Nanomaterial safety for microbially-colonized hosts: Microbiota-mediated physisorption interactions and particle-specific toxicity

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Summary

The external tissues of plants and animals are colonized by microbes that collectively form the host-associated microbiota. Antimicrobial substances that reach microbially-colonized tissues, can kill certain members of the microbiota, including members that contribute to host health and development. This forms a concern for nanomaterials that consist of antimicrobial nanoparticles. Many of these particles have been found to disrupt microbiota-dependent biophysiological homeostasis, which raises questions about the safety of antimicrobial nanomaterials for microbially-colonized hosts.

In addition to the effects of nanoparticles on microbiota, microbes can also affect the physicochemical properties of nanoparticles, and can modulate the biophysiological responses of hosts to nanoparticles. The work presented in this thesis investigates this using a combination computational techniques and zebrafish larvae experiments, as introduced in **chapter 1**. This chapter first of all presents an overview of the growing body of work that demonstrates the effects of nanoparticles on host-associated microbiota. Additionally, the chapter describes what chemical transformations of nanoparticles have been found to be mediated by host-associated microbiota. Thereafter, two knowledge gaps are identified, which are investigated in this thesis. Firstly, physisorption interactions between colonizing microbes, their metabolites and nanomaterials are investigated in **chapter 2** and **chapter 3**. Secondly, the influence of colonizing microbes on particle-specific toxicity is quantified, and underlying mechanisms are investigated, in **chapter 4** and **chapter 5**. Combined, these chapters address the research question: '*What mechanisms govern the physisorption-driven and particle-specific effects of colonizing microbiota on nanomaterial toxicity to microbially-colonized hosts?*'

In **chapter 2** we investigate physisorption interactions between ingested nanomaterials and metabolites from enteric microbiota in the gastrointestinal tract. As a starting point for this investigation, we generated a concise overview from the literature of 170 unique enteric microbial metabolites that are produced, modified or regulated by enteric microbiota. Using quantitative structure-activity relationship (QSAR) models we subsequently predicted the adsorption affinity (log k value) of 60 of these metabolites to 13 metal nanomaterials, 5 carbon nanotubes and 1 fullerene. For a case study on four vitamins, a silicon dioxide nanomaterial, and multiwalled carbon nanotube, we moreover performed molecular dynamics (MD) simulations to obtain direct molecular information of these nano-bio interactions. Correlations between QSAR predictions and descriptors from the biological surface adsorption index indicated that hydrophobicity-driven interactions contribute most to the overall

adsorption affinity, while hydrogen-bond interactions and polarity/polarizability-driven interactions differentiate the affinity to metal and carbon nanomaterials. Unconstrained MD simulations provided excellent support for these main interaction types. Additionally, the simulations showed how large and flexible metabolites can gain stability on the nanomaterial surface via conformational changes. Combined, these results provide qualitative and quantitative insight into biologically relevant interactions that could occur between microbial metabolites and ingested nanomaterials in the gastrointestinal tract.

In **chapter 3** we zoom out to study physisorption interactions at the scale of microbes, focusing on the earliest life stages of zebrafish. In this chapter we test the hypothesis that the adsorption of antimicrobial titanium dioxide nanoparticles ($nTiO_2$) onto zebrafish eggs can harm the developing embryo by eradicating early colonizing microbiota. To assess the effectiveness of the eggs' membranes in preventing particle uptake, we first used two-photon microscopy to localize gold nanorods in and on exposed zebrafish eggs. Since no detectable amounts of particles crossed the protective membranes, we continued to explore the effects of adsorbed $nTiO_2$ to microbiota colonizing the zebrafish egg surface. Using particle-induced X-ray emission analysis, we inferred that the TiO_2 could cover 25–45 % of the zebrafish egg surface. Both imaging and culture-based microbial identification techniques revealed that particle adsorption resulted in an overall increase of microbial abundance, despite its antimicrobial effects. Pathogenic *Aeromonas* bacteria tolerated the antimicrobial properties of the nanoparticles. This formed a risk to the larvae that hatched from $nTiO_2$ exposed eggs, which also comprised higher microbial abundance, even without continued exposure to $nTiO_2$. This demonstrates that the adsorption of suspended antimicrobial nanoparticles onto aquatic eggs could facilitate the spatiotemporal dispersal of pathogenic bacteria in aquatic ecosystems.

