Through the magnifying glass: The effects of size and shape on the uptake, biodistribution and (eco)toxicity of nanoparticles

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Chapter 6

Discussion

Safe by design Nanoparticles

Nanomaterials have proven their advantage in both consumer products as well as in the medical world. However, our understanding of the (unwarranted and wanted) toxicity of these materials is limited. Despite the tremendous advantages that nanomaterials provide for both the manufacturing industry as well as the pharmaceutical industry, we have started to realize that we need to take care of the safety for environment and health. To fulfill this need, the idea of 'safe by design' nanoparticles (NPs) was introduced: building NPs that fulfil the wishes of the manufacturer/developer and that are at the same time the safest option[1]. Typically, there are two strategies for 'safe by design' nanoparticles: reduce the hazard of the particle, or reduce the exposure to the particle by limiting the release into the environment[2]. In this thesis, we focus on the hazard assessment of the particles. To prepare a hazard assessment, we need to figure out what particle characteristics can influence the toxicity of NPs. By providing manufacturers with detailed information on hazard, as derived from relevant parameters, they can make educated decisions. Ideally, this whole selection process for the safest NP should already occur during the Research and Development (R&D) phase. During this phase, generally only limited amounts are produced since the process is yet still small-scaled and costly. Since standardized OECD (standard Organization for Economic Co-operation and Development) tests typically require vast amounts of materials, testing during the R&D phase under the currently standardized methodologies is usually impossible. To contribute to the tackling of this hurdle, we modified the OECD ZebraFish Embryo Test (ZFET) in order to test small quantities (Figure 1; **Chapter 4**). With this, we made a first step towards more 'safe by design' NPs.

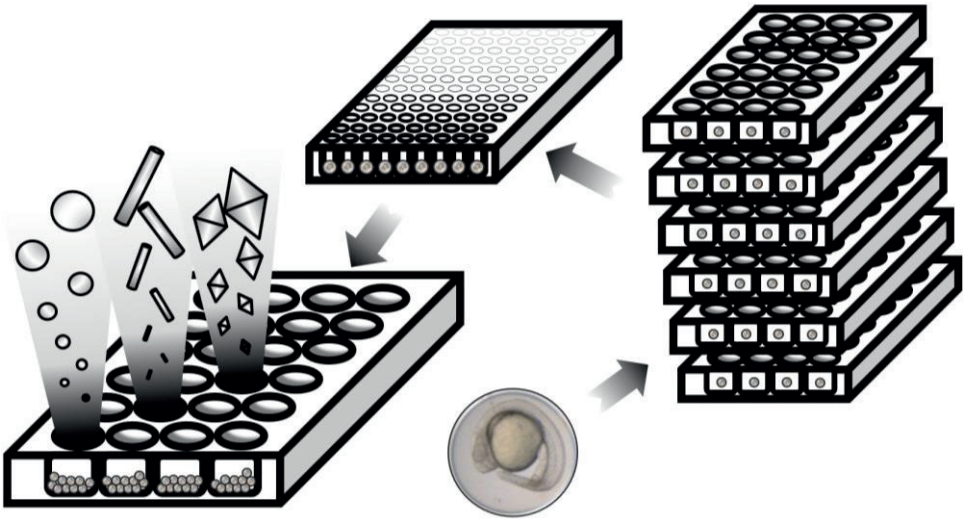

Figure 1. *Schematic overview of the standard OECD Zebrafish Embryo Test (top right), the adjusted version proposed by Hua et al (2014; top left) and the method proposed in* ***Chapter 4*** *(bottom left). The OECD test demands one 24-well plate per test-concentration with one individual per well (and 40 ml per tested concentration), the method proposed in Hua et al (2014) uses sixteen wells of a 96 well plate per test-concentration (again with one individual and uses 4 ml per concentration) and our method as described in* ***Chapter 4*** *exposes one well with 10 individuals per test-concentration (and 2 ml per concentration).*

The effect of single particles

In order to evaluate toxicity, the most standard way is to perform a dose-response test. By using a defined range of concentrations, the toxicity of a particular compound can for instance be addressed as a lethal or effect dose where 50% of the population responds to the exposure (LC_{50} and EC_{50}, respectively). Knowing that NPs differ from soluble molecules, this approach might not be sufficient for all particles. It has to be borne in mind that single particles may induce more or other effects than expected from the concentration tested as compared to dissolved chemicals: if one soluble particle is capable of penetrating the organism and can shed of ions over there, toxicity is induced in a different way (different mode of action) and possibly at a lower concentration than what would have been expected from previous knowledge from the molecular-form. This indicates that NP toxicity is not always as strongly correlated with concentration as is the toxicity of dissolved chemicals[3].

