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

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Chapter 1

General Introduction

1

Nanoparticles: what they are and where they come from

Nanomaterials have been present since the beginning of the Earth. Main sources of naturally occurring colloids (suspensions of nanoparticles) are dust of volcano eruptions and bush fires, and corrosion of river beddings and sea floors. In early civilization, men began to use these extraordinary and very small particles, without extensive knowledge about their characteristics. In the fourth century A.D. the ancient roman glass industry produced for instance the famous Lycurgus Cup: a ruby red cup colored with colloidal gold[1]. In the 19th century, modern technologies such as microscopes allowed for the rise of the nanotechnology: the production of well-defined and well-characterized nanomaterials[2]. Through the development of advanced techniques, the production of colloids started to become more sophisticated.

In nanotechnology, nanomaterials are defined as being materials that have one or more dimension within the 1 to 100 nm size range[3]. More specifically, if the nanomaterial has three dimensions within the nano range, it is classified as nanoparticle (NP)[4]. Although these definitions remained the same, different generations of nanotechnologies have been developed. The first generation of technologies (pre 2005) can be found on the market, with products containing particles either as individual nanomaterials or as mixtures with other materials. Examples of products are antibacterial socks (silver)[5], sunscreen (titanium dioxide and zinc oxide)[5] and solar cells (e.g. copper and silicon)[6]. Because they are widely used on the market, most research has been done and is still being done on these particles. Technologies developed from 2005 until 2010 are considered second generation: functional structures of products as based on nanoscale elements. The third generation technologies (2010-2015) start to layer their materials, making a combination of macro-, meso-, micro- and nano-scales. This can also be in a three-dimensional setting. Finally, the fourth generation (from 2015 onward) focuses on so called 'molecular manufacturing': multi-functionality and control of function at molecular level.[5]

Due to their unique properties, nanomaterials have gained interest from producers and entered the global market. Potentials that are ascribed to nanotechnology are: stronger, more efficient, cleaner and compact materials that allow for small yet complex products[5]. Currently, nanomaterials are used in numerous products, although exact numbers are lacking. In 2014, it was estimated that the market contains more than 13000 nano-based products[7]. The variety of products is large,

ranging from sunscreens and paint, to textiles, medicines, electronics and many more[5,8,9], covering many sectors.

With more and more nanotechnology entering the market, the amount of waste increases as well. Since most of the products are still in use, only an estimation can be made about the impact of all the nanomaterials in waste. The most important route from nanoparticles to the environment is via wastewater, with different types of nanoparticles released to water[7]. Man-made nanoparticles have already been detected in wastewater[10] and waste leachate[11]. With the fast growing nanotechnology, the amount of new and unknown materials that are introduced into the environment will increase concomitantly, which may lead to unpredictable long-term consequences on human and environmental health[12].

Nanoparticles in the aquatic environment

Once nanomaterials have entered the environment, a multitude of effects can occur. Nanoparticles are fairly small, and with decreasing size their surface to volume ratio increases rapidly (see Figure 1). Due to their large surface to volume ratio, the nanomaterial surface becomes more reactive in itself and to its contiguous environment[13]. However, the size of the particles can change over time. In the aquatic environment, non-stable metallic nanoparticles dissolve slowly over time, releasing ions to the environment while simultaneously the particle decreases in size[14]. On the other hand, agglomeration (loose clusters) and aggregation (irreversible clustering) processes result in an increase in size[15].

Furthermore, with their relative large surface area, nanoparticles are prone to react with organic and inorganic materials. These interactions influence the stability of the particles in the water, which in turn influences whether the particles remain in the water column and thereby determining which type of organism faces the highest exposure. For instance, when particles sediment and settle down, bottom dwellers are much more exposed than organisms that live at the water surface.

