Developmental effects of polystyrene nanoparticles in the chicken embryo

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Chapter 1. General introduction

Problem statement

A new category of potentially harmful substances is attracting the attention of researchers. These substances are so-called nanomaterials, which are various substances in particulate form, and with a predominant particle size on the nanoscale. One group of nanomaterials are the nanoplastics (NPs). These are either intentionally manufactured for example for use as research reagents; or arise naturally in the environment from the breakdown of plastic waste (Hernandez et al., 2017; Sangkham et al., 2022). The breakdown of plastics first releases particles called microplastics (MPs) which are in the size range ≤ 5 mm (Ray et al., 2022) and these in turn can break down into smaller fragments including NPs.

NPs are widely present in the environment as pollutants in water bodies, soil or the air, and this provides a potential route for wildlife and humans (Sangkham et al., 2022). NPs are being considered as potential drug-delivery agents in the field of nanomedicine (Boehnke et al., 2022). This could open up an additional route through which humans could be exposed. The toxicity of NPs has mainly been studied using aquatic invertebrates and fish model species. In this thesis, I will use a warm-blooded vertebrate model, the chicken embryo, to investigate NP toxicity.

The origins and fate of nanoplastics

Origins of nanoplastics

Nanoplastics are small fragments of plastic — that is, polymer-based materials synthesized from petroleum products (Desai and Galage, 2015; Geyer et al., 2017). Plastics have a broad range of applications in manufacturing, the electronics, packaging and food industries, and in agriculture and medicine and surgery (Gourmelon, 2015). This is because they are durable, cheap, water-resistant, have

relatively low energy requirements for their manufacture, and are relatively light in weight (Gourmelon, 2015; Panda et al., 2010). Moreover, plastics can be significantly altered by heating (Becker and Locascio, 2002; Levine and Berman, 1995), allowing them to be molded into required shapes. Since the 1950s, the annual global growth rate in plastics production has remained at an average of 8.5% (Platt, 2003). By 2018, global plastics production reached 359 million tonnes. According to some predictions, this value will have doubled by 2025 (Gibb, 2019). This thesis is concerned with a particular form of plastics called small plastic particles. In a sense, plastics, and small plastic particles, are typical representative 'fossils' of human activity (Ng et al., 2018), that is to say, they are enduring artefacts in the environment that are **anthropogenic** (uniquely created by human activity).

Classification of small plastic particles

Small plastic particles include microplastics (MPs) whose particle size is ≤ 5 mm (Arthur et al., 2008); and nanoplastics (NPs), whose particle size is either ≤ 100 nm (Zhang and Webster, 2009) or ≤ 1000 nm (Gigault et al., 2018) depending on the opinion of the author.

Other than classifying them by their size, small plastic particles can be classified according to to their origins. Thus, MPs and NPs can be classified as being primary or secondary (Ziajahromi et al., 2017). **Primary** MPs and NPs are small plastic particles manufactured for a specific purpose, in the case of MPs: personal-use products including toothpaste, cosmetic, shampoo (Hernandez et al., 2017); and in the case of NPs as potential drug-delivery vehicles in the emerging field of nanomedicine (Galafassi et al., 2019; Patel et al., 2009). The potential route of administration of NPs to human patients might include intravenous injection or inhalation (Chai et al., 2020).

By contrast, **secondary** MPs and NPs are fragments coming from larger pieces of plastic such as plastic bags, bottles and fishing nets (Boucher and Friot, 2017). They are generated through the natural processes of physical, biological, and chemical

degradation in the environment, for example, the action of UV light, ocean waves, and wind abrasion (Stevens, 2015). Another source of MPs and NPs is the microfibers generated by laundering of synthetic textiles (Center and Wash, 2017), and the accidental discarding of plastic products during manufacturing or transport (Rezania et al., 2018). Surprisingly, even plastic teabags can release nanoplastics (Hernandez et al., 2019).

