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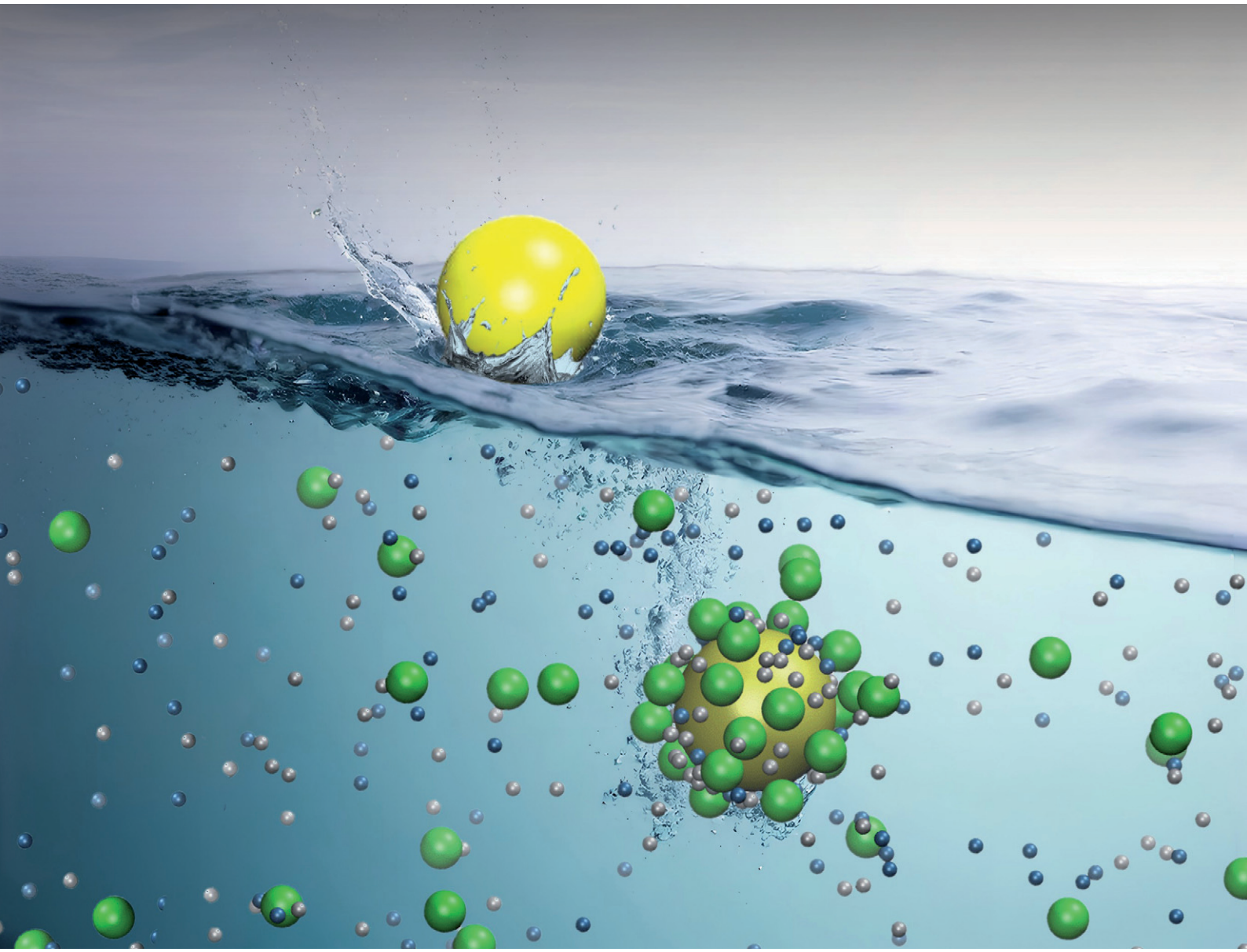

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CRITICAL REVIEW

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Understanding the role of biomolecular coronas in human exposure to nanomaterials

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Nanomaterials (NMs) are increasingly used in medical treatments, electronics, and food additives. However, nanosafety—the possible adverse effects of NMs on human health—is an area of active research. This review provides an overview of the influence of biomolecular coronas on NM transformation following various exposure routes. We discuss potential exposure pathways, including inhalation and ingestion, describing the physiology of exposure routes and emphasising the relevance of coronas in these environments. Additionally, we review other routes to NM exposure, such as synovial fluid, blood (translocation and injection), dermal and ocular exposure, as well as the dose and medium impact on NM interactions. We emphasize the need for an in-depth characterisation of coronas in different biological media, highlighting the need and opportunity to study lung and gastric fluids to understand NM behaviour and potential toxicity. Future research aims to predict better *in vivo* outcomes and address the complexities of NM interactions with biological systems.

Environmental significance

This critical review reports on the environmental implications that the human route of exposure have on nanomaterials (NMs) and highlights the important role of biomolecular coronas in shaping NM behaviour. As NPs are increasingly applied in many fields, including medicine, construction, and food, the concerns about their nanosafety are increasing. In this work, we review biomolecular coronas in diverse biological fluids, taking into account the exposure routes to NPs in order to introduce more detail. Recognizing inhalation and ingestion as primary exposure routes, we emphasize the need for an in-depth characterisation of coronas in different biological environments to achieve a thorough understanding of NM toxicity. This work contributes substantively to the field of environmental science by remarking on the interplay between nanoscale materials' behaviour and the role of biomolecular coronas to understand the impact on human health.

Introduction

During the last few decades, engineered nanoparticles (NPs), *i.e.*, materials smaller than 100 nm in at least one dimension, have received considerable attention because of their unique properties, such as their chemical reactivity, electrical conductivity, fluorescence, and magnetism.[1–5] Owing to the technological advancements in the last years, they have rapidly been incorporated in a wide range of products to improve the properties of the final products. The medical sector is the fastest growing NP market[6] where NPs have found application in both the diagnosis and therapy of several diseases.[7] Electronics is a big market, and technology is evolving at high speed owing to the properties of metals at the nanoscale.[8] Meanwhile, painting, coating, and construction industries have recently been driven by the introduction of nanomaterials (NMs) into existing products. This results in more hydrophobic or antimicrobial surfaces[9] or more durable and stronger concrete due to the addition of nanosilica and carbon nanofibers.[10] The global NM market size was valued at $11B in 2022 and is expected to grow by ~15% annually in the next decade, eventually surpassing the $50B market size.[6,11,12]

Because of their increasing use, human exposure to NMs has become a matter of concern as workers or end users could be indirectly exposed to them with unpredictable

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outcomes. Moreover, exposure to humans occurs following NM release into the environment during the life cycle of products.[13] During the last few years, safe and sustainable by design (SSbD) strategies have been developed to modify products and processes involving advanced NMs to reduce their potential for release or hazard potency without changing their technological properties.[14,15] In the same direction, a wide range of multicomponent NMs have been recently developed. These advanced hybrid materials are formed by two or more functional components such as organic molecules conjugated by molecular bonds to NPs or by a NM modified by hard or soft coatings.[16,17]