In the next two chapters, we study the effects of colonizing microbiota on the particle-specific toxicity of nanomaterials. For this purpose, in **chapter 4** we optimize protocols for comparing the acute toxicity of nanomaterials to germ-free and microbially-colonized zebrafish larvae. By combining this methodology with the response addition model, we found that colonizing microbiota protect zebrafish larvae against the particle-specific toxicity of silver nanoparticles (nAg), but do not affect the acute toxicity of zinc oxide nanoparticles (nZnO) to zebrafish larvae. By isolating microbiota from zebrafish larvae at the end of the two day-exposure period, we additionally found that nAg eradicated most of the larvae-associated microbiota, while nZnO did not affect the abundance of larval microbiota significantly. These results show that, at least for certain nanomaterials, it is important to take host-microbe interactions into account when assessing toxic effects of nanoparticles to

microbially-colonized hosts.

In **chapter 5** we focus on the specific case of nAg toxicity to zebrafish larvae, to further dissect how members of colonizing microbiota can protect hosts against nanomaterial toxicity. Using an *il1ß*-reporter line, we characterized the accumulation and particle-specific inflammatory effects of nAg in the total body and intestinal tissues of the larvae. This showed that silver gradually accumulated in both the total body and intestinal tissue, yet specifically caused particle-specific inflammation on the skin of larvae. Subsequently, we used three mutant lines to assess if the recognition of microbiota by toll-like receptors (TLRs) contributes to the microbiota-dependent protection against nAg. Both a zebrafish mutant for TLR2, and a mutant for TLR2-adaptor protein TIRAP (Mal) were more sensitive to nAg than their wild type siblings under microbially-colonized conditions. In contrast, both of these mutants were equally sensitive to nAg as their wildtype siblings under germ-free conditions. Irrespective of the presence of microbiota, the sensitivity of a third mutant, for TLR2-adaptor protein MyD88, did not differ from that of its wildtype sibling. Combined, these results suggest that the recognition of microbiota by TLR2 protects zebrafish larvae against nAg toxicity via TIRAP-dependent downstream signaling. More generally, by differentiating between the effects of host-microbiota and microbe-particle interactions, the results of this chapter support the conclusion that host-microbiota interactions affect nanomaterial toxicity to zebrafish larvae.

In **chapter 6** we discuss how the results of this thesis can support microbiota-inclusive nanomaterial safety assessment for humans and the environment. Focusing on microbiota-mediated physisorption interactions, we explain under what circumstances microbial metabolites can particularly be hypothesized to affect exposure scenarios for nanomaterials via biocorona formation. We moreover discuss how test systems can account for the formation of heteroaggregates consisting of microbes and particles, potentially facilitating the dispersal of pathogenic microbes. Thereafter, we discuss implications of the obtained insight into the microbiota-dependent and particle-specific toxicity mechanism for nAg to three common nanosafety testing strategies. Firstly, with regard to the aim to predict chronic exposure outcomes, our results indicate that antimicrobial agents might sensitize hosts to nanomaterials due to the loss of protective microbiota. However, the immunomodulatory effects of other common environmental pollutants could mask this. Secondly, for grouping and read-across approaches, the results of this thesis suggest that enhanced *il1ß* expression could potentially serve as a marker for microbiota-dependent toxicity. If so, this could help predicting microbiota-dependent toxicity outcomes for nanomaterials. Thirdly, concerning cross-species extrapolation, the conservancy of microbiota functioning across hosts, rather than taxonomic microbiota composition, indicates that especially functional measures of

microbiota composition constitute promising targets for cross-host extrapolation. Altogether, I envision that the test approaches, results and insights obtained in this thesis can contribute to the safe and sustainable design of nanomaterials by supporting a transition to microbiota-inclusive nanomaterial safety assessment for humans and the environment.