Some cosmetic products contain both primary and secondary small plastic particles. One study (Hernandez et al., 2017) analyzed commercially-available facial scrubs, and found 'microbeads' of around 200 μm diameter which were ingredients added intentionally by the manufacturer — along with nanoplastic particles of 24 ± 6 to 52 ± 14 nm. It is not clear why nanoplastics were present, because they were not part of the product formulation. It is possible that they arose from the breakdown of the larger microbeads.

Small plastic particles are also distinguished by their composition; for example they may be made of polystyrene (PS), polypropylene, polyethylene, low-density polyethylene, polyacrylates, polyvinylchloride, polyamide, polyethylene terephthalate and polyvinyl alcohol (Anderson et al., 2016; Avio et al., 2017). Finally, MPs and NPs can be categorized according to their shapes which include: beads or spheres, fibers, fragments, film, pellets and foam (Burns and Boxall, 2018).

Plastic waste as a source of small plastic particles

We mentioned above that secondary plastic particles are released from manufactured plastics when they break down (Stevens, 2015; Zhang et al., 2021). For example, at their end of usable life, plastics end up forming a large component of domestic garbage and industrial waste (Subramanian, 2000). The great majority of this plastic waste is dumped into land-fills, while some is burned and a smaller amount is recycled (Geyer et al., 2017). Dumped plastic waste can been found in freshwater bodies, oceans, soil, air, and even in remote, uninhabited islands (Bergmann et al., 2019). For these and other reasons, plastic pollution to our

environment is a matter for great concern (Chauhan and Wani, 2019). Plastic waste can persist for years in the environment due to the low speed at which plastics undergo degradation (Webb et al., 2013). One reason so much plastic is dumped rather than being recycled is that recycling technology is not currently optimal. For example, it is common practice to collect all types of recyclable plastic into one mixed batch, making it difficult to produce a high-quality recycled product (Geyer et al., 2017). Furthermore, plastics can only be recycled twice (Geyer et al., 2017). Because of the size of nanoplastics it is not practical to remove them from water by filtration, nor is not it practical to try to sort them into different plastic types for recycling (Nguyen et al., 2019). In any case, no matter how plastic wastes are disposed, of they can potentially break down and release small plastic particles into the natural environment.

Distribution of MPs and NPs in the environment

After the breakdown of waste plastic, and its release of small plastic particles into the natural environment, MPs and NPs become widely distributed in freshwater bodies, sediments, air and the sea, from the equator to the polar regions (Browne et al., 2011; Wan et al., 2019a). Furthermore, MPs and NPs persist in aquatic system because the waste-water technology to deal with these kinds of materials is lacking. Even though membrane bioreactors have been created by scientists to remove the particles, the high cost of implementation results in feasibility problems for large-scale, public application (Westphalen and Abdelrasoul, 2017). As a result, a large amount of MPs and NPs enter the ecosystem (Browne et al., 2007; Fendall and Sewell, 2009).

Trophic transfer of MPs and NPs

MPs and NPs are transferred between species that are part of food chains (Shruti and Kutralam-Muniasamy, 2019; Vom Sqaal et al., 2008). This is called trophic transfer, and is one of the most important uptake routes of MPs and NPs (Toussaint et al., 2019). MPs and NPs can be taken up by wildlife passively or actively (Vom Sqaal et al.,

2008). For instance, fish can take up MPs and NPs while feeding or drinking (passively), or when foraging by accidental ingestion (actively; (Roch et al., 2020).

Animals tend to accidently ingest plastic items in their food (de Sá et al., 2018). In Gall & Thompson's (2015) report, large pieces of plastic have been found in the digestive tract of 208 species of marine organisms worldwide including 86% (6/7) sea turtle species, 39% (122/312) of seabird species, 0.3% (50/16,754) fish species and 26% (30/115) marine mammals (Gall and Thompson, 2015). Such large pieces of plastic (plastic bottles, plastic bags etc.) can cause adverse effect to wild animals, resulting in starvation and even death (Nkwachukwu et al., 2013). In addition, animals (including sea turtles, seals and sharks) can become entangled in plastic fishing nets and fishing lines and this may impair the movement of the animals, and even cause asphyxia (Butterworth et al., 2012).