Unintentional NM exposure generally occurs *via* inhalation (during the manufacturing process or release), by ingestion, or even by dermal or ocular exposure, while intramuscular or intravenous injection is more common for intentional exposure to bio–nano materials with therapeutic effect (Fig. 1).[18] Moreover, the mechanisms associated with the bio-physicochemical interactions of NMs in humans are complex. In particular, the initial interactions between NMs and biological fluids are difficult to accurately model.[19] When dispersed in aqueous solutions containing electrolytes, NMs first interact with the ions present in the media, forming a thick solvation sheath that gives their hydrodynamic appearance. Likewise, in biological media, they are quickly and spontaneously covered by biomolecules, forming what is called biomolecular corona. While this paradigm initially attracted attention from the nanotoxicity context, it is now widely studied in the field of nanomedicine and environmental science.[20] Additionally, initial corona studies mostly focused on identifying the protein component. However, recent studies have revealed that the corona is also made of other metabolites,[21,22] such as lipids[23] and glycans,[24,25] and the concept is often referred to as the biomolecular corona.[19,26–28] The interaction between biomolecules and NMs is governed by many factors, including particle size, shape, surface chemistry, hydrophobicity and surface charge.[29–31] Additionally, affinity-based interactions of proteins towards the NM surface and protein-to-protein interactions are responsible for changes in the corona composition over time.[32] Accordingly, two stages define the formation mechanism of the biomolecular corona on a NP. Firstly, engineered NPs enter the biological medium and come into contact with biomolecules, which adsorb onto the NPs' surface within seconds, forming the initial corona.[33] Secondly, competition between proteins takes place, evolving the corona composition over time, until a stationary or slowly-evolving state is reached.[33] The biomolecular corona is usually divided into hard and soft corona, depending on factors such as the binding strength and rate of exchange of biomolecules from the surface of the NP. The hard corona (HC) is typically made of proteins directly adsorbed onto the NP's surface, which therefore exhibit long/constant exchange time with the surrounding molecules in the medium. On the contrary, soft corona (SC) proteins are known to be loosely attached to the NP surface, or most likely attached to an already formed hard corona *via* weak protein–protein interactions. Between them, the intermediate (or displacement) corona (IC) has been recently described as the transition layer made of biomolecules with moderate binding affinities and exchange rate with the NP surface compared to the HC and SC.[34] Consequently, the proteins of the HC are arranged in the inner layer of the corona, whereas the SC proteins are distributed in the upper layer of the corona. This makes them more dynamic and easily exchangeable over time with other biomolecules in the surrounding medium (Fig. 2).[35,36] This is the paradigm for inorganic NMs, which typically form robust coronas due to higher surface energy and charge density.[37] Recent studies have reported that the biomolecular corona is also formed in organic NMs and exosomes,[38–40] which in turn affects the biological fate. It is expected that the corona will have a different half-life and exchange on organic and inorganic NPs, mainly due to the difference in binding interactions. In fact, the presence of hydrophobic or hydrophilic groups dictates the type and amount of proteins that bind. Hydrophobic surfaces tend to bind more proteins, as has been observed in the case of both

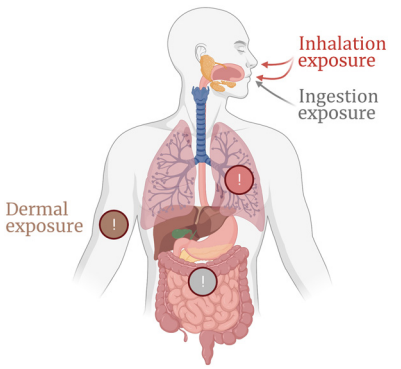

Fig. 1 Common routes of exposure to NMs. Schematic view of the human body with pathways of exposure to NMs, and the organs through which NMs can translocate to the bloodstream.

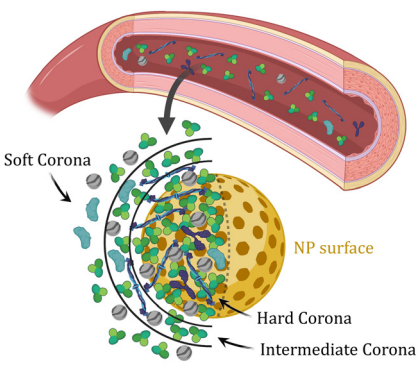

Fig. 2 Biomolecular corona structure on top of NPs. Representative picture of corona formation onto a mesoporous silica NP when exposed to biological fluids such as blood plasma. Different proteins may adsorb to the surface of the NP, depending on several factors such as NP surface chemistry and buffer pH.

organic and inorganic NMs.[41] Moreover, the addition of surface functional groups, ligands or linkers – especially those providing steric hindrance or specific chemical functionalities – can tailor protein binding, usually reducing the amount of biomolecules adsorbed and influencing the overall composition and behavior of the corona.[42–44] Charge also plays a crucial role, with positively charged NPs generally attracting more proteins due to strong electrostatic interactions.[41] Therefore, more comparative research is needed to take all these factors into consideration and closely study the potential differences in the formation of the biomolecular corona between inorganic and organic NMs.

The interaction between NMs and biomolecules can alter their properties and thus influence protein function, but also the NMs' fate *in vitro* and *in vivo*. For instance, binding proteins to NPs may induce structural changes,[45] potentially exposing new epitopes. Epitope mapping has been a valuable approach to investigating these newly exposed epitopes.[46,47] Research in the field has provided insights into the protein structural alterations caused by the adsorption to NPs,[48,49] such as the NP-induced unfolding of fibrinogen promoting Mac-1 receptor activation and inflammation.[50] In some occasions, this protein structural change has been reported to be as big as losing its enzymatic activity, disturbing biological processes, and even accelerating pathogenic events.[51] On the other hand, the adsorption of proteins onto the NP surface can alter the colloidal characteristics of NPs, including their aggregation characteristics and/or hydrodynamic diameter, which may impact the cellular response to the NPs exposure, leading to accumulation, toxicity, and clearance.[52]

The colloidal characteristics of NMs are significantly influenced by the type of biological fluid, its composition, and the concentrations of biomolecules.[53] The formation of a biomolecular corona on the surface of NPs plays a critical role in modulating their interactions with biological systems.[35] In fact, this corona can attenuate the surface energy of the NPs, potentially reducing their toxicity. For example, studies have shown that pristine NPs may disrupt cellular membranes and induce necrosis, whereas NPs coated with a biomolecular corona do not exhibit this effect.[54,55] Additionally, the corona can delay the dissolution of NMs, further mitigating their toxic impact.[55,56] Therefore, the risk assessment of such NPs must evaluate their colloidal properties in various biological fluids by exploring different routes of exposure to NMs. Common routes of exposure include inhalation (lungs) or ingestion (gut), and each could potentially lead to different biodistribution and translocation into different organs, resulting in different outcomes.[57] For instance, inhaled NMs can be deposited deep into the lungs where they could induce oxidative stress and promote inflammation,[58,59] or travel into the bloodstream and accumulate at inflamed vascular sites.[60] A potential link between diesel exhaust NMs and cardiovascular damage has been recently reported, based on the induction of oxidative stress *via* the NRF-2 pathway.[61,62] Moreover, particles suspended in the air are also capable of adsorbing some compounds. One example is bacterial lipopolysaccharide (LPS), a strong pro-inflammatory agent commonly found as an environmental contaminant in some parts of the body like the gut. It was observed that the co-incubation of LPS with TiO_2 NPs significantly increases its pro-inflammatory impact on mouse macrophages, suggesting that LPS's activity is amplified when forming part of a NP bio-corona.[63] The concept of an ecological corona or eco-corona arises in this context to understand the nanoparticle interactions within biological and environmental systems.[64] In contrast to the biomolecular corona, the eco-corona forms in environmental settings, like water, soil, or air, and is composed of natural organic matter, extracellular polymeric substances, and humic substances. This pre-formed eco-corona also influences the nanoparticles' behaviour, bioavailability, and potential effects on human health and ecosystems.[65] For instance, proteins released by *Daphnia magna* create an eco-corona around polystyrene NPs, which causes heightened uptake of the NPs and increases toxicity.[66] Recent studies have emphasised the significance of eco-corona formation in determining the fate and ecotoxicity of NMs in aquatic environments.[67,68] Understanding the eco-corona is therefore important to assess the environmental and health risks associated with NP exposure, as well as the associated molecular mechanisms.[69] Other examples contributing to this "exposome"[70] include the release of NMs from medically implanted devices such as the leaching of NMs from hip implants or dental composites, which may have a long-term toxic impact on patients.[71] Thus, for a proper assessment of the engineered NP hazard, it is necessary to focus on all body systems that are potential targets for NMs.

