparticles in food is tedious and uncertain work. There are many steps in which the particles may change structure, charge, surface functionalities or aggregation state, as compared to those in its natural environment. Avoiding those artifacts is not straightforward. Aiming to combine methods for analysis that are reproducible and non-invasive, with scant sample preparation, provides best conditions for a reliable characterization.

The immediate presentation below of common analytical techniques will simply in a sentence mention the purpose of each method. For a more thorough description of each technique, the curious reader is encouraged to read Appendix I. The methods for analysis has been divided into three main parts; 1) separation techniques, 2) size/structure/ chemical composition and 3) surface area and charge. Note that some techniques may be used for different purposes.

1) Separation techniques

Chromatographic methods may be used both for separation of solutes, according to for example size, charge, polarity or affinity, and as an analytical tool for quantification of different phases of a sample.

Chromatographic techniques:

- Size exclusion chromatography (SEC)
- Hydrodynamic chromatography (HDC)
- Field flow fractionation (FFF)

Size exclusion chromatography (SEC) separates molecules, as the name implies, according to (hydrodynamic) size.

Hydrodynamic chromatography (HDC) separates molecules according to their hydrodynamic size, much as SEC. The hydrodynamic radius refers to the radius of a molecule in solution including the solvent surrounding it, taking into account the dynamics of the molecule.

Field flow fractionation (FFF) is similar to chromatographic methods, by some called one-phase chromatography (Calzolai *et al*., 2012). The separation depends solely on diffusion rate, which in turn depends on size (nanolytics.de).

Analytical ultracentrifugation (AU)

Analytical ultracentrifugation (AU) may be used for fractionation and size determination, as well as detection of interactions between molecules (Luykx *et al*., 2008).

2) Size/structure/chemical composition measurements

Electron microscopy (EM) has been the most widely used method for characterization of nanomaterial (Calzolai *et al*., 2012).

Electron microscopy (EM):

- Transmission electron microscopy (TEM)
- Scanning electron microscopy (SEM)
- Environmental scanning electron microscopy (ESEM)

In transmission electron microscopy (TEM) an electron gun shoots electrons through a very thin section of the specimen (Tiede *et al*., 2008, Luykx *et al*., 2008) and the electrons then form an image that is visualized.

Scanning electron microscopy (SEM) differs from TEM in that it instead of shooting electrons through a specimen shoots electrons at its surface. This gives an image of the surface with a depth, similar to a 3D image (Luykx *et al*., 2008).

Environmental scanning electron microscopy (ESEM) allows the sample to stay wet, thereby reducing the amount of preparation that may perturb the sample (von der Kammer *et al*., 2011), thus allowing the sample to remain in a more natural environment (Tiede *et al*., 2008) and maintain its structure.

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) is by far the most used technique for size measurements of monodispersed samples (Calzolai *et al*., 2012, Luykx *et al*., 2008).

Atomic force microscopy (AFM)

Atomic force microscopy with its resolution down to 0.1 nm is an appreciated tool for a 3D-visualization of structure and aggregation state of nanoparticles (Tiede *et a*l., 2009).

Spectroscopic techniques:

Spectroscopic techniques all have one thing in common; they measure any interaction between electromagnetic radiation and matter. The interaction may be absorption, emission or diffraction, and the frequency of radiation may vary from nanometer length (X-ray diffraction) to radio waves (NMR) (Penner M, 2010).

- X-ray diffraction (XRD)
- UV-Vis spectroscopy
- Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS)
- Nuclear magnetic resonance (NMR)

X-ray diffraction gives information on the crystal structure and composition of solids (Tiede *et al*., 2008, Luykx *et al*., 2008).

UV-Vis spectroscopy is grouped into absorption and fluorescence spectroscopy, which can be divided into quantitative and qualitative (Penner M, 2010).

Mass spectrometry is an applicable tool in the characterization of a known, or unknown, compound regarding its elemental composition (including isotopes), mass concentration (Tiede *et al*., 2008, Smith J., 2010) and sometimes particle size (Calzolai *et al*., 2012).

In ***single particle inductively coupled plasma mass spectrometry (SP-ICP-MS)*** the peak obtained in the analysis reflects the size and concentration of single atoms, as opposed to only concentration of fragments (Miller D, 2010, Calzolai *et al*., 2012) gained from other mass analyzers.

Nuclear magnetic resonance (NMR) provides information on composition and 3D-structure as well as the dynamics of molecules (Luykx *et al*., 2008).

3) Surface area and charge (zeta-potential)

Gas adsorption (BET)

In the ***Brunauer-Emmet-Teller (BET)*** method, gas particles adsorb to the surface of the particle to be measured, which gives the whole area, internal and external, of solid samples (Tiede *et al*., 2009, Mihranyan *et al*., 2012).

DLS uses laser to measure the external surface in liquid samples. The area measured by DLS is therefore smaller than that obtained by BET (Tiede *et al*., 2009).

Capillary electrophoresis/electrophoretic mobility/zeta potential

The electrophoretic mobility of molecules depends on parameters such as size, charge/mass ratio, hydrophobicity, isoelectric point and zeta potential.