MPs and NPs are difficult to detect in terrestrial environments owing to the complex composition of soils. Fortunately, analytical methods for measuring MPs in soils have been developed (He et al., 2018). Large amounts of MPs and NPs have been found in agricultural soils and in industrial regions (Galloway et al., 2017; Zhang and Liu, 2018). The accumulation of MPs and NPs in the soil cause negative effects through altering its biophysical properties (Navarro et al., 2008; Wan et al., 2019b).

Toxicity of small plastic particles to living organisms

MPs and NPs pose a number of health threats to wildlife and humans. For example, once they have entered the natural environment, they can adsorb further toxic chemicals such as polycyclic aromatic hydrocarbons (PAHs) (Maes et al., 2017), polychlorinated biphenyls (PCBs) (Velzeboer et al., 2014) and dichlorodiphenyltrichloroethane (DDT) (Bakir et al., 2014), which can potentiate any intrinsic toxicity they may have (Souza et al., 2022; Ziccardi et al., 2016).

Any of these toxins may later get leached out and released into the environment when the plastic is degraded into particles (Burns and Boxall, 2018; de Souza Machado et al., 2018; Horton et al., 2017; Smith et al., 2018). Adsorption and leaching, respectively, are influenced by internal factors, namely: particle composition, size and shape, and external factors such as temperature, pressure and pH in the environment (Bakir et al., 2014; Frere et al., 2017). In this context, it is important to note that NPs have a very high surface-area-to-volume ratio.

Like all plastics, NPs may contain toxic plasticizers and pigments from manufacture (Unar et al., 2010). Toxic chemicals used in the manufacture of some plastics include nonylphenol, bisphenol A, and vinyl chloride, and these are able to leach from the plastics when they degrade in the environment (Gallo et al., 2018; Koelmans et al., 2014). Also, MPs and NPs can affect the food chain by transferring biocide (pesticide and herbicide) residues in agro-ecosystems (Ng et al., 2018). Other pollutants such as heavy metals and organic chemicals can also be adsorbed to the surface of the MPs and NPs (Zeng, 2018). In summary, MPs and NPs are widely distributed in biotic and abiotic matrices (Karlsson et al., 2017), and can show enhanced toxicity due to physical and chemical changes.

Toxic effects of MPs and NPs in experimental animals

The biological effects of MPs and NPs have been widely studied in aquatic organisms including crustaceans such as *Daphnia* sp. (Brun et al., 2017), and teleost fish such as the zebrafish (*Danio rerio*) (Bashirova et al., 2023), its adults or embryos/larvae (Veneman et al., 2017), reviewed by (Jiang et al., 2020). When MPs or NPs enter into the body of animals, they can accumulate and cause significant effects. These effects may be on **higher levels of the biological hierarchy**, such as changes to the populations, communities and ecosystems of animals (Eerkes-Medrano and Thompson, 2018; Galloway et al., 2017). Pollution by MPs and NPs can affect a community if a an invasive species contaminated by MPs and NPs is introduced; this

can result in alterations to the composition of species in ecosystem (Gall and Thompson, 2015; Lusher, 2015).

Effects of MPs and NPs on **lower levels in the biological hierarchy**, include deleterious effects at the molecular, cellular and organismal levels such as reproductive and development toxicity (Wang et al., 2019; Zhang et al., 2019). Such organismal effects have been seen in the common goby (*Pomatoschistus microps*) (Ferreira et al., 2016), the Pacific mole crab (*Emerita analoga*) (Horn et al., 2020) and the water flea (*Daphnia magna*) (Aljaibachi and Callaghan, 2018). Furthermore, polyethylene MPs accumulated in gill and intestine of the adult zebrafish can lead to abnormal behavior and movements, including seizures (Brun et al., 2019; Mak et al., 2019). More seriously, it has been reported that 40 nm and 200 nm polystyrene NPs can increase the risk of cardiovascular disease by disrupting the cellular components and extracellular matrix in human induced pluripotent cells (Bojic et al., 2020; Prata, 2018).