In this context, this review summarizes the latest results on the specific mechanisms of NP interaction with different biological systems. The biological identity of the NMs depends on their surrounding environment. Therefore, the main routes of human exposure are thoroughly explained throughout the text. Moreover, the subsequent implications of embedding different NMs in these environments are reviewed in detail, highlighting the impact of the exposure route on the biomolecular corona, as well as the recent advancements and challenges in nanosafety.

NM exposure through inhalation

Physiology of the exposure route

The respiratory system, which is the main route of unintentional exposure to NMs, encompasses various parts, including the nasal cavity, pharynx, larynx, trachea, bronchi, bronchioles, and alveoli.[72] As shown in Fig. 3, the conduction airways of the lung (bronchus and bronchiole) and the epithelium tissues are protected by a viscous layer of mucus (mainly composed of glycoproteins), which makes them a relatively efficient barrier where particles and microbes are retained by the mucus and transported back to the oral cavity

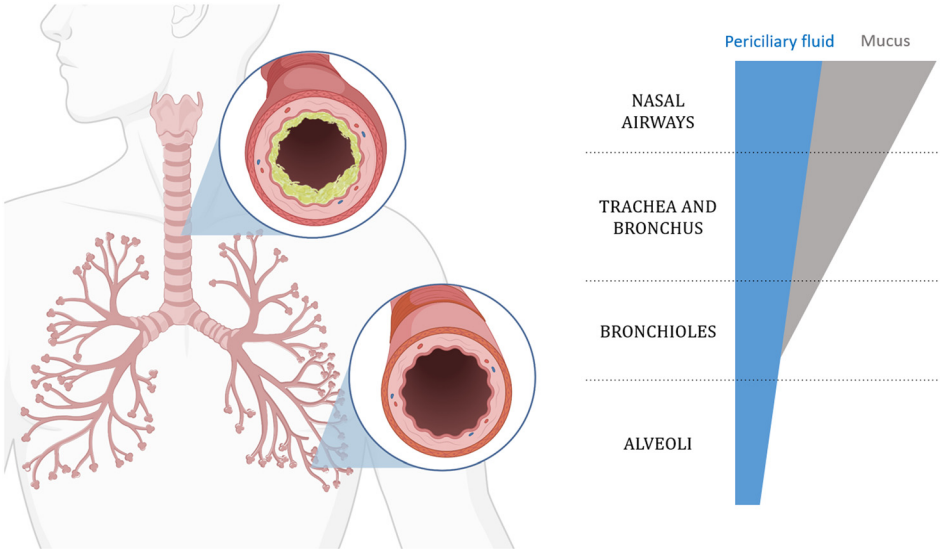

Fig. 3 Different parts of the respiratory system and their fluid composition. As represented, the composition of the different components changes along the respiratory airways. On the right, a schematic of the component changes occurring in the LLF that are present in different regions of the respiratory tract, from the nasal airways to the alveoli. In the upper airways, the LLF is composed of two layers: a periciliary watery sol layer covered by a viscous mucous layer, which decreases its presence until disappearing in the bronchoalveolar region.

by the cilia located on the lung epithelial cells. The alveoli are responsible for the exchange of oxygen and carbon dioxide in the blood, and they have a simple squamous epithelium tissue with large surface area and intense air–blood contact compared to conduction airways.[73] In fact, studies that utilised airway casting – a method for creating detailed 3D models of lung structures – reported an approximate alveolar surface area of 8900 m^2, which is significantly higher than the 1.78 m^2 reported for the nasopharyngeal regions.[74] This makes the alveoli more exposed to airborne pathogens and pollutants, a common target of particles and one of the most permeable barriers to blood circulation. For NMs to come into contact with epithelium tissues, they need to either penetrate the mucus barrier or move down in the direction of the inhaled air to the alveolar region, and encounter the last thin liquid amphipathic film composed of surfactants.[75] The alveolar epithelium is structurally simple to ensure a high surface area for oxygen/carbon dioxide exchange into/from the circulatory system. The surface tension at the air/liquid interface is controlled to prevent the collapse of the alveoli, and it is maintained by the presence of biomolecules, such as surfactants, proteins, and phospholipids. The lung lining fluid also exerts functions in protection against pathogens' translocation into the bloodstream.[76]

Lung fluids have been extensively simulated to reproduce the characteristics of biological lung fluids. A wide range of artificial respiratory tract lining fluids (RTLFs), also known as lung lining fluids (LLFs), have been developed to emulate the biological fluid that overlays the underlying epithelial cells.[77] The lungs comprise different cell types, such as epithelial, endothelial, and inflammatory, all having an impact on the composition of the LLFs. The components they secrete lead to significantly varying compositions along all of the respiratory airways. In the upper airways, it is particularly enriched in mucus, which is made of lipids, glycoproteins, and salts, and is the primary barrier for the clearance of bacteria. However, it was also shown to be effective in the retention of inhaled NMs.[78] Moving from the nasal cavity to the inner parts of the lung, the diameter of the airways decreases and the mucus layer thickness decreases, as shown in Fig. 3. When the alveolar region is reached, no mucus is present anymore and it is replaced by surfactants secreted from type II pneumocytes, which lower the surface tension and prevent alveolar collapse.[78]

However, the complexity and heterogeneity along the respiratory tract of the lung present a major challenge. To obtain lung fluids, the usual procedure is to perform a bronchoalveolar lavage (BAL), first used to treat several airway diseases by pumping out the LLFs. This medical procedure involves the introduction of saline fluid into the lung airways, followed by the collection of this same fluid, along with any biomolecules present in the airways using suction.[79] This practice, despite being invasive, continues to be a common clinical diagnosis tool that is able to monitor diverse airway disorders. However, its main drawback is the loss of the regional composition of the different parts of the lung while pumping out the lung fluids, as well as the alteration of the salt concentration, as most BAL procedures consist of the previous introduction of large volume aliquots of saline solution.[80,81] These drawbacks lead to the complexity of characterising the biological fluid, as there is still not enough data and techniques able to track how this change in the concentration and composition of substances occurs along the respiratory system.[82]

Several studies have been focused on understanding the NM behaviour after inhalation, and the development of simulant LLF has become largely studied. The addition of various components to mimic the LLF holds critical significance in physiological contexts. Salts play a pivotal role by ensuring proper protein folding, crucial for maintaining biological functions. In this context, buffers, such as phosphate-buffered saline (PBS), are essential to keep the correct pH and buffering capacity, creating a physiologically stable environment for protein stability and cellular processes.[83] Proteins in the LLF have a dual role, as they not only contribute to the protective layer but also participate in immune responses, safeguarding against pathogens.[84,85] Additionally, lipids emerge as fundamental constituents, particularly as pulmonary surfactants, where they are vital for lowering surface tension, preventing alveolar collapse at the air–liquid interface, and enabling efficient gas exchange.[86] In regards to simulating the composition of the LLF, two of the most established protocols were developed decades ago: Gamble's and Hatch's solutions. Gamble's solution was first developed with compositions similar to the extracellular fluid in the skeletal muscle.[87] The basic components are cations (magnesium, sodium, calcium, and potassium) and anions (proteins, bicarbonate, sulphate, organic acids, chloride, and phosphate), as well as some non-electrolytes such as glucose, reaching a pH of approximately 7.4.[88] Lately, the original Gamble's solution has been modified to add proteins, amino acids and phospholipids constituting lung surfactants, such as dipalmitoylphosphatidylcholine (DPPC).[89–91] The other widely established method to simulate the LLF is Hatch's solution. In this protocol, the composition of the mucous layer of the respiratory tract is considered, and the simulated lung fluid contains a high number of proteins, enzymes, lung surfactants, and complex organic molecules.[92] Recently, Kumar *et al.* have also included lipids when building a biocompatible synthetic lung fluid based on human LLF composition.[93]