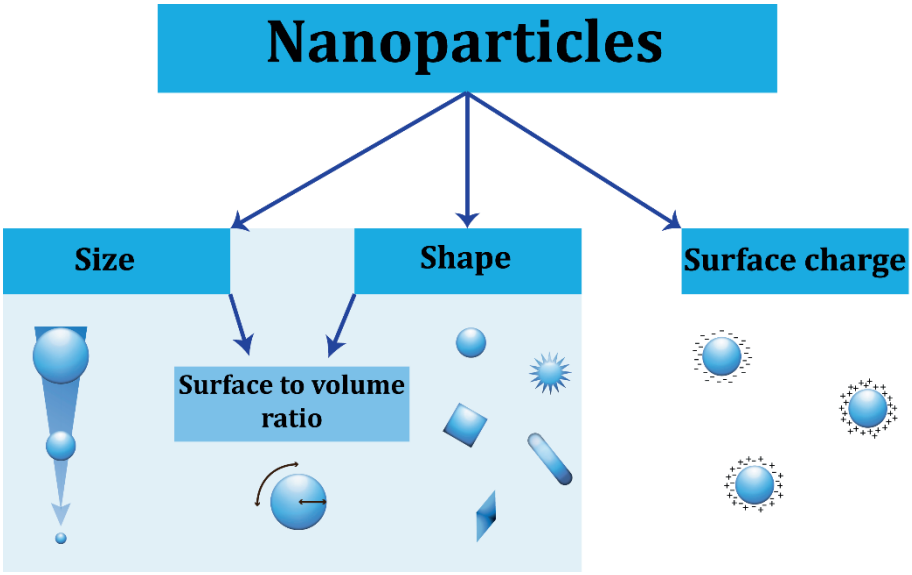

Figure 1. Most important aspects of nanoparticles*. Different aspects of nanoparticles are important for their uptake in and toxicity to cells and organisms. Besides size and shape, which are the focus points of this thesis, surface charge (negative, positive or neutral) influences the uptake op particles.*

Uptake of nanoparticles

Once organisms are surrounded by particles, the impact is based on the effective exposure: the fraction of the nanoparticles that is accessible for the organism. More importantly: the effective exposure determines whether the particles are taken up. With the large surface to volume ratio, membranes can attach to the relative large surface area, wrap around the particle and transport it inside the cell[16,17]. In general, the surface charge also influences the uptake of the particles (Figure 1). This charge is based on the material properties, as well as the layer (corona) formed by organic materials[18]. Different charges have been found to behave differently: positively charged particles are in general found to be taken up much faster than negative or neutral charged particles. The reason for this is supposedly the slightly negative charge of the cell membrane, causing uptake by electrostatic attractions[19]. The surface charge also results in the adhesion of proteins to the particle. Particles with surface ligands (e.g., peptides, antibodies, etc.) are able to target specific organs[20]. The amount of proteins that absorb onto the surface can for instance be reduced by decorating the particle with

a coating[21]. This layer improves stability and therewith prolongs the *in vivo* circulation time and subsequently the duration of the effective exposure[21].

Besides size, the surface to volume ratio is also influenced by shape (Figure 1)[22]. The surface to volume ratio (which is related to the aspect ratio) has large influences on the membrane wrapping[22,23]. Flat, disc-like shapes adhere to the outside of the membrane, forming aggregated rather than internalized particles[24]. For other shapes, rotation during endocytosis is important in order to find the most optimal position for cell entry[22,25,26]. With increasing aspect ratio (i.e. with increasing particle length), cellular uptake becomes more difficult[26,27]. This also explains why the flat particles remain on the outside of the cell membrane. Therefore, on average, small and elongated particles are easily taken up by cells, whereas big, long and flat particles are a much bigger challenge for cells to internalize[28]. Even within the cell, the shape of the particle influences its position[29].

Due to the difference in cellular uptake of nanoparticles and larger mixed materials of metallic compositions (from now on called bulk), different exposure routes are possible. The bulk form (for metals) tends to dissolve in free ions, which can only pass certain ion channels and or ion pumps[30]. However, nanoparticles can, as previously discussed, either pass through membranes or enter the cell via membrane wrapping. In nanoparticle grouping efforts, a distinction is made between metallic particles that are either stable (inert) or non-stable (dissolving ions)[31]. At this point, non-stable nanoparticles start to act like a Trojan horse being an uptake route for colloid metals (see Figure 2): while dissolving, their shed ions reach places that they would have never reached when present in their ion form[30,32]. Another type of Trojan horse principle can also occur: not the shedding of ions, but other molecules that adhere on the surface of the particle accompany the particle inside the cell (Figure 2)[33]. This mechanism of co-exposure is beneficial for medicines delivered to cancer cells[34,35], but can result in unpredicted exposures of organisms in the environment.