In ***capillary electrophoresis*** a sample migrates through a capillary between a source-vial to a destination-vial where the rate depends on its charge (Luykx *et al*., 2008).

Zeta potentiometer is an instrument that converts particle velocity to zeta potential (Brookhaven instruments). It may be used instead of electrophoresis to measure the zeta potential.

Conclusions

Along with the evolution of nanotechnology and nanomaterial, a revolution of our society may follow. But there are many sides to the story of nano. Below follows a short compilation of pros and cons of this revolution in the food and supplements sector.

TiO_2 in nanosize is added to sunscreens because of its ability to capture UV-radiation, thereby diminishing the risk of skin cancer. This is unequivocally positive. Putting TiO_2 in toothpaste, candies, food, supplements and medicines, to make them brightly white, does not seem unquestionably positive, given the potential health risks that have been put forward.

Dendrimers and nanoliposomes added to foods for increased uptake of nutrients or controlled release of additives, or, smart food packaging with contaminant alarm, are some examples of what is being explored by the food industry. But, not enough is known of their health effects, and so far they have not been approved by EU. Hopefully, within a near future, more is known of the effects, so their potentials may be fully, and safely, used.

The possible relation between particle intake and inflammatory bowels diseases has been discussed and, if true, that seems like a high price to pay for white food. One might question why, and how, TiO_2 has been allowed in food in the first place. Among the references on

which JECFA 1969 based their decision allowing TiO_2 in food, not one assay is to be found with a specified particle size that is in the same size range as E171. They were either bulk material, 10-35 micrometers or simply unspecified. The same applies to the NCI carcinogenesis bioassay. This seems a bit peculiar, as it was never a question of adding TiO_2 as bulkmaterial to foods, since the wanted whiteness is gained at roughly 200 nm. Also quite enigmatic, JECFA wrote in their comment to the lack of toxicity that "*Titanium dioxide is a* ***very insoluble compound"*** and "*studies on* ***soluble titanium compounds have therefore*** *not been reviewed*". It would be interesting to know what soluble forms they were referring to. Could modified E171 be one?

But, it must be emphasized again that the results of the toxicity of TiO_2, or silver, are not unambiguous, not at all, and the main part of the studies is *in vitro* assays. They are dependent on many factors, and comparisons in between are difficult. What seems to be a pattern though, is oxidative stress and inflammation. Also suggested by many, complications of nanoparticles in general in food could be due to a Trojan-horse effect. The nanoparticles may both bring unwanted substances with them, but they may also induce morphological changes of epithelial cells, thereby diminishing their barrier function.

Even if the toxicity and inflammation induced by nanoparticles are of a low and chronic kind, rather than acute, a constant intake of particles in food and medicines must be deemed unnecessary, since inflammation is a well-known contributor to cancer and many other illnesses.

From what has been learned during the writing of this report, of particular interest for the National Food Agency, are the potentially hidden effects of E171. The occurrence, size, and toxicity of modified E171 need to be fully investigated.

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Appendix I

Techniques and methods for characterisation and determination of nanoparticles

In the compressed version of "Techniques and methods for characterisation and determination of nanoparticles in food" the purpose of each technique was briefly given. What follows below is a more thorough description, for a somewhat deeper understanding of the basis of the techniques and their usage.

As the identity and reactivity of a nanomaterial are dependent on a plethora of properties (both intrinsic and those of its surroundings, such as concentration, chemical composition, functional groups on the surface, particle size, surface area, redox state, crystal structure, solubility, aggregation/agglomeration state as well as ion concentration, pH and temperature of the media (Tiede *et al*., 2008, von der Kammer *et al*., 2011), a thorough characterization thereof calls for an array of methods. Some of the methods that may be used in analyses of nanomaterial will be discussed in this chapter. Together they confront aspects of; how to separate the nanomaterial, confirmation of fractions, size distribution, chemical composition, surface area, crystal structure, surface characteristics, zeta potential, aggregation state and dynamics.

It should be kept in mind that the extreme reactivity of nanomaterial, and the, often invasive, sample preparation, taken together with a lack of standards (von der Kammer *et al*., 2011), make a reliable characterization difficult. For example, information gained from one assay may not be applicable to the very same nanomaterial if, say, the ion concentration or protein content of the media would be different in the other sample (Blasco *et al*., 2010), nor may any results be compared if different preparation techniques have been used, as they might have influenced the nanomaterial differently (Calzolai *et al*. 2012).

1) Separation techniques

Chromatographic methods may be used both for separation of solutes, according to for example size, charge, polarity or affinity and as an analytical tool for quantification of different phases of a sample. The foundation of chromatography lies in the inherent physico-chemical properties of a solute, which determines its partition between two phases: stationary and mobile (Ismail B, 2010). These may be either gas, liquid or solid and many combinations thereof may be used depending on the sample to be analyzed. The separated fractions that pass through, elutes, may be analysed with spectroscopy (Luykx *et al*., 2008) both for confirmation of a proper separation, as indicated by narrow peaks, as well as for

quantification. Chromatographic techniques are commonly combined with other methods, such as mass spectrometry, to gain more information about composition and concentration.