Nanotoxicology: a mixture of ions and particles

Although nanoparticles can be made out of a large variety of materials, a large portion of the manufactured NPs consists of metallic nanoparticles. Most of these metallic NPs (except for e.g. gold and titanium dioxide) have one feature in common: they show dissolution behavior (i.e. non-stable particles). This dissolution behavior adds another dimension to the understanding of the NP toxicity, since now the suspension does not only include particles but also ions[4]. This mixture of particles and ions makes it also important to know the toxic effects of the ions alone: by knowing the ion-toxicity, the relative contribution of both the ions and subsequently the particles can be modeled (**Chapter 4, Chapter 5)**. Since the dissolution behavior differs per specific core material as well as the characteristics of the particle, it is important to measure the dissolution of the NPs in the test medium in order to address the actual particle effect rather than the ion effect.

General aims

Since we have adjusted the standard toxicity tests in order to work with small amounts of materials, we are in a position to focus on defining the most important factors determining toxicity. In this thesis, the overall aim was to get more insights in the effect of nanoparticle characteristics on the biodistribution and the subsequent toxicity in zebrafish embryos. As with any exposure, there is a difference between the nominal exposure and the effective exposure. Questions that arose were: Are the particles taken up in the organism, or do they only adsorb to the outside of the organism? And if they are taken up, where do they go to into the organism? For that reason, we investigated the effect of size (**Chapter 2**) and shape (**Chapter 3**) on the biodistribution of NPs in zebrafish embryos. On top of that, we investigated what the effect of shape is on short-term toxicity of zebrafish embryos (**Chapter 4**). In **Chapter 4**, we went even deeper into the topic of shape related toxicity. We asked ourselves the question: does the shape of particles induce a specific pattern in toxicity, irrespective of the core material? With this knowledge, we aim to predict the toxicity of a new shape, based on the information we have from other shapes. As a final step, we aimed to focus more on the interaction effects on the fate of nanoparticles in mixtures. Knowing that the dissolution behavior is important with regard to the overall toxicity of the particle suspension, we wondered what will happen when we add a stable nanoparticle to the suspension. The nanoparticles added, may interact with the dissolving particle (**Chapter 5)**?

Biodistribution and accumulation

Short-term experiments do not always give definite answers in toxicity assays (hence environmentally relevant concentrations may not induce short-term effects), but knowing where particles accumulate may give an indication of where effects will occur on the long term. Therefore, accumulation can also be classified as a sub-lethal effect. By locating where particles accumulate, so which target organs the NPs have, we can obtain a sub-lethal endpoint that gives an indication of where effects eventually may occur and what type of long-term endpoint will be affected. For this reason, a large part of this thesis focusses on the biodistribution of particles: in which organ(s) do they accumulate?

Knowing the biodistribution patterns of particles does not only give an idea about the possible long-term implications of NPs for that particular organism, but also provides a predictive tool for assessing the impacts of nanoparticles in the environment. Particle build-up in particular organs may result in effects once a threshold has been reached, or when the organ is overloaded with particles and is unable to perform its function. In order to evidence accumulation of nanomaterials, the uptake rate of the particle should be higher than the elimination rate. For that reason, it is important also to include the clearance capacity in the toxicity assessments. Unfortunately, it was not feasible within our experiments to include both an exposure period as well as a clearance period. However, the capability of organisms to clear certain particles influences the long-term toxicity of these particles. When particles are cleared within a short period of time, then the overall toxicity will be low, especially when the exposure was only for a short duration. In contrast, when the organisms cannot clear the particles, the internal concentration will continue to increase over time. Not only will the exposure concentration increase over time, causing much more toxicity after a longer period of exposure than expected, this build-op of particles will also have environmental consequences. The absence of clearance classifies the particles as persistent, causing them to transfer through the food chain and in some cases even to build-up along the food chain (biomagnification). Whereas the small (test) organisms may not perceive any toxic effects, the top predator may.