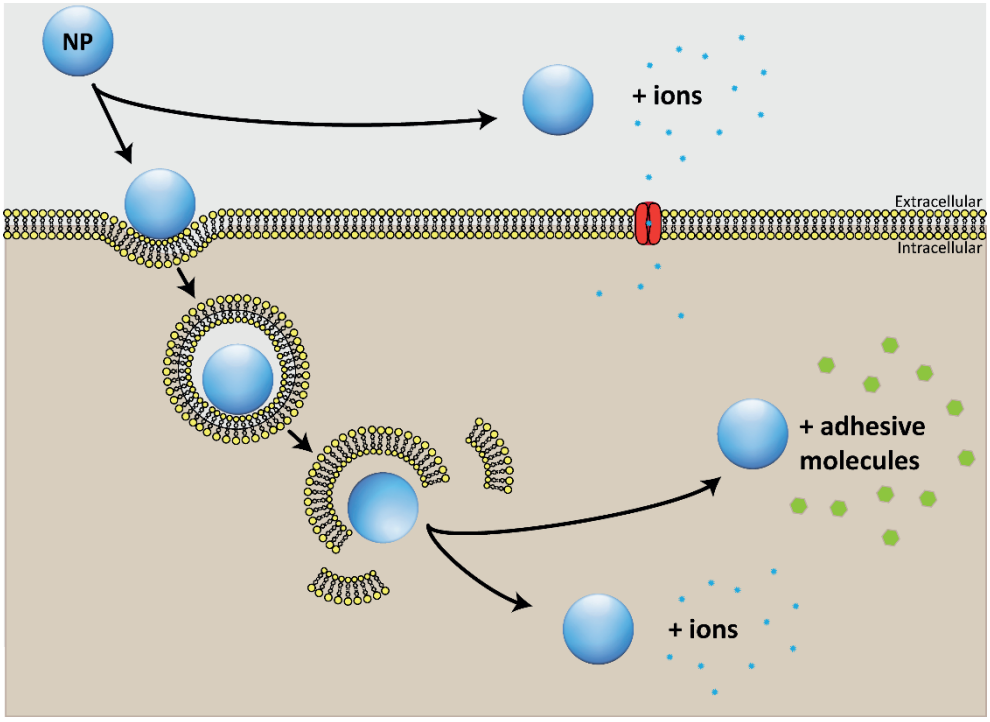

Figure 2. Trojan horse principles. *Metallic nanoparticles can shed of their ions in their surrounding media, from where they can enter the cell via channels. However, the particle itself can also work as a Trojan horse: either it can shed of ions once it is inside the cell, resulting in much higher concentrations of ions, or the particle carries adhesive molecules inside the cell, where they are released.*

Besides effects of the material, the particle itself can induce effects as well. Once the particles are inside the cell, interactions with the biological processes can occur. If the particles are small enough, they can enter the nucleus and bind to the DNA, causing DNA damage[36]. Moreover, their presence in the nucleus may also interfere with the DNA replication[37]. Particles that are not entering the nucleus, might bind to proteins and disrupt their function by protein unfolding, fibrillation, thiol cross-linking and enzymatic activity loss[37–39]. For instance, thiol groups in enzymes like NADH dehydrogenase are popular binding places, causing disruption of the respiratory chain and subsequently generate reactive oxygen species (ROS)[36]. This in turn can induce oxidative stress, cell damage and eventually cell death[40–42]. Both *in vitro* and *in vivo* studies have shown that particle toxicity might be related to ROS formation[43–45]. Since particles induce toxicity via other pathways than the ions of the core material, distinct adverse outcomes can be found for the particles and the ions[38].