In general, chromatographic techniques are quick and easy to perform, do not require any expensive equipment or expert personnel. They are also non-destructive and quite sensitive. A drawback would be that the sample often needs to be dissolved in a media different from that of its natural surrounding which, as previously mentioned, might affect its structure (Tiede *et al.*, 2008).

Chromatographic techniques:

- Size exclusion chromatography (SEC)
- Hydrodynamic chromatography (HDC)
- Field flow fractionation (FFF)

Size exclusion chromatography (SEC) separates molecules, as the name implies, according to (hydrodynamic) size. The stationary phase in the column contains porous beads where the pores match the size of the smaller molecules in the mobile phase, which are trapped. The larger molecules are eluted first as they are too big to fit in the pores and hence pass through with the mobile phase (Ismail B, 2010).

Some (Luykx *et al*., 2008) consider SEC an appropriate method for separation of nanomaterial as size is one of the critical features. However, a so-called "ideal SEC" (Ismail B, 2010) requires the sample do not interact with the stationary phase but rather is separated solely on size. As the hallmark of nanomaterial is their enhanced reactivity, as compared to the bulk material, it is advised by Tiede *et al*. (2008) to use the method cautiously as there is a great risk of interactions between the nanomaterial and the stationary phase, in which case the separation would be "nonideal" (Ismail B, 2010). Von der Kammer *et al*. (2011) takes it even further and states that SEC is not applicable to separating nanoparticles due to interactions between sample and stationary phase. As nanomaterial tend to aggregate into larger structures, SEC would not handle the potentially vast difference in sizes in one sample (Tiede *et al*., 2008).

Defying that statement of von der Kammer, SEC has been used for separation of molecules as varying as quantum dots, polystyrene, single walled nanotubes (Tiede *et al*., 2008), proteins (Ismail B, *S.S Nielsen, Food Analysis*, ch.27) and nano delivery systems (NDS) made of lipids or large polysaccharides. If the latter is followed up by MALDI-TOF- (matrix-assisted laser desorption/ionization-time-of-flight) or ICP (inductively coupled plasma)–MS, information may be acquired on composition and the repeating units of the saccharide (Tiede *et al*., 2008, Luykx *et al*., 2008) and coupling the eluate to dynamic light scattering (DLS) offers size measurements.

Hydrodynamic chromatography (HDC) separates molecules according to their hydrodynamic size, much as SEC. The hydrodynamic radius refers to the radius of a molecule in solution, including the solvent surrounding it, taking into account the dynamics of the molecule. This gives that an extended molecule tumbling around in solution has a larger hydrodynamic radius, as compared to a denser one of the same mass. While the stationary phase in SEC consists of porous beads allowing entrapment of smaller molecules, in HDC it is made of non-porous beads that instead form channels (Tiede *et al*., 2008). A pressure pushes the sample through a channel where the smaller molecules by force flow closer to the wall of the channel and are slowed down by this. The larger molecules that stay in the center move faster and are hence eluted first (Tiede *et al*., 2008, Luykx *et al*., 2008). With the help of calculations and models, the peaks from the chromatogram may be compared with known peaks from other substances and the retention time, (how long it took for the substance to pass), may be converted to particle size (Luykx *et al*., 2008).

While SEC has a narrow size-acceptance, determined by the porous beads, HDC allows for analysis of molecules ranging from 5-1200 nm. As larger molecules are able to pass through, even larger aggregates of the nanomaterial may be analyzed. Due to the fact that the beads are not porous in HDC, any interaction between the solute and stationary phase is limited, which is not the case in SEC. On the down side is the low resolution, indicated by broad elute peaks, due to sub-optimal separation (Tiede *et al*., 2008) and also frequent clogging of the column (Luykx *et al*., 2008).
After separation with HDC, the elute may be further analyzed by UV-vis spectroscopy or DLS for size measurement (Tiede *et al*., 2008, Luykx *et al*., 2008).
The HDC has been used in the analysis of for example lipid nanocapsules (which can be used as carriers of molecules), various biomolecules and colloidal solutions of nanoparticles (Tiede *et al*., 2008).

Field flow fractionation (FFF) is similar to chromatographic methods, by some called one-phase chromatography (Calzolai *et al*., 2012). The method has many advantages over the separation techniques mentioned above.
Much like chromatographic methods it is based on a flow through a channel where particles of different sizes travel with different rate. The separation depends solely on diffusion rate, which in turn depends on size (nanolytics.de). A field, applied perpendicular to the flow, corresponds to the stationary phase of other chromatographic methods in that it decides how fast the fractions will pass the channel. The molecules are forced to one side of the channel but the smaller ones tend to move back to the center (Calzolai *et al*., 2012). There the flow is faster and the smaller molecules pass through first (Luykx *et al*., 2008). The reason the smaller molecules move back to the center is the so-called Brownian motion, which decides how molecules diffuse in solution. The Brownian motion counteracts the force that pushes them to the side and as smaller molecules move faster they find equilibrium closer to the center (Tiede *et al*., 2008, Luykx *et al*., 2008). Fig 12 below illustrates the separation of smaller and larger molecules according to their movements in the channel.
After separation, the fractions are often further analyzed by for example UV-Vis absorbance, ICP-MS or DLS (Tiede *et al*., 2008, Luykx *et al*., 2008).