In the rainbow trout (*Oncorhynchus mykiss*), PS-Pd NPs (Palladium-doped polystyrene nanoplastics) can adhere to the gut epithelium, and can be taken up into the liver, kidney, gills and muscles (Clark et al., 2023). In another study it was found that NPs of 51 nm can be transferred into the gallbladder, liver, pancreas, heart and brain of zebrafish larvae (Pitt et al., 2018). Moreover, it has been found that MPs and NPs can cause inflammation in the liver, heart, lungs, kidney and brain in the Medaka fish (*Oryszia latipes*) (Kashiwada, 2006).

It is not clear whether they then enter the blood stream or the lymphatic system, both of which drain the gut. It is possible that MPs and NPs might be cleared from the circulation by the spleen, and some might be secreted in the urine reviewed by (Bouwmeester et al., 2015; Li et al., 2022; Zhu et al., 2022). Zhu *et al.* (2020) also found that MPs of 10 µm diameter accumulated in the gills and gut of the medaka (*Oryzias latipes*) (Zhu et al., 2020). The distribution of the NPs *in vivo* appeared to be influenced the size of the NPs. Thus, when zebrafish were exposed to 70 nm, 5 µm,

and 20 µm diameter polystyrene particles, the 5 µm particles could be found in the gills, gut, and liver whereas the 70 nm and 20 µm particles were not taken up (Lu et al., 2016). In this kind of study, it is important to realize that, with increased particle size, the number of particles decreases, for any given concentration of the plastic in the carrier vehicle (Wang et al., 2023).

A recent study in zebrafish larvae suggests that fluorescein-tagged 20 nm polystyrene NPs are able to cross the blood-brain barrier, and are subsequently accumulated in the brain itself, of the zebrafish larvae (Sökmen et al., 2020). The blood-brain barrier appears to develop in the normal zebrafish sometime between 2- and 10-days post fertilization, depending on the molecular size of the marker studied (Quiñonez-Silvero et al., 2020). The uptake, accumulation and transportation of MPs and NPs can have deleterious effects on the immune system, nervous system, and on metabolism in mammals (reviewed by (Yong et al., 2020). Another zebrafish study found that 5 µm polystyrene particles can induce inflammation and oxidative stress in the gut and changes in the metabolome and microbiome of the gut (Qiao et al., 2019).

Potential risks of MPs and NPs to humans

Relatively little has been published about the biological effects of small plastic particles on humans, not least because of the ethical difficulties in doing research on human subjects. Among their possible toxic effects on humans, are those affecting the embryo or fetus (reviewed by (Hougaard et al., 2015). Little is known about the possibility of placental transfer in any mammalian species, but one study used the BeWo cell line (which is used to model placental cells), and found that they could take up 50 nm polystyrene NPs (Dusza et al., 2022).

In addition, Ragusa *et al.* (2021) found a few fragments of MPs in human placentas including on the maternal side, the fetal side and in the chorioamniotic membrane (Ragusa et al., 2021). As a result, it has been speculated that MPs and NPs might be able to cause decreased birth weight, autoimmune lung disease, and a series of central nervous system abnormalities (Bates, 2019). Another recent study using the

perfused human placenta found that the NPs affect the gene expression related to inflammation and iron homeostasis (Chortarea et al., 2023).

MPs and NPs potentially cause effects on humans *via* various other exposure routes (Galloway, 2015; Powell et al., 2007). For example, MPs and NPs can be transferred to humans from food species (Chang et al., 2020). Thus, they have been widely found in fish, shellfish and shrimps (Garrido Gamarro et al., 2020; Li et al., 2015; Smith et al., 2018); in honey, salt and sugar (Liebezeit and Liebezeit, 2013; Yang et al., 2015); and in food and beverage packages such as teabags and soup cups (Du et al., 2020; Hernandez et al., 2019). Cox *et al.* revealed that people eat at least 50,000 MPs particles per year on average (Cox et al., 2019). Hence, there is at least the potential for humans to suffer harmful effects from exposure to MPs and NPs.