Although these simulated fluids do not have the protein complexity of their biological counterpart, as long as the main BAL corona biomolecules are present in the LLF corona, the approach will be appropriate to evaluate and study the impact of lung fluids on the corona composition of inhaled NPs and their possible translocation in blood.[94] Another approach to recreate the LLF complexity is using the secreted media from an *in vitro* 3D cell culture of human lung epithelial cells as a representative fluid.[95] After the particles have been inhaled into the lungs, the resulting corona is mainly formed by phospholipids and proteins and is fundamental to reproducing the biological fluid impact on the NM.[96] It has been recently reported to allow the particles to be phagocytised and expelled from the lung, but might also be used as a target for anticancer drugs to increase their antiproliferative effect.[97,98]

Bio–nano interactions

Inhalation of NMs *via* the respiratory system represents an important route of human exposure. In addition to the major risk of exposure in occupational settings, anthropogenic atmospheric pollution coming from vehicle exhausts, industrial emissions, ore extraction, and energy production has widened the risk exposure to the greater population.[99] In some cases, exposure to NMs can be intentional for medical purposes, such as with colloidal silver sprays, which have been recently used to treat conditions like chronic rhinosinusitis.[100] Depending on the NM characteristics, the particles can become deposited into the different structures of the human lungs if they are not cleared from the upper airways. This can potentially lead to chronic inflammation, epithelial injury, and further pulmonary diseases, especially fibrosis.[101] The particle deposition in the lung may occur through five different mechanisms: sedimentation (gravity), inertial impaction, interception (particle–surface contact), electrostatic deposition, and diffusion.[102] Many factors can affect the deposition of NMs, including properties such as size, surface charge or hydrophilicity, aggregation state after the interaction with the LLF, concentration, and surface reactivity, as well as physiological characteristics of the respiratory system, such as air volume, breathing frequency, and flow rate (Fig. 4).[103–105] Soliman *et al.* investigated the effects of exposing iron oxide NPs (IONPs) to SmallAir-HF™ cultures and MucilAir-HF™ cultures. These cultures represent models of two different anatomical sites: large bronchial airways for MucilAir-HF™ and small airways for SmallAir-HF™. During the incubation time, particle aggregation was observed. This is most likely because they became trapped in the viscous mucus.[95]

Several studies also assessed the effects of inhaled NPs' properties *in vitro* (using either diffusion chambers or more

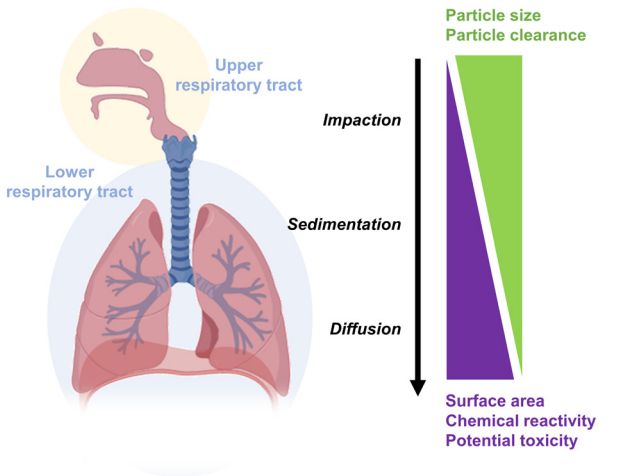

Fig. 4 The interaction of particles with the human respiratory tract. At the left, a scheme of the respiratory system with the upper and lower respiratory tract. At the right, the different interactions between the particles and the respiratory tract, depending on the particle characteristics.

advanced devices in static or dynamic exposure) and *in vivo* (through whole-body exposure, nose/head-only exposure, or lung-only exposure).[106] For instance, it was reported that the neutral small particles can penetrate the mucus layer better than charged large particles, which will most likely be trapped and removed by the cilia.[107,108] The *in vitro* effects of silica NPs (SiO_2 NPs) with different sizes (10, 150, and 500 nm at concentrations of 50, 100, and 100 μg mL^{-1}, respectively) were investigated in human lung submucosal cells (Calu-3). Results showed that 10 nm SiO_2 NPs induced ROS production, lipid peroxidation, and loss of viability, but no toxic effect was detected in the case of 150 and 500 nm. They also observed limited bioavailability of both fisetin and catalase to prevent toxicity as a result of biomolecular corona formation.[109] Another factor that can enhance the particles' toxicity is the adsorption of surfactants on the NPs' surface, as reported by Kasper *et al.* This *in vitro* study investigated the effect of different surface charges of amorphous SiO_2 NPs (unmodified, negatively or positively charged modified) on lung toxicity in the presence and absence of lung surfactants – *i.e.*, the phospholipid fraction from bovine lung extracts, which is used for the treatment of respiratory distress syndrome (RDS) in neonates.[110] The alveolar epithelial cell line A549 was used in both mono- and co-culture with the microvascular cell line ISO-HAS-1. As a result, high toxicity was detected in the presence of surfactants with unmodified or positively charged NPs. The increased cytotoxicity is believed to result from the formation of the reactive silanol groups (Si–O–H) in the presence of surfactants. In other words, the biomolecular corona formed in surfactant-containing media becomes less biocompatible, with its composition varying based on the surface properties of the NPs.

Investigation of the lipid and protein corona composition after exposure to pulmonary surfactants from porcine lung lavage was reported by Raesch *et al.*[23] The study was performed for three different NP coatings: phosphatidylcholine coating (lipid-NP), PEG5000 coating (PEG-NP), and poly(lactic-*co*-glycolic acid) coating (PLGA-NP). It was found that the amount of lipids adsorbed was dependent on the NPs' surface properties. In particular, the highest amount of lipids was detected for lipid-NPs, while the smallest amount was detected for PEG-NPs. However, the total lipid composition did not show any relevant differences in both coronas, in contrast to the changes observed in the protein composition. These results indicate that the corona might be of great importance for further interactions with the lung cells, especially in the alveolar region, since some proteins induce agglomeration of NPs, which in turn favours phagocytosis by macrophages and/or protein-mediated binding for particle internalisation. Gasser *et al.* also used porcine lung lavage to understand how the presence of pulmonary surfactant influences the adsorption of biomolecules to multi-walled carbon nanotubes (MWCNTs) and MWCNTs functionalised with positively ($-NH_2$) and negatively (–COOH) charged side groups.[111] They showed that surfactant lipids exhibit nonspecific binding to different functionalized MWCNTs. This behavior contrasts with the selective and characteristic binding patterns observed in the blood plasma proteins during corona formation. In later research, they used the same particles to demonstrate that the pulmonary surfactant coating of the nanotubes decreases their oxidative and pro-inflammatory potential, highlighting the relevance of the corona. Moreover, this surfactant coating not only affects how NMs interact with lung cells, but also affects their biodistribution and toxicity in secondary target organs.[112] *In vivo* studies also support the latter findings. For instance, it was reported that large NPs (>5 μm) can be trapped in the upper airway, while small NPs of 1–5 μm can enter the lower airway and reach the alveoli, within the deepest region of the lungs.[106,113,114] Surface activity/treatment of titanium dioxide (TiO_2) NPs by alumina or amorphous silica showed mild toxicity *in vivo* (lung inhalation) compared to reference NPs (without surface treatment).[115] Similar results were obtained in a case of whole-body inhalation by TiO_2 NPs in terms of their moderate toxicity.[116] Regardless of the exposure mechanism, the toxicity of inhaled particles could be attributed to the formation of a NP corona in LLF and their accumulation/retention in the lungs for a long time. Reasons for the prolonged retention include deep penetration into the mucus and a slow clearance mechanism, prolonging interaction with the lung cells. However, the retention time could differ depending on the particle size, as reported by Kreyling *et al.*[117] It was found that gold NPs (AuNPs) with a core diameter of either 5, 18, 80, or 200 nm had longer retention times than the NPs with core sizes of 1.4 and 2.8 nm. This short retention time for the two smallest particles is because they can cross the air–blood barrier more easily. Other studies have indicated that the translocation of NPs from the lungs to extrapulmonary compartments is also governed by their size.[118,119] For instance, organic and inorganic NPs with a hydrodynamic size of ≥34 nm demonstrated rapid translocation from the lungs to the mediastinal lymph node, regardless of their chemical composition, shape, or conformational flexibility. In contrast, for NPs smaller than this threshold (34 nm), surface chemistry becomes a critical factor in facilitating their rapid translocation from the lungs to regional lymph nodes. This is probably due to the formation of different coronas.[119] In the case of colloidal silver nasal sprays, despite having no reported adverse health effects on humans,[120] recent studies have pointed out that the administration of silver nasal spray leads to NP accumulation in rat brain tissues.[121]