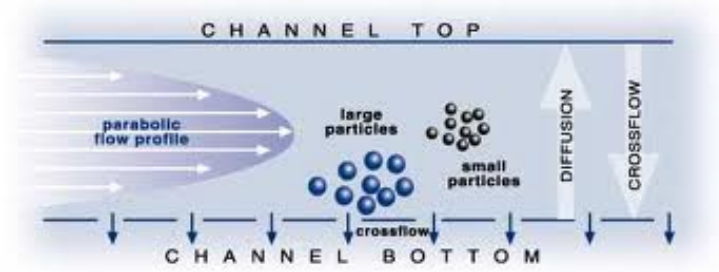

Fig 12: Field flow fractionation. The white arrow pointing down illustrates the applied field that pushes the particles to the side, and the white arrow pointing up illustrates the diffusion driving the smaller particles back to the center, where they pass through the channel faster than the larger ones that are pushed to the side. (Picture taken from www.nanolytics.de)

There are some critical benefits gained from using this method in analysis of nanomaterial. Keeping the nanomaterial in its natural environment, like biota or food, is very important to ensure the structure does not change. Thus, the possibility to use a carrier solution of varying ionic concentration or a pH in the range from 2-11, thereby allowing a mimic of nature, gives FFF an upper hand, as compared to other techniques. Add to that the superior resolution of molecules above 100 kDa (Luykx *et al*., 2008), the wide size range of analyte (Tiede *et al*., 2008) from a few nm to µm (Tiede *et al*., 2009) and the ability to separate molecules in complex media (Calzolai *et al*., 2012). These features make FFF the preferred choice by many researchers, but it is not all good. The solute may interact with the membrane, which would distort the result. This can, however, be overcome to a certain extent by choosing an appropriate ion concentration and pH. Also, FFF may not tell the difference between nanomaterial and aggregates of the same size, but that applies to other chromatographic techniques as well. A technical problem may be clogging of the channel and although it may be overcome by diluting the sample, that might in turn cause problems for the subsequent analysis, especially if the concentration to begin with was low.

Given the wide size range possible for separation with FFF and the ability to separate a complex mixture, an array of molecules have been separated from smaller ones such as TiO_2, carbon black and SiO_2 (Tiede *et al*., 2008) to larger like proteins, liposomes, emulsions and polysaccharides (Luykx *et al*., 2008).

Analytical ultracentrifugation (AU)

Analytical ultracentrifugation (AU) is a useful technique not only for fractionation and size determination, its most common applications; it may also reveal interactions between molecules (Luykx *et al*., 2008).

In the ultracentrifuge, which may accelerate to 1 000 000 *g,* three different forces govern the movement of the particles. The centrifugal force, which is proportional to the mass of the particle, brings the sample down to sediment while the boyant force (pushing upwards) together with frictional force (time dependent diffusion effects) counteracts the centrifugal

force. These forces reach an equilibrium during the centrifugation within a fraction of a second and the resulting force, which is proportional to the size, determines how fast the molecule will sediment (Calzolai *et al*., 2012, Luykx *et al*., 2008). Due to the relation between mass and force, smaller molecules will sediment slower while larger go first. This is exploited in fractionation. During centrifugation UV-absorbance can be measured simultaneously which provides information on the concentration of the size fractions. By using time data on sedimentation, the sizes of the molecules can be calculated.

Analytical ultracentrifugation is a powerful tool in nanomaterial research. For one, the wide size range possible for analysis, accepting small molecules with a size of a couple of nanometers, or 100 Da, up to molecules with a mass of 10 GDa (Tiede *et al*., 2008) is quite unique. Another benefit would be the possibility to analyze samples in aqueous or other solution. The analysis is rather simple, not expensive, not destructive and the samples may be re-used (Tiede *et al*., 2008, Luykx *et al*., 2008).

One drawback, as discussed by Calzolai and coworkers (2012), is the difficulty to calculate the particle size if the density is unknown, based on the relation between density, mass and volume. This is even more challenging if the sample form aggregates or agglomerates. Even if the density of the primary particle is known, the density of an aggregate is not easily obtained.

2) Size/structure/chemical composition measurements

Electron microscopy (EM) has been the most widely used method for characterization of nanomaterial (Calzolai *et al*., 2012) as it has the ability to visualize the molecules in the sample (von der Kammer *et al*., 2011). Specifically, TEM (transmission electron microscopy) has most successfully characterized nanomaterial (Blasco *et al*., 2010). The ability of EM to visualize the nanomaterial regardless of aggregation state, size or shape makes it superior to other techniques (Tiede *et al*., 2008), such as SEC or DLS, which rely on a more uniform sample.