Factors influencing biodistribution and the subsequent effects

In this thesis, we focused on the two particle characteristics that are known to influence cellular uptake: size and shape[5,6]. As we saw in **Chapter 2**, size not only influences the

uptake, but also the biodistribution of particles. Whilst 50nm particles were able to distribute to the eye, 250nm particles remained in the gut system and adsorbed to the skin. This indicates that there is a size limit in between 50 and 250nm above which the particles become too large to be taken up by cells and subsequently by the organism. If the particles have reached this size (or beyond), then the toxicity will most likely decrease significantly: toxicity can now only be induced by hindering the organism (adsorption) or by release of ions from the NPs. In addition, also the shape of the particle influences the biodistribution. In **Chapter 3**, we saw the particles flowing through the blood vessels, after which they were removed via the mononuclear phagocyte system (MPS). Although all shapes were found in the same clearance organs, the ratio in which they were found over these organs differed. This indicates that some shapes are better in evading the immune system than others, prolonging their circulation time in the organism and therewith enabling themselves to reach other organs in higher quantities.

Based on our knowledge on how size (**Chapter 2)** and shape (**Chapter 3)** influences the biodistribution of particles, we aimed to assess the importance of these characteristics for NP toxicity (**Chapter 4).** By subtracting the effects induced by the ionic form from the total observed toxicity, we obtained the effect induced by the shape of the particle. Each differently shaped particle gave a different toxicity, which we tried to explain by different parameters. The parameter that explained the shift in toxicity the best was the minimal diameter of the particle: the smallest diameter that can be found on the particle. This is in line with our earlier findings that demonstrated that size is an important driver determining the efficiency of uptake of NPs (**Chapter 2)**. To illustrate this: a rod with the same volume as a spherical particle will have a smaller diameter. Therefore, the rod might penetrate the membrane of a cell easier than its equally weighted spherical counterpart might. When particles can penetrate the membrane easier, the uptake in the organism will occur faster, which will lead to a higher internal concentration and thus higher internal exposure concentration within the same period.

However, not only the minimal diameter influences the toxicity of the particles. While looking at the data generated for Ag NPs, we see that some data points (each representing another shape) deviate more from the modeled line than others do (Figure 3c, **Chapter 4**). Whereas the possibility to penetrate is mostly dictated by the minimal diameter of the particle, sharp edges of the particles may also contribute to higher penetration rates. Since it is quite difficult to express this feature in a specific (numeric)

characteristic, we were not able to add this characteristic/dose-metric to our comparison. Nevertheless, it would be interesting to group particles in categories ranging from 'very sharp' to 'blunt' and see how these groups are distributed in Figure 3c (**Chapter 4**). When the categories that lie close to 'very sharp' show the largest deviation from the model line, then this feature (as observable via TEM imaging) can be taken into account when predicting toxicity. On the other hand, particles with sharp edges might also show higher dissolution rates (which can be verified via chemical measurements). Our model is based on the particle-toxicity, so the toxicity of the material itself (induced by the released ions) is extracted. When the dissolution rate increases, the relative contribution of the particle might decrease, resulting in a lower suspension toxicity (so a higher LC_{50} value). The same might be true for other characteristics of the nanoparticles (e.g. coatings), which may influence the dissolution rate of the particles. Additionally, the shape may influence the mode of action of the particle, influencing the slope of the dose-response curve and therewith the relative contributions at different concentrations. Whereas at low concentrations the ions contribute the most to the total toxicity of the NP, at higher concentration the particle effect may have much more impact in a relative sense.