From a toxicological point of view, both positively and negatively charged small NPs (<34 nm) have the potential to translocate into the bloodstream and thereby have a direct impact on cells and molecules in the circulatory system, as well as reaching other organs.[119] To our knowledge, no study has been reported to evaluate the specific effect of lung mucus on the physico-chemical properties of NPs following exposure to it, which is definitely of interest. Additionally,

inhaled NPs have the potential to be adsorbed through the olfactory epithelium, transported alongside the olfactory bulb, and reach the brain. For instance, following exposure to Al_2O_3 NPs at a concentration of 0.5 mg m^{-3} for 28 consecutive days, levels were found to be significantly increased in the cortex of mice.[122] A study by Oberdörster *et al.* reported the direct transfer of radiolabelled nanoparticulate carbon from the nose of rats into the brain.[118] Following transportation, NP deposition and interaction with brain tissue can take place.

The interaction between NMs and proteins might affect their physiological functions through conformational changes, as well as through either inhibiting or enhancing enzymatic activities. Shim and colleagues analysed the interactions between NPs and proteins from brain homogenates —a mixture created by thoroughly blending brain tissue. They later identified the adsorbed biomolecular corona from this solution. Two different sizes (20 and 100 nm) of SiO_2 NPs with two different surface charges (positive and negative) were used and incubated with brain homogenate collected from adult rats. The mixture was incubated for 1 h at 37 °C. As a result, it was found that a greater number of proteins (>140) were bound onto the positively charged 20 nm SiO_2 NPs than onto their negative counterparts in brain homogenate, while almost the same number of proteins were adsorbed to the 100 nm NPs regardless of their surface charge. This could be attributed to a larger surface area/volume ratio of the 20 nm NPs compared to the 100 nm ones, which allowed for greater numbers of proteins to bind onto the surface. In addition, it was found that different proteins involved in different physiological pathways, including the acetyl-CoA metabolic process, endocytosis, protein folding, glycolysis, energy-coupled proton transport, protein polymerisation, regulation of neurotransmitters, blood coagulation, and the acute inflammatory response, were attached to the NPs' surface.[123] This indicates that the biomolecular corona may play a key role in the NMs' interactions with cells for endocytosis and their involvement in tissue, leading to the overall toxicity or beneficial effects to an organism. Furthermore, a proportion of inhaled NMs can be mobilised up the trachea *via* the mucociliary escalator and thus reach the gastrointestinal tract (GIT), which will be discussed in the following section.

Despite the lung has been considered for long a sterile organ, it was found that the trachea and the bronchial tree can contain more than 2000 organisms per cm^2 surface (calculated as the number of bacterial/fungal/viral genomes).[124] The alveolar region is characterised by a thin layer of squamous epithelial cells, Alveolar Type I Epithelial Cells, that are covered by a thin layer of alveolar surfactant, which is secreted by alveolar type II cells and contains free fatty acids, phospholipoproteins, phosphatidylcholine-containing lipids and surfactant proteins. It has been shown that free fatty acids have antibacterial activity against Gram-positive bacteria, while Surfactant Proteins A and D exert bacteriostatic action against Gram-negative bacteria, increasing the permeability of the microbial membrane.[125,126] The lung microbiota is complex, and varies in composition between healthy and diseased conditions. Its functions include immune-modulation, support to nutrient uptake, and protection against other airborne pathogens.[127]

Most nanotoxicological studies have focused on the potential for NMs to induce respiratory inflammation. However, little is known about the direct effects that inhaled NMs have on the lung microbiota, and how this can affect the entire body.[128] Inflammatory processes, such as those potentially induced by inhaled NMs, can lead to increased alveolar permeability, which in turn can increase the local nutrient levels, alter the growth conditions, and favour bacterial adhesion. Once in the lung, NMs can modulate the body's immune response, leading to the recruitment of additional immune cells to the site of inflammation. This can lead to disruption of the microbiota homeostasis. Alternatively, NMs can directly affect the resident bacterial population, *i.e.*, by releasing bactericidal ions (*e.g.*, Ag or Zn ions), inducing oxidative stress (*e.g.*, nano TiO_2), or the bound NMs can induce rupture of the bacterial cell. The resulting imbalance in the microbial population could potentially lead to the proliferation of other, potentially pathological strains.

NM exposure through ingestion

Physiology of the exposure route

Ingestion is an important route of exposure for NMs since they are intentionally present as food additives,[129] contained in pharmaceutical and cosmetic products that can be accidentally ingested,[130] and unintentionally present in food from materials that are in contact with it.[131] Moreover, NMs ingestion might be accidental, or it can follow inhalation, since NMs can reach the gastrointestinal tract *via* the mucociliary escalator.[132] The mucociliary escalator is a key defence mechanism in the respiratory system based on the coordinated movement of cilia from cells in the respiratory tract to push mucus that traps particles and pathogens up toward the throat. This mucus can then be swallowed or expelled, helping to protect the lungs from infections and clear inhaled particles.[133] Despite its relevance, the number of studies assessing the hazard of ingested NMs is still relatively low if compared to inhaled NMs. One of the reasons can be found in the poor availability of *in vitro* models able to simulate the complexity of the gastrointestinal apparatus. This apparatus, whose main functions are motility, secretion, and absorption, is composed of accessory organs and the alimentary canal or oral-gastro-intestinal tract (OGIT).[132,134] The accessory organs are salivary glands, liver, gallbladder, and pancreas, which produce the secretions that comprise digestive fluids.[135] The alimentary canal consists of the mouth, oesophagus, stomach, and intestine. It is responsible for the motility and digestion of food, as well as nutrient absorption. This is possible owing to the mucosal barrier, having a surface area and length of approximately 200 m^2

and 5 m in adult humans, respectively.[135] All areas of the OGIT are mechanically protected by a layer of mucus of variable thickness and composition that is produced by specialised OGI epithelial cells (*i.e.*, goblet cells).[132,136] This mucus layer represents the first barrier through which ingested substances, including NMs, must diffuse and pass before coming into contact with the OGI epithelial cells. To add more complexity, a heterogeneous community of microorganisms colonise the OGI tract. The gut microbiota is formed by more than 10^{14} symbiotic microbial species, and it is known to play a major role in human health and disease.[137]