Electron microscopy (EM):

- Transmission electron microscopy (TEM)
- Scanning electron microscopy (SEM)
- Environmental scanning electron microscopy (ESEM)

In ***transmission electron microscopy (TEM)*** an electron gun shoots electrons through a very thin section of the specimen (Tiede *et al*., 2008, Luykx *et al*., 2008) and the electrons then form an image that is either visualized on a photographic film or formed by a sensor. Biological samples are not allowed a thickness above 1µm (Luykx *et al*., 2008). The widespread use of TEM is based of its high resolution, down to 0.2 nm, and the fact that it

visualizes any size, aggregation state or shape in its environment (Calzolai *et al*., 2012). However, in spite of its popularity there are many drawbacks to the method. As the resolution is so high with a magnification of 1000000 it means only a small portion of the sample can be analyzed and from that it follows that the image might not be representative of the whole sample (Luykx *et al*., 2008). Also, the procedure is very tedious, requiring expert personnel and costly (Blasco *et al*., 2010, Calzolai *et al*., 2012, Luykx *et al*., 2008). More importantly, it is probable that both preparations and analysis alter the sample. The analysis is done in vacuum, which requires drying of sample, quite possibly altering it. Also, the electrons that are shot through the sample may affect its structure or charge. To overcome these problems, the sample is often fixed in for example plastics, however, this may also change the structure or its interaction with other material (von der Kammer *et al*., 2011). To avoid the alterations induced by fixation one can use cryogenic SEM/TEM, which is rapid and leaves the sample in its natural state.

Having said that, if the sample survives the extensive handling and preparation, the true size is obtained, as compared to the hydrodynamic size measured by other techniques (Calzolai *et al*., 2012).

Because analyses using TEM is such tedious and complex work it will never make it as a standard procedure in every lab. However, it is suggested being used to develop standards that may aid optimizing other techniques (Calzolai *et al*., 2012).
Food related samples analyzed with TEM spans from nanotubes made of milk proteins, nanoparticles of albumin, liposomal NDS (Luykx *et al*, 2008) to TiO_2, CeO_2 (cerium dioxide) and ZnO in rainbow trout (Blasco *et al*., 2010).

Scanning electron microscopy (SEM) differs from TEM in that it instead of shooting electrons through a specimen, shoots electrons at its surface. This gives an image of the surface with a depth, similar to a 3D image (Luykx *et al*., 2008). When electrons are shot at the surface, so-called secondary electrons from the sample are scattered and these produce flashes of light (when detected by another material) and these flashes are subsequently detected and an image is formed. That electrons are scattered has implications on the detection of atoms as lighter ones do not scatter electrons to the same extent as heavier, more electron dense (Tiede *et al*., 2008).
Although the resolution of TEM is ten times higher, that of SEM still suffice for a good characterization and adding to that the 3D –like structure SEM is a helpful means to examine surface characteristics and structures.
Many of the disadvantages of SEM are shared with TEM but because the surface is under investigation in SEM any surfactants that cover the nanomaterial may distort or destroy the image (Luykx *et al*., 2008). Most techniques dealing with characterization of nanomaterial are sensitive to what material is under investigation. For example, characterizing carbon nanotubes in an environment dense with carbon is problematic, as EM cannot tell the carbon nanomaterial from the background carbon (von der Kammer *et al*., 2011). In general, the applications and limitations of SEM and TEM are similar. Both of them are destructive methods so the sample cannot be further analyzed (Tiede *et al*., 2008). They have been used

for similar samples, such as polysaccharides, proteins and liposomes (Luykx *et al*., 2008) but can also be used for smaller nanoparticles of for example gold or titanium (von der Kammer *et al*., 2011) and a cryogenic alternative exists for both of them. Cryo-SEM for example has been used to look at aggregated nanoparticles and their interaction with cells (von der Kammer *et al*., 2011).

Environmental scanning electron microscopy (ESEM) evades some of the limitations of SEM. In ESEM, the sample is allowed to stay wet, thereby reducing the amount of preparation that may perturb the sample (von der Kammer *et al*., 2011) and also allows the sample to remain in a more natural environment (Tiede *et al*., 2008). Also, it does not require any coating as for SEM. The price is lower resolution, around 30 nm (von der Kammer *et al*., 2011). Just as for SEM and TEM, it is best fitted for electron dense elements (Tiede *et al*., 2009).

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) is by far the most used technique for size measurements of monodispersed samples (Calzolai *et al*., 2012, Luykx *et al*., 2008). Using a laser beam, a small region in a solution is illuminated for only a fraction of a second. The light is scattered by the diffusing particles in the solution and the pattern of the scattered light depends on the rate of the diffusion. This rate of diffusing particles is in turn related to their size, as there is a relationship between Brownian motion (mentioned in the section of FFF) and spherical particle size. Hence, by analyzing the scattered light, the particle size is given (Luykx *et al*., 2008).
It is preferred by many because it is easy, quick, handles varying concentrations, cheap, non-invasive, non-destructive and hence reproducible. However, it is preferred, not perfect. It cannot handle a solution of varying sizes (less than a factor of 3-4 in difference) as the larger particles would cloud the measurements of the smaller. An illustrative example of this is an assay by Calzolai where gold nanoparticles in the sizes of 5, 15 and 45 nm, in a ratio of 350:15:1 were tested and surprisingly (or not) the result showed that the sample mainly contained particles of 45 nm. This implies that the method cannot be used for measuring complex matrices of varying sizes but suitable for monodispersed solutions (Calzolai *et al*., 2012).