Even though the skin functions as a biological barrier, it is still another potential route of human exposure (Prata *et al.*, 2020) because MPs and NPs are commonly contained in personal products such as sun cream, toothpaste and shower gel (Sharma & Chatterjee, 2017) in many countries (except the EU, Canada and USA, where personal care products containing such plastic 'microbeads' are banned (Hernandez et al., 2017). It has been shown that other classes of nanomaterials, namely the so-called 'quantum dot' nanoparticles, can penetrate the skin of mice (Mortensen et al., 2008). There is evidence that 40 nm polystyrene NPs can enter through hair follicles in human skin (Vogt et al., 2006). Both 40 nm and 200 nm polystyrene NPs were found to be able to penetrate into mouse skin (Mahe et al., 2009). In addition, poly(l-lactide-co-glycolide) nanoparticles of 70 nm and 300 nm diameter were found to accumulate more, and penetrate deeper, in inflamed skin compared to healthy skin, in mouse and pig models (Try et al., 2016). Moreover, the degree of penetration of NPs into the skin was influenced by their size (Try et al., 2016).

The penetration and accumulation of NPs in skin can, in principle, result in the exposure of epidermal and dermal cells to the NPs. However, the epidermis does not

contain blood vessels or lymphatic vessels (Lund et al., 2016). Therefore, in order for the NPs to spread to other parts of the body, from the skin, they will have to cross the epidermal basement membrane and enter the blood vessels or lymphatic vessels in the dermis. Furthermore, it has been shown that exposure to NPs can lead to oxidative stress in human cerebral and epithelial cells *in vitro* (Schirinzi et al., 2017).

While it is difficult or impossible to perform the necessary experiments in humans with MPs and NPs, we can get insight from other warm-blooded vertebrates such as rats and birds (De Jong et al., 2008; Wang et al., 2023). We can also get indirect evidence about plastic nanoparticles from experiments with metal or other sorts of nanoparticles (De Jong et al., 2008; Yan et al., 2020; Yan et al., 2021), although great care should be used in extrapolating from one nanomaterial to the other. In summary, there is growing evidence that MPs and NPs might be a potential threat to human health.

The potential advantages of using chicken embryos in nanoplastic toxicity research

The aim of this thesis was to use a model closer to humans than the fish and invertebrates that have often been used for testing the toxicity of MPs and NPs. This model is the embryo of the chicken (*Gallus gallus*). Birds are warm-blooded, 'higher' vertebrates with a physiology quite similar to humans (Pozio, 2005). Birds share a most recent common ancestor with humans that lived approxinately 319 million years ago (Pardo et al., 2020; Rezania et al., 2018; Sánchez-Villagra, 2012; St John et al., 2012). By contrast, the most recent common ancestor of humans and the zebrafish (*Danio rerio*) lived 450 million years ago (Hedges and Kumar, 2009). Furthermore, the chicken embryo can be directly exposed experimentally to NPs *in ovo*, and then the egg returned to the incubator (Tickle, 1993). Such experiments are

not possible to perform in mammalian model species because of the placental barrier.

Other reasons for using the chicken embryo (often called the chick embryo) are that there is a genome sequenced and the chicken embryo has been for decades an important model in developmental and toxicology studies (Bryda, 2013; Davey and Tickle, 2007; Hamburger and Hamilton, 1951; Korhonen et al., 1982; Rashidi and Sottile, 2009; Romanoff, 1960). Another argument in favor of using the chick embryo model is that the chicken is a bird, and there is a need to understand the effects of pollutants on wild birds, as shown by Rachel Carson in *Silent Spring* (Carson, 1962).

Before hatching, the chicken embryo is not subject to the European Laws on animal welfare licensing (European Union (EU) directive no. 2010/63/EU). At Leiden University, we have chosen to use the embryos no further than day 14, which is ⅔ of the incubation period. Additionally, the chicken embryo is a relatively cost-effective model, and at the time of writing, in our laboratory, we buy fertilized chicken eggs costing approximately €1.00 each (including transport costs) from a commercial hatchery.