The mouth and stomach are responsible for the breakdown and mixing of food, and the initial partial digestion of lipids, starches, and proteins.[138,139] Meanwhile, the intestine is the main organ responsible for the final digestion and absorption of nutrients and water. Water reabsorption occurs in the large intestine (*i.e.*, colon, rectum), while the other nutrients are absorbed in the small intestine (*i.e.*, duodenum, jejunum, and ileum) after being digested by pancreatic enzymes.[139] To ensure efficient absorption of nutrients, the intestinal wall of this anatomical portion presents particular structures, such as folds, villi, and microvilli, which increase the available area for absorption.[139] The units making up the villi structures are specialised cells that are divided into 5 different types: enterocytes, which is responsible for absorption, goblet cells that produce mucus, enteroendocrine cells that are responsible for the hormone production, Paneth cells that maintain intestinal homeostasis in different ways, and microfold cells (M-cells) that phagocytise large foreign particles and present antigens to the immune system.[140] After the mucous layer, epithelial cells represent the second physical barrier of the intestine. Epithelial cells are connected by an intercellular junctional system represented by tight junctions (TJs), adherent junctions (AJs), and desmosomes.[140] They regulate the absorption of nutrients, and prevent the passage of bacteria and other particles throughout the epithelial barrier.[141] Altogether, epithelial cells maintain the integrity of the epithelium by forming strong adhesive bonds.[140,142] AJs are located below TJs and contribute to their assembly, while TJs are the most apical and adhesive junctions that strongly seal the intercellular space. Several proteins and biomolecules are involved in their formation: transmembrane proteins, such as claudins and occludin; peripheral membrane proteins like zonula occludens (ZO) proteins, specifically ZO-1 and ZO-2; and junctional adhesion molecules (JAMs),[140,142] in which ZO-1 acts as a scaffold for the others proteins.[143,144] TJs and AJs are directly connected to the cytoskeleton, and can be regulated *via* actin and myosin.[140,142] Owing to TJs thickness (less than 0.4 nm), just water and solutes can pass through the paracellular route[145] in the case of an intact barrier.[146–150]

To avoid the diffusion of pathogens and other dangerous particles or molecules, the epithelial barrier is linked to a competent lymphoid tissue called the gut-associated lymphoid tissue (GALT). GALT includes Payer's patches, mesenteric lymph nodes, and competent immune cells present in both epithelium and lamina propria.[151] The main components are dendritic cells (DCs) that transport the antigens to mesenteric lymph nodes[152,153] and stimulate T-lymphocytes,[154,155] macrophages that present antigens to T-cells[156,157] and contribute to maintaining the immune tolerance,[158,159] B-lymphocytes that produce antibodies and cytokines[160] and activate T-cells,[161] and T-lymphocytes that are the effectors of the immune response.[151] This specialised tissue is indirectly in communication with the intestinal lumen *via* M-cells, which can phagocytise luminal antigens (including bacteria, viruses, fungi, toxins, prions), and transport them to the GALT components by transcytosis.[162–164] The microbiota also plays a role in gut immune function, contributing to the maintenance of immune tolerance and promotion of intestinal homeostasis by producing molecules such as short-chain fatty acids.[165,166]

Simulated digestion systems are essential tools for studying the exposure to NMs through ingestion. At the moment, there are no validated models for NMs. This is mainly due to the difficulty in mimicking the different variables of the OGIT. Initial studies, such as those described in Minekus *et al.*,[168] settled the groundwork for creating these artificial fluids, focusing on the non-fasted state to better mimic the physiological conditions during food ingestion. These efforts led to the development of the INFOGEST protocol, a standardised and comprehensive approach widely adopted for simulating gastrointestinal conditions.[169] Published in 2019, the INFOGEST protocol represents a significant advancement by offering a more accurate representation of digestive processes. It incorporates various physiological parameters, such as enzyme activities and pH levels typically observed postprandially, and fixes the ratio between the ingested amount (bolus), simulated saliva fluid, simulated gastric fluid, and simulated intestinal fluid, ensuring consistency and reproducibility across studies. Other protocols, like those used by Sohal *et al.*[170] and Antonello *et al.*,[171] focus on the fasted state, which is crucial as well for different types of digestion studies. These protocols address specific research needs and complement the INFOGEST guidelines by providing alternative conditions under which the biomolecular corona of NMs might exhibit different physicochemical and biomolecular characteristics.

Bio–nano interactions

Upon reaching the gut and being internalised by cells, NMs can have various fates, *i.e.*, i) secretion back into surroundings; ii) accumulation in the cells; iii) degradation in the cell; and iv) gain access to the blood and other organs.[134] Therefore, ingested NMs may induce both local and systemic effects. The barrier crossing can occur *via* a transcellular route mediated by M-cells or epithelial cells.[172–176] An example can be found for polystyrene NPs that were found to be able to cross an *in vitro* epithelial barrier

model (Caco-2 cells) cultured both on porous inserts and on an organ-on-chip device.[177] Alternatively, barrier crossing may occur *via* a paracellular route if the barrier's integrity is perturbed. For example, some NPs have been reported to interact with cytoskeleton proteins, leading to the disruption of TJs.[149,178] It is thus clear that the set-up of *in vitro* models able to reproduce the complexity of the gut and to monitor the different possible effects of NMs is not straightforward. During the transit in the OGIT, NPs experience conditions that can dramatically modify their bio-identity, such as a pH ranging from 2 to 8, strong ionic strength, interaction with mucus, and several proteins (including enzymes). Consequently, dissolution, enzymatic degradation, aggregation, and surface modifications, comprising corona formation, may occur depending upon the chemical nature of the NMs, thus affecting the absorption and biological response, including the uptake rate, uptake pathways, and toxicity (Fig. 5).[170,179,180]

The dissolution of NMs after the exposure to biological fluids is a widely studied process. For inorganic NMs, the solubility and dissolution rate determine their bio-durability (in turn, affecting their bio-persistence)[132,170] in the biological systems.[181] On the other hand, the solubility and dissolution rates may increase the hazard of NMs when the released species are toxic. Dissolution is less important for organic NMs. In this case, enzymatic degradation is the most relevant process affecting their bio-persistence. For example, the enzyme-mediated partial degradation of lipid NMs was shown by Antonello *et al.*[171] Interestingly, because of the extreme conditions of the OGIT fluids, NMs might be formed following precipitation processes. For example, following the exposure of an intestinal model to $AgNO_3$, NMs were detected in the cell fraction.[182,183] Similarly, hydroxyapatite NMs dissolved in simulated gastric fluid were found to form novel NMs in simulated intestinal fluid.[171] Particle agglomeration and aggregation are highly probable in the OGIT. This is due to the high ionic strength of the fluids and the presence of the glycoprotein mucin. This process is known to modify the bioactivity of NMs. Particle size can actually modulate the uptake of NMs and their ability to cross biological barriers, including the intestinal one. For example, a study reported by Jani *et al.*[184] showed much more absorption in the colon mucosa for 50 nm polystyrene NPs compared to 100 nm ones, while 300 nm NPs were not absorbed. Additionally, 100 nm particles remained within the submucosa (deeper connective tissue) of OGIT, while 50 nm NPs entered the bloodstream and accumulated in the liver and spleen. This is because small particles can more easily diffuse through the pores of the mucus network, and gain more access to the systemic circulation.

The NMs dissolution and agglomeration/aggregation were reported in a previous study by Sohal *et al.* In this study, a series of inorganic particles (TiO_2, Fe_2O_3, ZnO, and SiO_2) with different size ranges were selected from commercial food products, and analysed for their dissolution and agglomeration using an *in vitro* system simulating the OGIT (saliva, gastric, and intestinal).[170] Upon incubation in simulated saliva fluid, SiO_2 and ZnO were shown to have a lower biodurability and persistence in comparison to TiO_2 and Fe_2O_3. No significant changes in agglomerate size were observed for TiO_2, ZnO, and SiO_2 in simulated saliva fluid. However, a large agglomeration was detected in the case of Fe_2O_3 when evaluated by DLS and TRPS (tuneable resistive pulse sensing). Dissolution can be predicted to be more relevant in gastric fluid for metals and metal oxides due to the strong acidic pH. As expected, SiO_2 particles were reported to only partially dissolve in simulated gastric fluid, whereas ZnO particles were dissolved completely after 2 h. On the contrary, TiO_2 and Fe_2O_3 particles showed large agglomeration, but no significant ion release.[170] Rapid aggregation in simulated gastric fluid was reported also for silver NPs (AgNPs) in other studies.[185,186] Dissolution and agglomeration/aggregation were reported to occur also in intestinal fluid. In this case, the basic pH condition promoted the dissolution of SiO_2, while no significant dissolution was reported for TiO_2 or Fe_2O_3.[170]