DLS has been extensively used by many and as the size range varies from 5 nm to several µm (Tiede *et al*., 209) it has wide applications. It has been used for size determination of NDS of polysaccharides, liposomes and gelatine. One specific example relating to food could be the measurement of nanosized liposomes used for encapsulation of anti-microbial peptides added to food (Luykx *et al*., 2008). It is suitable for aggregation analysis of samples (Tiede *et al*., 2008) and is frequently used by researchers in size characterization of nanoparticles like TiO_2, SiO_2.

Atomic force microscopy (AFM)

Atomic force microscopy with its resolution down to 0.1 nm is an appreciated tool for visualization of structure and aggregation state of nanoparticles (Tiede *et a*l., 2009).

The technique is based on a cantilever (like a bar) that scans the surface of molecules. As the cantilever moves over the surface of a sample, electrostatic forces in the range of 10^{-12} N are produced between the tip of the cantilever (which is in nanometer size) and the surface (Tiede *et al*., 2008). These forces make the cantilever move and with the help of a laser, that reflects the movements of the cantilever, a 3D- image is produced (Luykx *et al*., 2008).

Its advantages derive partly from the ability to be used on both wet and dry samples, and partly on its unprecedented high resolution. It may also reveal structural information in the z-dimension (depth) giving a 3D-image, although to 0.5 nm resolution (Tiede *et al*., 2008). As the size of atoms is in the range of 1 Å, AFM is able to visualize individual atoms and small molecules (Luykx *et al*., 2008). The fact that AFM can be used on wet samples makes it possible to image samples in their native environment (Luykx *et al*., 2008). There is a risk though that molecules floating around may stick to the tip of the cantilever and distort the image. This can be prevented by fixation of the sample, but that, in turn, may destroy its natural shape. Moist samples however are very well visualized (Tiede *et al*., 2008).

AFM has for example been used to look at natural colloids and how their structure changed with changing pH (Tiede *et al*., 2008). In the context of food research, AFM may be used to visualize liposomes, polysaccharides, proteins and nano delivery system thereof (Luykx *et al*., 2008).

Spectroscopic techniques:

Spectroscopic techniques all have one thing in common; they measure any interaction between electromagnetic radiation and matter. The interaction may be absorption, emission or diffraction, and the frequency of radiation may vary from nanometer length (X-ray diffraction) to radio waves (NMR) (Penner M, *S.S. Nielsen, Food Analysis*, ch. 21).

- X-ray diffraction (XRD)
- UV-Vis spectroscopy
- Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS)
- Nuclear magnetic resonance (NMR)

X-ray diffraction gives information on the crystal structure and composition of solids (Tiede *et al*., 2008, Luykx *et al*., 2008). In crystalline structures the atoms are placed with a few angstroms distance, which is the same length as the wave of an X-ray, therefore a diffraction pattern can be seen (Luykx *et al*., 2008). The fundament of a successful XRD analysis, and a prerequisite for high resolution, is crystals of good quality without impurities.

In crystals the atoms are placed in discrete layers and the X-rays that are beamed thereupon are reflected. Throughout the process the beams are directed at the crystal from all possible angles giving a pattern of the crystal. It is only when the reflected rays are in phase, so called constructive interference, which shows the sum of the phases, they are detected as a peak, as opposed to destructive interference during which the phases take each other out. Each molecule has a characteristic pattern, and the molecule is identified using databases with patterns of known molecules (Luykx *et al*., 2008).

XRD is an informative way of studying structures of solid materials in crystal form that is also non-destructive. One example, relevant for of this work, is the crystal structure determination of TiO_2 to verify if it is anatase or rutile. Another example is the identification and structural analysis of zero-valent iron nano particles whose ability to be used for remediation of polluted water is under evaluation (KemI Rapport, 2007, Tiede *et al*., 2008).

UV-Vis spectroscopy is ubiquitously used in laboratories as it is both simple and informative. It is grouped into absorption and fluorescence spectroscopy, which can be further divided into quantitative and qualitative - with quantitative absorption spectroscopy being the most used (Penner M, 2010).
The UV spectrum spans from approximately 200 nm to 350 nm where the visible light begins, which spans to around 700 nm, where infrared picks up.
In quantitative absorption spectroscopy, the concentration of a sample is easily attained by putting the sample in a cuvette through which a beam of a certain wavelength is aimed. The wavelength chosen should be that at which the sample has highest absorption. By comparing the incident (incoming) beam and the exiting beam, the absorbance is measured which, put in Beer's law, gives the concentration of the sample (Penner M, 2010). As mentioned above, coupling UV-Vis spectroscopy to, for example chromatography, measures the concentration of the separated fractions.
In fluorescence spectroscopy, by contrast, the emission of radiation of a sample is measured. When molecules relax from a higher energy state to ground state they emit light, fluoresce, as photons (light), which are entities of energy, are released (Penner M, 2010).
Since the sample must first absorb radiation to reach a higher energy level from which it may fall, fluorescence spectroscopy requires both an excitation beam and an emission beam, the latter being longer than the former.
The examples mentioned apply to quantitative spectroscopy in which the concentration of a sample is sought. However, a qualitative estimation of a sample can also be done. For qualitative measurements the aim is not to find the most appropriate wavelength for maximum absorbance, but rather, the whole spectrum of absorbance or emission of a sample is analyzed. Each sample has its own characteristic spectrum, which is used to determine its purity.