The effects of nanoparticles on organisms

Cellular uptake gives an indication of the possible effects induced by nanoparticles, but the picture is incomplete. With regard to upscaling of *in vitro* results to effects on whole organisms, the key issue is that particles do not induce effects at only one location. Epithelial tissues like skin and intestine generally protect organisms from hazardous materials. However, nanoparticles are small enough to penetrate into cells[46]. Once they are inside the cells of the barrier tissue, they may start to distribute throughout the body[47,48]. Because of this biodistribution, particles can accumulate in secondary organs[12,49]: accumulation in the body. Besides the biodistribution, the time that the particle stays within the organism also influences nanoparticle toxicity: the residence time. This duration is most importantly influenced by size: particles smaller than 6nm can quickly be excreted by the kidneys, whereas particles with a size larger than 200nm accumulate in spleen and liver, after which they are processed by mononuclear phagocyte system (MPS) cells[19]. This knowledge emphasizes the importance of understanding the factors that determine whether particles cross membrane barriers and subsequent distribute throughout the body. Therefore, if we want to know where the particle ends up irrespective of the material, *in vitro* studies can provide only half of the story.

Just as for particle uptake, shape is also an important factor for uptake, biodistribution and toxicity. Particle shape has been found to influence both the circulation time of the particles, as well as their distribution. Wires, discs and lamella, compared to spherical nanoparticles, were found to have a longer circulation time in the body, whereas cylindrical shapes display the longest circulation time[50]. With regard to the distribution, rod shaped particles distribute much further inside the tissue, whereas spheres and disk-like particles stay on the edge of the tissue[51]. Furthermore, the length of the rod is important for biodistribution: short rods are trapped in the liver, bigger rods are trapped in the spleen[52]. This knowledge is gathered in cancer research, where the study is performed by injecting rodents: biodistribution via the blood and / or lymphatic system is guaranteed. Studies in which environmentally relevant exposure routes are used still focus mainly on the effect of size. However, examples like given by Dai et al., (2015) provide nice evidence that also in environmentally relevant exposures, rod shaped nanoparticles display much higher uptake rates than plates and spheres.

Different types of organisms result in different types of exposure routes and biodistribution. In the animal kingdom, there is a division made in two distinct types of organisms: invertebrates and vertebrates (representatives can be found in Figure 3, top left + bottom right and top right + bottom left respectively). In general, invertebrates are smaller than vertebrates. This results in a larger surface to volume ratio and subsequent more relative exposure than animals with a smaller surface to volume ratio. Additional organs like gills may increase the amount of surface even further. However, not only do their sizes differ. Especially insects and arthropods exhibit different external properties compared to vertebrate species. Where vertebrate species obtain their name from their spine (consisting of vertebra), invertebrates usually have an exoskeleton that supports their body. This exoskeleton is for instance composed of chitin, and is much harder to penetrate by chemicals than the soft surface burdens of vertebrates. Therefore, hard-body invertebrates are usually exposed via specific structures like pleopods or via the digestive tract[54].

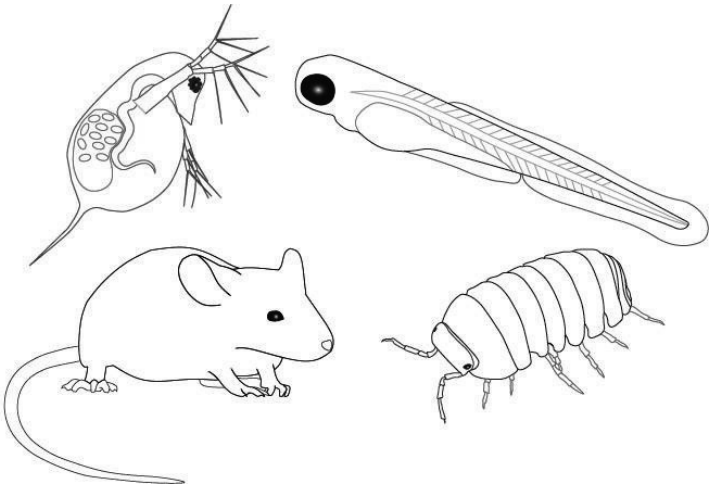

Figure 3. Model species. *In (eco)toxicology, model species are used to test chemicals. In this picture, an overview of two vertebrate species (Zebrafish larvae: Danio rerio; Mouse: Mus musculus) and two invertebrate species (Waterflea: Daphnia magna; Woodlice: Porcellio scaber). The top represents aquatic organisms, whereas the bottom row represents terrestrial organisms. Note: animals are not on scale.*