A standard series of embryonic stages has been described for the chicken (Hamburger and Hamilton, 1951) from laying to hatching (21 d). These stages allow for the standardization of research between laboratories. Duman *et al.* found that the chicken embryo model can be used to mimic human tissues and can be considered as a platform for the study of teratogen-induced malformations (Duman et al., 2019). For example, exposure of the human embryo to valproic acid (a teratogenic drug used to treat epilepsy) can harm the developing embryo, producing fetal valproate syndrome (Ornoy, 2009). The same exposure has also been shown to be teratogenic in chicken embryos, producing a pattern of defects similar to those in humans (Nanau and Neuman, 2013; Tanoshima et al., 2015). Thus, the chicken embryo is likely to act as a practical model with relevance to humans.

The chicken embryo model has been used for examining the effects of nanomaterials in a small number of studies (reviewed by (Ghimire et al., 2022). These include studies of nanoparticles made out of polystyrene (Nie et al., 2021; Wang et al., 2023), zinc oxide (Yan et al., 2020; Yan et al., 2021), titanium oxide (Patel et al., 2018), carbon (Kurantowicz et al., 2017; Samak et al., 2020), gold (Zielinska et al., 2011), platinum (Prasek et al., 2013), silver (Grodzik and Sawosz, 2006), magnetic iron oxide (Patel et al., 2019). We do not claim that the chicken embryo is a model for environmental exposure, nor do we make any claims about the uptake of particles into the bird egg in the field. We are simply using it as a convenient experimental model to unravel the cellular and molecular effects of nanoplastics in a warm-blooded, higher vertebrate model.

Outline of this thesis

The main objective of this thesis is to explore the developmental toxicity of nanoplastics and the underlying mechanisms of that toxicity. Another objective is to determine whether the chicken embryo a good model for ecotoxicology. My specific research questions are these:

- Do MPs and NPs cause developmental toxicity in chicken embryos?
- If so, what are the cellular and molecular mechanisms underlying the toxic effects?

I use a multi-technique approach in this doctoral work to: (i) unravel the effects of surface embryonic exposure to different concentration of 25 nm nanoplastics in the stage 8 chicken embryo using experimental embryological techniques; (ii) explore the teratogenic effects induced by nanoplastics in the chicken embryo using MicroCT and synchrotron x-ray tomography; (iii) determine the cellular and molecular mechanisms of toxicity of nanoplastics exposure by *in situ* hybridization in the chicken embryo.

In **Chapter 1**, I review the relevant research literature relating to nanoplastics, their origins and harmful effects, including their potential (but poorly understood) risks to humans. I also explore the literature justifying the my use of the chicken embryo as a model for this research.

In **Chapter 2**, I describe the research in which I exposed chick embryos to nanoplastics and looked at their dose-dependent and size-dependent developmental toxicity (and teratogenicity). I found that the the nanoplastics have a dose-dependent harmful effect on the neural tube, eye, tail and other organs. I could not make any conclusions about the possible size-depency of these effects because of confounding variables (surface area, number of particles) which also change with particle size.

Chapter 3 consists of a detailed analysis of the effects of polystyrene nanoparticles (PS-NPs) on the cardiovascular system of the chicken embryo. Using synchrotron scanning, I showed that 25 nm PS-NPs cause ventricular septal defects and abnormal numbers of aortic arches. Video recordings of live, exposed chicken embryos showed that PS-NPs caused bradycardia. Gene expression studies and immunochemistry showed that malformations in the cardiovascular system might be understood in terms of abnormal migration of the cardiac neural crest.

In **Chapter 4**, I conducted transcriptional profiling of neural crest marker-genes in early chick embryos. I found that exposure of chick embryos to 25 nm PS-NPs caused disrupted migration of the trunk and cranial neural crest. Using fluorescein-tagged PS-NPs I showed that the nanoparticles co-localised to the neural crest (dorsal midline of the neural tube). TUNEL staining showed that exposure to PS-NPs caused a wave of cell death in the dorsal cells of the embryo.

Chapter 5 presents a summary, discussion and perspective of my research. I conclude that PS-NPs pose a potential health risk because they bind to, and disrupt the development of, neural crest cells. I argue that more work is needed to understand the mechanism underlying these effects, and to map the biodistribution of PS-NPs in the body of animals and humans.

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