Using this knowledge on the effect of size and shape on the uptake and biodistribution of particles, we aimed to contribute to the knowledge needed to obtain 'safe by design' nanoparticles. With the knowledge that the size influences the uptake efficiency of particles (**Chapter 2)**, it might be ideal to produce particles that are around 250nm and therewith limiting the amount of particles taken up in the organism. However, this may result in particles being too big for the purpose of the manufacturer. In those cases, it is recommended to use particles that are as big as possible, while remaining within the useful range of the manufacturer. The same is true for the shape of the particle. As we saw that sharp edges of particles influences their uptake (**Chapter 3**) and toxicity (**Chapter 4**), the safest choice would be for the particles to have the bluntest shape: spherical. However, sometimes the shape determines the functionality of the particle for the manufacturer. Therefore, the best option would be to opt for the bluntest shape that still provides the desired functionality. In general, based upon the knowledge we obtained, the particle most suited for the label 'safe by design' would be the largest and bluntest particle of the possible options suited for the manufacturer.

The pace of technological innovations gives increased applicability

In order to be able to visualize particles, they either need to be dyed/labeled, need to have optimal optical properties or need to be measured by using highly specialized equipment. Each of these requirements brings it owns restrictions. For instance, the dye or label of the particle should not get detached; otherwise, it is impossible to know whether one is imaging the particles or the detached dye/label. Additionally, attaching a label to a particle may influence its behavior inside the organism, resulting in a different biodistribution pattern as compared to the bare particle. To continue, optimal optical properties are spares among the full range of nanoparticle. This limits the amount of testable NPs significantly. In order to visualize the full range of available NPs, highly specialized equipment is needed. Unfortunately, this type of equipment is not always within reach, restricting researchers to the previous two options. Luckily, technology develops with an astonishing pace. Where we started to investigate the use of the two-photon confocal laser microscope at the beginning of this PhD project, only able to distinguish injected nanoparticles in the 3 cell-layers thick tailfin, we are able to fully visualize and track injected particles inside the whole organism (**Chapter 3**) just 3 years later. Where most technologies are currently able to visualize clusters of particles, future technologies will enable researchers to track single particles inside living organisms. Development of faster microscopes with higher resolution, such as light-sheet microscopes, will further increase our knowledge on the biodistribution of particles in living organisms. Probably within even shorter time window, methods like single-particle ICP-MS will make it possible to routinuously measure very low amounts of particles in different organs, giving a quantitative image of the biodistribution. The largest benefit of all these new technologies is that we can gain more knowledge on single, non-modified particles.

Key events and AOPs

Knowledge on mode of action, or in mechanistic pathways 'mechanisms of action', of nanoparticles informs us on how the particles induce toxicity. In order to describe this mode of action, 'key events' are being formulated: measurable endpoints that are specifically linked to exposure[7]. Using these key events, Adverse Outcome Pathways (AOPs) can be build[8]. Within these AOPs, a chain of events is described that eventually results in the adverse effects observed in the organism. For instance, a disturbance at the molecular level might eventually lead to effects on the growth or survival of organisms[9]. With the knowledge obtained in this thesis on the biodistribution of

nanoparticles, building blocks were formed for further research and the eventually development of AOPs. For instance, the knowledge which size is small enough for uptake provides a first step in an AOP. This will answer the question whether there is actual internal exposure.

Although we have obtained a glimpse of what can be important for developing key events and subsequent AOPs, there is still a lot of lacking knowledge. Most of our knowledge is built upon experiments with stable particles, especially concerning biodistribution. Yet, the dissolution behavior of particles plays also an important role in their toxicity. It is key to understand which characteristics of the particles influence the dissolution behavior, and how this subsequently influences the toxicity. Looking more into the effect of coatings may be an important aspect here, as well as how multi-exposures (mixtures of different NPs) influence the particle. As seen in **chapter 5** the relationships between NPs in co-exposure are then not always easy to explain. Obtaining more information about which characteristics influences the fate of the mixture and therewith the toxicity, showed to be a relative unexplored field.