Being a surface-driven process, dissolution could be affected by the presence of a biomolecular corona. For example, this was shown by Ngamchuea *et al.* for AgNPs, when they reported that coating AgNPs with organics/proteins reduced the access of dissolved oxygen to the NP surface, thus minimising the release of Ag^+ ions.[187] A similar protective effect was reported by other authors on AgNPs[170] or hydroxyapatite NPs.[171] The latter material was completely dissolved in simulated gastric fluid deprived of the proteins, but only partially when proteins were present. The protective effect of the biomolecular corona is expected to be dependent on the surface chemistry of NMs, which in turn determines the nature of the protein–surface interaction. For example, Abdelkhaliq *et al.* reported that coating AgNPs with lipoic acid or with citrate affects the cellular uptake. This is likely due to the formation of a different corona because of the different surface chemistry.[188] Another study in simulated

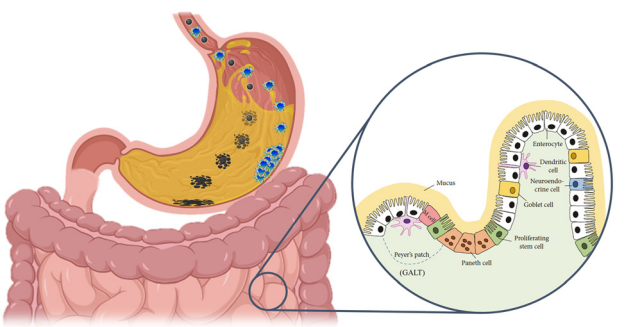

Fig. 5 NM transformation through the oral-gastro-intestinal tract. Ingested NMs might undergo dissolution/degradation, aggregation/agglomeration, and surface modification following formation of a biomolecular corona. These transformations show great potential in modifying their bioactivity toward the gut epithelium. In the inset, a scheme of the complex structure of the intestinal barrier is shown. Adapted from Kong *et al.*[167]

gastric fluid using AgNPs of 20 nm and 110 nm with two different coatings – citrate and polyvinylpyrrolidone (PVP) – indicated a significant increase in diameter, especially in the presence of pepsin, suggesting aggregation. Despite this, there was minimal impact on pepsin's proteolytic function and no detectable changes in its secondary structure.[186] Extensive work has been done with TiO_2 NPs due to their use as a food additive (E171), despite their use in some countries having been recently banned.[189] For instance, the interactions of TiO_2 NPs with casein, a major milk protein, were characterised in one study. The formation of a protein corona around the TiO_2 NPs led to the dissociation of casein micelles and the creation of NP–protein complexes, which extensively aggregated. This aggregation hindered gastric digestion by reducing the accessibility of pepsin to its substrate, influencing the bioavailability and toxicity of the NPs.[190] Another article examined the biomolecular corona on food-grade TiO_2 NPs in different food models. After digestion, a biocorona rich in lipids was observed, with its composition varying significantly with the fat content of the diet. This lipid-rich corona appeared to reduce oxidative stress and toxicological impacts, suggesting a protective role.[191]

The effect of the biomolecular corona on the colloidal properties of NMs is only one of the possible mechanisms potentially leading to a modification of the NMs bio-identity. In fact, the biomolecular corona can contain molecules that have a biological activity. In a study by Antonello *et al.*,[171] different kinds of NPs (lipid-surfactant NPs, carbon NPs, surface-modified Fe_3O_4 NPs, and hydroxyapatite NPs) were studied for their effect on an intestinal barrier *in vitro* model after treatment with a simulated digestion system. The formation of a bio-corona, which contains proteases, was demonstrated for Fe_3O_4 NPs. This was related to the ability of the NPs to upregulate the tight junction genes in the intestinal barrier, and to increase the expression of both pro- and anti-inflammatory cytokines (IL-1β, TNF-α, IL-22, IL-10). The INFOGEST protocol has also been used to study NMs, such as magnetite NPs, after gastric and duodenal digestion phases.[192] Using sucrose gradient ultracentrifugation, they isolated and characterised the size and protein composition of the NP–corona complexes, and conducted translocation studies on Caco-2 cell monolayers in a serum-free environment. The findings revealed that the NP corona differed in size, surface charge, and protein composition between the gastric and duodenal phases. The digestive protein corona enhanced NP cellular uptake, inducing morphological changes in the Caco-2 cell monolayer.[192] Brouwer *et al.* also used the INFOGEST protocol to explore how gastrointestinal digestion affects the protein corona on polystyrene micro- and nanoplastics, and their subsequent uptake by human THP-1-derived macrophages.[193] Researchers found that digestion creates a unique protein corona, which can persist in serum-containing cell culture medium. This digestion-induced protein corona significantly increases the uptake of uncharged plastics smaller than 500 nm, but does not affect larger or charged micro- and nanoplastics.[193] Other examples of molecules in the bio-corona that can modulate the biological effect of NPs exist. For example, transferrin[194] and bile acids[195–197] were found to promote transcytosis of NPs since they are able to interact with specific receptors present on the apical side of the enterocytes. The transformations described above are material-dependent. In a study performed on food-grade TiO_2, aggregation and formation of a hard corona were observed during simulated digestion. Nevertheless, these processes have little effect on the acute toxicity and genotoxicity of TiO_2 toward epithelial HCT116 cells, while a slight decrease of the surface reactivity and of the ability to induce oxidative stress was observed.[198] Furthermore, the composition of the biomolecular corona can be dramatically affected by food. For instance, an increase of the uptake of poly(acrylic acid)-coated AgNPs by Caco-2 intestinal cells was observed when digested in the presence of food.[199,200]

Finally, an increasing number of studies provided evidence of the effects that follow the interaction of NMs with the intestinal microbiota. NMs may induce modification of the microbial physiological community.[201–203] At the same time, molecules derived from the microbiota may modulate the biological effect of NMs. For example, *Pseudomonas aeruginosa* exotoxin A was found to enhance the transcytosis of NPs.[204] It is important to note that data obtained by *in vitro* studies concerning ingestion are in general poorly comparable. This is due to the lack of standardised methodologies and validated models for both OGI fluids and the intestinal epithelium.[205] On the other hand, the large variability of the composition of the fluids due to physio/pathological conditions and food makes the definition of standards particularly challenging.

NM exposure in the blood

Physiology of the exposure route

Blood is a specialised connective tissue responsible for transporting different components to different organs, playing a vital role in the physiological regulation and defence of the body. The most abundant element is red blood cells or erythrocytes, whose primary function is to transport oxygen from the lungs to the different tissues, and to carry carbon dioxide back to the lungs for exhalation. Red blood cells achieve this vital role through the protein haemoglobin, which binds to both oxygen and carbon dioxide.[206] They have a distinctive biconcave shape, an evolutionary approach – which is reminiscent of the one in nanoscience – to acquire larger surface areas, enabling them to exchange gases more efficiently.[207] White blood cells or leukocytes are also an important part of blood, being essential for immune responses and maintaining body health.[208] There are several types of white blood cells, each with specific roles in the immune system. The main types include neutrophils, lymphocytes, monocytes, eosinophils, and basophils.[209] However, to understand the bio-identity of NMs in blood, the

most important item is plasma. Blood plasma, the liquid component of blood, constitutes about 55% of its volume and is primarily constituted by water (92% of its volume), which acts as a solvent to transport proteins, electrolytes, nutrients, gases, and wastes throughout the body.[210] It supports vital functions such as maintaining homeostasis, regulating body temperature, and ensuring pH balance. Plasma proteins like albumin, globulins, and fibrinogen are essential in maintaining osmotic pressure, facilitating immune responses, and enabling blood clotting.[211] Abnormalities in these protein levels can indicate various diseases, such as nutritional deficiencies, liver disease, immune disorders, and clotting dysfunctions.[212] Thus, plasma plays an integral role not only in transporting substances but also in diagnosing and managing various health conditions, making it a central element in cardiovascular health and overall body maintenance.