Not only does the uptake route differ between vertebrates and invertebrates, but also their accumulation pattern. Structural and functional differences in organs for instance modify the biodistribution pattern. For instance, most invertebrates have a hepatopancreas where (bulk) metals accumulate[54]. In vertebrates however, this

structure continued to develop in two individual organs: the liver and the pancreas[55]. Indeed, a large number of particles have been found to accumulate in the liver and are being excreted in the bile[32,56–60]. Other specific excretion routes that can occur are for instance via the kidneys for vertebrates[61–63] and via the maxilla gland for invertebrate species[54].

As we have seen up to now, most of our knowledge on nanoparticle (eco)toxicity on vertebrate species is based on *in vitro* studies or *in vivo* injection studies. However, these studies do not provide information about the effective exposure in the environment, and do not answer the key question: are particles able to penetrate and enter the organism (uptake) at realistic environmental exposure conditions? As mentioned above, knowledge obtained on particle uptake in invertebrate species might not be applicable to vertebrate species due to their morphological differences. It is therefore important to assess the ability of particles to penetrate the borders of vertebrate species (epidermis, gut lining, etc.) under environmentally relevant conditions. By doing so, more relevant information about potential hazard for both ecological vertebrate species and for humans can be obtained.

After particles have crossed the exterior borders of the organism, particles will distribute throughout the organism. Although *in vivo* injection studies provide information on where particles distribute to once they have entered the blood stream, it is not warranted that every internalized particle travels through the blood stream. For instance, distribution via the lymphatic system is also a possibility. Additionally, most *in vivo* injection studies deal with induced tumor tissue, which influences the accumulation of the particles and the focus area of the researchers. It furthermore is known that size and shape influence the *in vitro* uptake of the particles, but it is largely unknown how these characteristics influence the particle distribution inside the healthy organism. Knowing where particles accumulate (biodistribution) may provide an indication of long-term effects.

Besides the usually stable nanoparticles used for imaging, nanoparticles can be made from various materials. Within the aim of the 3Rs, to Reduce, Replace or Refine animal testing, understanding the effect of particle shape on toxicity irrespective of the core material may provide valuable knowledge for modeling purposes. If there is a shape related effect irrespective of the core material, then the (eco)toxicological effects of an unknown/untested shape of a known material can be modeled rather than tested using animal tests.

The zebrafish as an example

A suited vertebrate model organism to screen for uptake and effect of nanoparticles is the zebrafish (*Danio rerio;* Figure 3, top right*).* This sub-tropical fish is due to its size (2-3 cm) easy to maintain. With 100 eggs or more in a clutch of one female, this model organism is ideal for high-throughput screening. The life-stages of the zebrafish embryos are well documented and therefore uniform to use for each researcher[64]. During the first 24 hours of development (see Figure 4), when the majority of the organs are formed, the embryos are transparent. This makes the embryos ideal for monitoring developmental processes. After the first day, pigmentation starts and the embryos lose their transparency. However, transparent fish lines, called *Caspers*, do not develop this pigmentation and are therefore ideal for imaging for instance uptake of particles at later life stages.

Although zebrafish are cold-blooded, they are well suited as a vertebrate model. With the full genome known, it has been found that at least 71% of the human proteins have a zebrafish orthologue[65]. This indicates that numerous genes are well conserved among the vertebrate group[66]. With a fully known genome and a generation time of 3 months, the zebrafish is an ideal model organism for mutant and transgenic fish lines. Moreover, transgenic fish lines with fluorescent-labeled proteins or cell types are enabling targeted screenings[67]. For instance, with the first innate immune system cell present after 24 hours post fertilization, immune system responses can be easily monitored using fluorescent microscopy.