Mass spectrometry is an applicable tool in the characterization of a known, or unknown, compound regarding its elemental composition (including isotopes), mass concentration (Tiede *et al*., 2008, Smith J, 2010) and sometimes particle size (Calzolai *et al*., 2012).

A mass spectrometric analysis can be divided into three separate processes; the sample is first ionized, after which it is separated according to its mass-to-charge ratio (m/z) and the separated particles are then analyzed (Smith J., 2010).
To this end, a mass spectrometer has an ion source, two common being electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Particularly useful for metals is an inductively coupled plasma torch (ICP) (Tiede *et al*., 2008) although any element may be ionized (Miller D, 2010). For the separation there are a number of mass analyzers to choose from, suited for different mass-to-charge ratios and with different resolution (Tiede *et al*., 2008). Examples of common mass analyzers are quadrupoles (Q), ion traps (IT), time-of-flight (TOF), Fourier–transform-based ion cyclotrons (FT-ICR) and a number of them may be combined for an expanded analysis (Smith J, 2010).

In ***single particle inductively coupled plasma mass spectrometry (SP-ICP-MS)*** the particles are atomized and ionized in an argon plasma torch and the peak obtained in the analysis reflects the size and concentration of single atoms, as opposed to only concentration of fragments (Miller D, 2010, Calzolai *et al*., 2012) gained from other mass analyzers. The size is not given by the instrument but may be calculated if enough data of the particle is known. Since the number of atoms of each element is shown by the peak, knowing the stoichiometry of the particle as well as the density, together with the ion-transfer efficiency, particle size may be calculated (von der Kammer *et al*., 2011).
Inside the torch is a flow of argon gas. This is exposed to radio frequencies, which induce a magnetic field that oscillates. With an electric spark the argon gas is ionized into argon ions and electrons. The energy applied in the form of radio frequencies is picked up by the electrons, which collide with argon atoms in the oscillating field, giving more ions and electrons, until 1% of the argon atoms are ionized. This process results in an extreme heat reaching 10000 K. When a sample is introduced into the argon torch the molecules are separated into atoms and ionized. Most atoms lose one electron giving an ion with a charge of +1. The ionized atoms are then transferred to a mass analyzer where they are separated according to their *m/z* ratio. The ions are then focused on a detector that counts the number of ions (Miller D, 2010).
By combining for example FFF or HPLC with ICP-MS a complex sample may first be separated into size fractions and thereafter analyzed regarding composition and concentration (Tiede *et al*., 2008). Possible applications are many such as measuring silver nanoparticles in waste water (Calzolai *et al*., 2012), or verification of nanoparticle silver, as opposed to ionic, in rats exposed to silver nanoparticles (68) as well as prevalence of TiO_2, CeO_2 and ZnO in rainbow trout (Blasco *et al*., 2010).

Nuclear magnetic resonance (NMR) is useful in analyses of compounds as it provides information on composition and 3D structure as well as the dynamics of molecules, not to mention the fact that both solid and liquid samples are candidates for analysis with NMR, which is also non-destructive (Luykx *et al*., 2008). Taking all these advantages into account it is obvious that NMR is a potent tool in nanomaterial research. The down sides would be the fact that only elements with the right spin is up for analysis, and there cannot be any

paramagnetic elements (like Fe (III)) in the sample as they would ruin the analysis (von der Kammer *et al*., 2011). The complex evaluation of the results, which requires skilled expertise, is also a limiting factor.

The basic feature of spectroscopy, the interaction of atoms with radiation of some sort, is applicable to NMR, albeit with some differences. The two most striking would be that in NMR it is the nucleus that absorbs the radiation (Reuhs B, 2010, Luykx *et al*., 2008) and it only does so when a magnetic field is applied (Penner M, 2010). The radiation absorbed is in radio frequency (Luykx *et al*., 2008).

In NMR analysis the proton (H) and ^{13}C are most studied (Reuhs B, 2010) but also ^{19}F, ^{15}N, ^{17}O and ^{31}P are common (Luykx *et al*., 2008). All nuclei used have a characteristic spin and charge which is exploited in NMR. When a charged nucleus is spinning there will be a magnetic field. When an external magnetic field is applied, the nuclei will align either in parallel or antiparallel to the field- the parallel being most popular (Reuhs B, 2010). How they align depends on the applied magnetic field (Penner M, 2010). This results in a net magnetic field that adds to the external field. After the sample has been exposed to energy of radio frequency it will emit this energy, which is detected as a signal, a signal of nuclear magnetic resonance (NMR), which is also in radio frequency. The magnetic field generated by the nuclei depends on its surrounding atoms, therefore information about molecules can be obtained by analyzing certain nuclei (Penner M, 2010).