Read across from zebrafish embryos to humans

When comparing zebrafish to humans, they have proven to be a relative good non-mammalian model organism. From all the human genes, roughly 70% has an identifiable counter gene in the zebrafish model[10]. Not only is the zebrafish model fully sequenced and annotated, the zebrafish offers a wide repertoire of genetic, molecular and cellular manipulation tools[11]. These tools are used to answer various biomedical and toxicological research questions, such as within research on cancer and ecotoxicology [12]. Additionally, due to the high conservancy between the molecular pathways, the development and internal structure of the zebrafish and mammalian species, the zebrafish provides a strong screening model in between cell/tissue cultures and higher animal models. For these reasons, knowledge obtained and the emergence of AOPs based upon zebrafish data can be used for the read-across from zebrafish to humans. During the exposures in **Chapter 2**, we noticed that the exposure route to a high extent determines the uptake of particles. Although the uptake of single particles cannot be excluded, no clear biodistribution pattern was observed until the mouth of the embryo opened at three days old. This indicates that (at least for the particles we tested) the addition of the intestine lining exposure route results in a much higher uptake than solely via the epidermal exposure route. Moreover, since the uptake via the

skin was limited, performing solely *in vitro* studies on dermal cell cultures might underestimate the uptake efficiency/capacity of particles. Due to these underestimations, particles may appear to be safe (for dermal exposure), while their uptake occurs somewhere else. Determining the most likely location for uptake will provide a more realistic view on the hazard of exposure.

In **Chapter 3,** we concluded that the shape of the particle influenced the ratio in which the particles were distributed over three organs related to toxicant clearance. These same organs have been found to accumulate NPs in humans[13], indicating that the same clearance processes (the MPS) are present in both zebrafish and humans. However, there are many more organs where particles can distribute to when the particles have escaped the immune system. Within cancer research, there has been a large emphasis on which particle shape is capable of evading the immune system in order to reach the highest accumulation rate of particles in tumors[14]. Since tumors are often tissues that demand high oxygen levels and usually contain relatively high amounts in capillary vessels, it is clear why tumors accumulate large quantities of NPs: large quantities of blood pass through this tissue over time, accelerating the accumulation of particles. However, healthy organisms in the environment do not contain tumor tissue. So the question remains: where do these particles go to when there is no tumor tissue present? And more specifically, how is the accumulation in other tissue/organs that are physiologically similar to tumor tissue (i.e. high in capillary vessels)? The brain is an organ that consumes a lot of oxygen and has therefor a high amount of capillary vessels. Obviously, it is unwarranted that NPs accumulate in our brain, where they might interfere with neurological and biological processes or even change the structure. The blood-brain barrier is effective in preventing most molecules from entering the brain. Important to know, is that molecules pass membranes via active uptake. In contrast, we do not know for sure which particles can cross the membrane barrier via passive diffusion. Particles that cross membranes via passive uptake are much more likely able to cross the blood-brain barrier. Fairly small particles can enter the brain[15], but perhaps also particles with sharp edges. This relatively unexplored field may be of high interest, especially concerning long-term effects.

Long-term testing and other experimental applications

Within this thesis, we have obtained more insights in the factors that are important for the uptake and biodistribution of particles. Ideally, this type of test will substitute long-

term tests, saving a lot of time, money and expensive materials. However, in order to make short-term tests a full substitute for long-term testing, it is important to understand the uptake and depuration efficiency, as stressed before. Without this knowledge, it is unsure whether the observed accumulation over short-term will lead to long-term effects. So, besides including an analysis of the depuration efficiency in the experiment, it should be validated whether the accumulation and biodistribution of nanoparticles is a genuine predictor of long-term effects and which endpoints are the best to predict. Furthermore, imaging techniques might be also interesting for other types of experiments. By using sensitive imaging techniques, it will be possible to examine the embryos for that have been taken up (internalized particles). Currently, it is impossible to distinguish quantitatively between internalized particles and particles that adhere to the outside of the organism. The benefit of imaging techniques is that they will enable researchers to distinguish between internal and external particles.

Ultimately, building detailed AOPs on the knowledge obtained via improved biodistribution patterns will provide a more comprehensive view of the actual hazards posed by NPs. Using these AOPs, we will have a more refined knowledge of the toxic effects on both the environment and on humans.

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