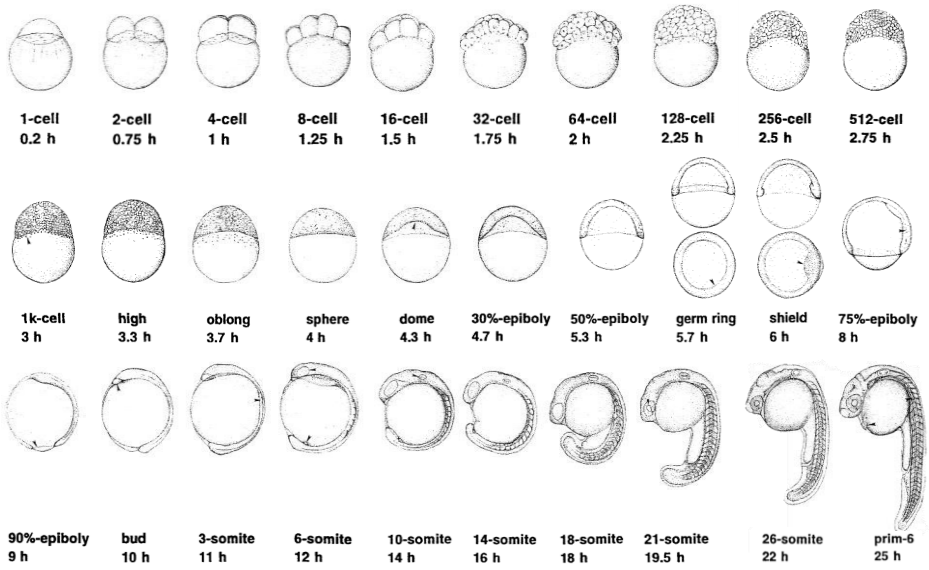

Figure 4. Embryonic development. *In this picture, the developmental stages of a zebrafish embryo during the first 25 hours post fertilization (hpf) are depicted. Reprinted from Kimmel et al., 1995.*

Zebrafish, both larvae and adults, have been used in (bio)medical and toxicological research for a while. Early medical researcher Dr. George Streisinger started using the zebrafish as a model in the 1970's[68]. With the establishment of the zebrafish as a (medical) model organism, the zebrafish became also a representative for the aquatic vertebrates in toxicological assessments[69,70]. Standardized tests were developed to be used for systematic testing of chemicals under the European REACH initiative[71,72]. With a variety of endpoints ranging from lethality to malformations to behavioral effects[73,74], the zebrafish presents itself as a diverse model for toxicity testing. Due to the high rate of conservancy in the genome of vertebrate species, adverse outcome pathways for emerging contaminants in the environment can be extrapolated from zebrafish to different species of ecological relevance[75].

Outline of this Thesis

The work described in this thesis focusses on the uptake and biodistribution of nanoparticles in zebrafish larvae (up to 5 days post fertilization). For this, we covered different characteristics of the nanoparticles and studied the corresponding influence

on the uptake in and also their effect on the zebrafish larvae. The aim of this thesis is to understand the main drivers behind uptake and the subsequent biodistribution of nanoparticles, and how the effects they induce translates to ecotoxicological effects.

Chapter 2 focusses on the influence of the size of particles on the uptake and subsequent biodistribution in zebrafish larvae. In addition, we studied the importance of the exposure route on the uptake of particles. Using fluorescent polystyrene nanoparticles, we investigated where the particles were located inside the organisms after waterborne exposure.

Continuing on uptake, we shifted in **Chapter 3** our focus from size to shape. Using the exposure route that resulted in the highest uptake in Chapter 2, we continued our work with differently shaped particles. Since shaped polystyrene particles are not commercially available, we used gold nanoparticles that we imaged using two photon multi-focal laser microscopy and we imaged the subsequent immune response with stereo fluorescence microscopy.

In **Chapter 4**, we took a closer look at the impact of differently shaped particles on zebrafish fitness. By testing different shapes of silver nanoparticles for toxicity effects, we determined the effect of particle shape. In addition, by combining our data with existing data, we made an effort to model the particle effect irrelative to the core material.

Until now, we have focused on the uptake and induced effects of mono-material nanoparticles. However, as described earlier in this introduction, nanotechnology is advancing and building multi-material nanoparticles. For this reason, we investigated in **Chapter 5** what the interaction effects between TiO_2 nanoparticles and differently dissolving nanoparticles are on the mixture toxicity.