Possibilities are many with NMR but it is particularly suitable for protein analysis and nano delivery systems based thereupon. Also nano-formulations of lipids may be analyzed. However, NMR analysis is restricted by size, so it cannot take on the analysis of large structures (Luykx *et al*., 2008).

3) Surface area and charge (zeta-potential)

Gas adsorption (BET)

Surface area is, as discussed in the introduction, a cardinal feature of nanoparticles. With decreasing diameter, the surface area in relation to mass increases, which has a stupendous impact on the behavior of the particle. Knowing the surface area of a nanoparticle is equally, or sometimes more, important than knowing its concentration in a solution.

Surface area can be measured with gas adsorption, such as the frequently used the Brunauer-Emmet-Teller (BET) method, or DLS. Gas adsorption measures the whole area, internal and external, since the gas particles adsorb to the surface of the particle (Mihranyan *et al*., 2012). Knowing how many gas particles that bind to the surface gives information about the complete surface. This area measurement is not the same as that obtained with DLS, which uses laser to measure the external surface, not detecting any holes or folds, which of course is smaller. When to use each technique is given by the context, not to mention the fact that DLS is for liquid samples and BET for solid samples (Tiede *et al*., 2009). If, for example, the surface area of an agglomerate is sought, gas adsorption like BET should be used, as that

surface is equal to the sum of the surfaces of all the primary particles. Would instead the ability of a particle to bind to something (like a protein) or pass a barrier of some sort be of interest, the internal area should not be in focus, but rather the outer surface, as measured by DLS (Mihranyan *et al*., 2012).

Capillary electrophoresis/electrophoretic mobility/zeta potential

The electrophoretic mobility of molecules depend on parameters such as size, charge/mass ratio, hydrophobicity, isoelectric point and, important for nanoparticles, the zeta potential. The zeta potential is also one of the cardinal features of nanoparticles, affecting its reactivity and bioavailability, among others, and knowing the zeta potential of a nanoparticle may give insights into its tendency to aggregate. It is therefore relevant in an evaluation of nanoparticle fate and its stability in solution (Tiede *et al*., 2008). If the particles are highly charged they will repel each other and not willingly aggregate, whereas particles with a charge close to neutral might do so (Tiede *et al*., 2008). Since we lingered a bit on the concept of zeta potential in the Background-section, it will not be discussed again, but the confused reader is welcome to revisit page 9-10.

In ***capillary electrophoresis*** a sample migrates through a capillary between a source-vial to a destination-vial, aided by an applied electric field. All ions, regardless of charge, migrate. However, they do so with different rate depending on charge. At the end of the capillary, a detector is connected (e.g measuring UV-vis absorbance) which will show peaks of the separated molecules as they pass. It should be kept in mind that they migrate according to both charge and hydrodynamic size (Luykx *et al*., 2008).

The method is quick, not expensive, simple and separates with a good resolution. On the negative side, samples may not be very dilute, thereby excluding trace compounds for analysis (Luykx *et al*., 2008). However, with an MS as detector even low-concentration samples may be analyzed.
Considering nanoparticles may be present at very low concentrations in a sample this might limit its use. Capillary electrophoresis may be used on samples varying in size from a few nanometers to microns (Tiede *et al*., 2008, von der Kammer *et al*., 2011). Documented applications vary from characterization of small silica spheres (Tiede *et al*., 2008) to larger nanomaterial based on liposomes, polysaccharides and chitosan (Luykx *et al*., 2008).

In ***electrophoretic mobility*** assays that aim to elucidate the zeta potential of particles, this is easily confounded with the surface potential, making analysis difficult (Tiede *et al*., 2008). Many researchers use ***Zeta potentiometers*** instead of electrophoresis to measure the zeta potential. They are easy and quick based on light scattering, which converts particle velocity to zeta potential (Brookhaven instruments).

A suitable combination of methods covering relevant data on nanoparticles has been put forward by von der Kammer and co-workers (2011). In their example, CeO_2 NP in soil could

be separated according to size by a chromatographic method after which the fractions are analyzed with DLS to ascertain proper fractionation. To identify chemical composition ICP-MS could be done followed by characterization of crystal structure and size distribution with TEM. According to Tiede *et al*. (2008), and apparent from the literature, the combination of FFF, ICP-MS, DLS and TEM is frequent in laboratories.

As a final comment on methods for analysis of molecules in food, the Nutrichip-project is worth mentioning (Vergeres *et al*., 2012). It is being developed with the purpose of understanding how functional foods affect humans throughout the digestive tract, from mouth to intestine, with relevant enzymes, pH, movements, etc. It particularly aims at investigating the inflammatory response after a meal (so-called post-prandial inflammation) induced by food and how functional foods may decrease that inflammation. It seems such a model could be suitable for evaluation of nanomaterial in food, especially since inflammation is a hallmark of nanoparticle toxicity and the inflammatory response is under scrutiny by the Nutrichip. Also, the difficulties in understanding nanoparticles toxicity depend on their ability to change properties as a response to the surrounding. Hence, a model that mimics all phases of digestion, and register the inflammatory response, could perhaps provide some additional understanding.