In **Chapter 6** the main findings of this thesis are summarized and discussed in context of environmental safety and future perspectives for nanotechnology.

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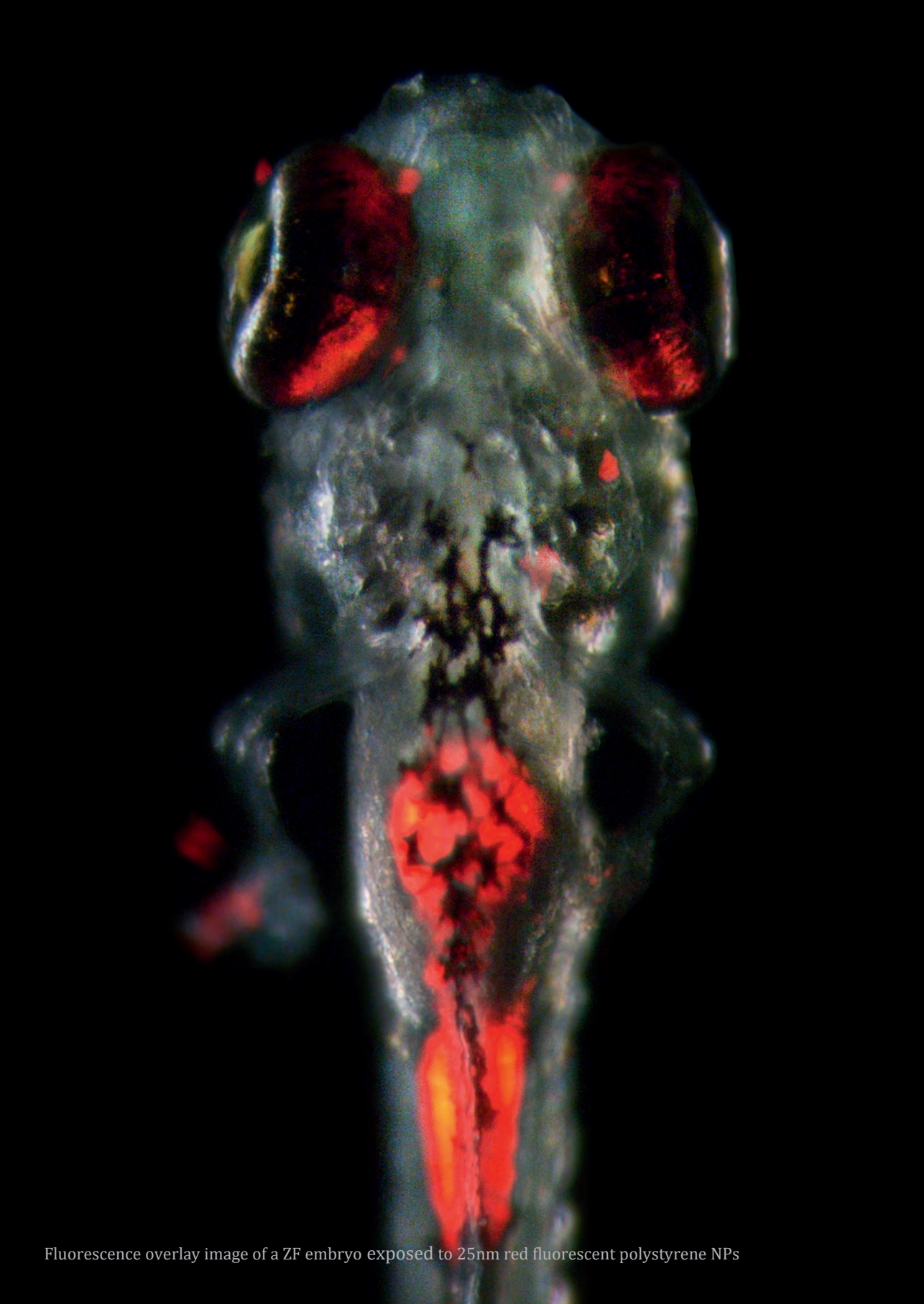

Fluorescence overlay image of a ZF embryo exposed to 25nm red fluorescent polystyrene NPs