Bio–nano interactions

As stated in previous sections, the translocation of NMs from different entry routes could end up in the bloodstream.[213] In addition, NMs can access the bloodstream either by direct injection,[214] or rarely, through the hair follicles of the skin.[215] When NMs come into contact with the bloodstream, they can interact with associated cells and biomolecules, and trigger a variety of potentially adverse effects. In this context, the potential toxic effects of NMs exposure in blood and the influence of the corona have been highlighted.

For example, a study reported by Kim *et al.* showed that SiO_2 NPs caused hemolysis, deformation, and aggregation of red blood cells (erythrocytes).[216] Negatively charged NPs can also induce hemolysis of erythrocytes because of the interactions with organic cations of their membrane.[217] NMs can also interact with platelets, leading to activation of the coagulation cascade, the formation of blood clots, and the partial or total occlusion of blood vessels by thrombi, as reported in the case of SiO_2 NPs, AuNPs, AgNPs, CdTe, and CdSe quantum dots, among others.[218–221] Therefore, hemocompatibility extends beyond hemolysis to encompass factors like erythrocyte aggregation, deformation, and vascular occlusion.[222] While hemolysis serves as an indicator, the formation of thrombi due to NPs aggregation poses significant risks, potentially leading to vascular blockages.[223] However, not all intravenous nanoformulations result in health risks. Some products that are already on the market, like nanoparticle-based intravenous injections indicated for the treatment of iron deficiency, seem to improve traditional products.[224] In this case, the particles are multicomponent NMs consisting of an iron core surrounded by a carbohydrate shell, emulating the structure of serum ferritin.[225] The shell formulation varies, depending on the product and the iron content, with concentrations ranging from 12.5 to 100 mg ml^{-1}.[225] To assess the NM safety, the physicochemical properties that modulate NP aggregation in blood have been recently studied.[226] The interaction of NPs with blood biomolecules results in the aforementioned corona, guided by the NPs' physico-chemical properties. For instance, smaller NPs adsorb relatively more proteins compared to larger NPs due to having a larger surface area-to-volume ratio in smaller NPs.[227] Additionally, hydrophobic NPs adsorb more than twice the amount of proteins of hydrophilic NPs (12 *vs.* 26 μg of proteins per mg of NP, respectively).[228] Other studies showed that hydrophobic NPs have a higher binding affinity for apolipoproteins, while hydrophilic NPs favoured the adsorption of fibrinogen, IgG, and albumin.[27,229] Researchers also found that hydroxylation can reduce the adsorption of certain proteins like HSA and IgE, which typically promote immune clearance, while maintaining the adsorption of ApoE, which prolongs circulation in the bloodstream.[230] Charged NPs can cause more protein denaturation than neutral NPs.[231] Studies on polystyrene NPs demonstrated that positively charged NPs have higher binding affinity to proteins with an isoelectric point of less than 5.5 (*e.g.*, albumin), while negatively charged NPs prefer proteins with higher values (*e.g.*, IgG).[232] Another study on various organic and inorganic NPs has demonstrated that their exposure to human serum albumin results in diverse corona formation and composition. These differences manifest in the hydrodynamic size of the NPs.[119] Porous particles decreased the amount of adsorbed proteins due to the size-exclusion effect.[227] In turn, coating NPs with polyethylene glycol (PEG), zwitterionic compounds or polysaccharides can also minimise protein adsorption.[233,234] However, when targeting ligands are attached to the NP surface, serum proteins interfere with their binding to target cells. Conjugating targeting ligands to an equilibrated corona is an alternative to overcome the issue and enhance their binding.[235]

On the other hand, the biomolecular corona can impact the NMs' physico-chemical properties and highly influence their circulation time, as well as their deposition in tissue. Binding proteins known as opsonins, such as immunoglobulins and complement proteins, facilitate the recognition and uptake of NMs by phagocytic cells, thereby reducing their circulation half-life.[236,237] In contrast, desopsonins, such as albumin and apolipoproteins, promote prolonged NM circulation times and enhance the potential for targeted delivery to specific tissues.[238–240] For instance, a study reported by Wang *et al.* outlined that blood proteins can mask the surface charge of cationic NPs, and thereby inhibit cell death caused by positively charged NPs.[241] The masking effects of several types of blood proteins, specifically, bovine fibrinogen (BFG), BSA, transferrin (Tf), and γ-globulin (Ig), can be extended to reduce NPs' toxicity compared to naked/pristine ones, as reported for poly(3-hydroxybutyrate-*co*-3-hy-droxyhexanoate) NPs and SWCNTs.[54,242] The superparamagnetic properties of magnetic NPs were reduced upon interaction with serum proteins because of corona formation, which prevented the core spins from aligning along the field direction.[243] The hydrodynamic size of AuNPs was increased by approximately 50 nm upon

incubation with blood plasma proteins for 30 min.[244] Such events can cause changes in the NPs' cellular internalisation and co-localization based on the new identity of the NPs. For instance, SiO_2 NPs under serum conditions exhibited stronger accumulation at lysosomes. Meanwhile, under serum-free conditions, they showed a higher degree of attachment to the cell membrane and greater internalisation (both lysosomes and cytosols).[245] In addition, translocated NMs from different entry routes may be subjected to a partial displacement of the original corona by new molecules from the new environment. Other biomolecules that are not replaced would serve as a corona 'memory' of the NMs' previous exposure environment.[246] This could be evolved to map the transport pathways utilised by NMs, and eventually explore the potential hazards from their exposure.

During the last few years, a wide range of the population has been voluntarily exposed to NPs in their blood.[247] The worldwide administration of vaccines during the COVID-19 pandemic led to a novel formulation, where the vaccines were based on encapsulated mRNA in lipid NPs.[248,249] Since then, the research on NP-based vaccines *via* intramuscular or intravenous injection has become a critical aspect of immunisation strategies,[250] but also for cancer treatment.[251,252] For example, Siemer *et al.* developed cisplatin-loaded, polysarcosine-based polymeric NPs that bypass the LRRC8A-mediated resistance mechanism through endocytic delivery, effectively eradicating cisplatin-resistant cells.[253] In this context, injection routes will need to be addressed for future human trials. Intramuscular injection, commonly performed in the deltoid muscle, offers advantages such as slower absorption and prolonged immune response.[254] On the other hand, intravenous injection represents a more direct route into the bloodstream, but can lead to rapid immune activation with severe effects on health, such as myopericarditis.[255] These recent advancements in vaccine delivery technology, however, still lack comprehensive characterisation of the NP stability and the potential biomolecular corona formation on top of the nanosized vaccines.

NM exposure in synovial fluid

Physiology of the exposure route

The synovial fluid is a viscous non-Newtonian fluid found in the cavities of synovial joints.[256,257] It is mainly characterised by its critical role in facilitating proper joint function by reducing friction between the articular cartilages during movement. Synovial fluid is primarily an ultrafiltrate of plasma containing hyaluronic acid, which gives viscous and elastic properties, and lubricin (or proteoglycan 4), crucial for lubrication to protect cartilage surfaces.[258,259] Additionally, it includes serum proteins such as albumin and globulins, cytokines, and growth factors that regulate synovial cell functions and respond to inflammation and healing,[260] as well as phospholipids and proteoglycans that contribute to the biochemical environment.[261] Beyond lubrication, synovial fluid serves as a transport medium for nutrients and metabolic wastes to support the avascular cartilage, and contains phagocytic cells for removing debris and microorganisms, therefore contributing to immune protection.[260] Alterations in the composition or quantity of synovial fluid can lead to joint diseases such as osteoarthritis or rheumatoid arthritis, where the fluid often becomes inflamed, losing its protective properties and leading to pain and reduced mobility.[262,263] Treatment for joint diseases such as rheumatoid arthritis focuses on reducing inflammation, managing pain, and maintaining joint function. Medications like non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease-modifying antirheumatic drugs (DMARDs) are commonly used.[264] In severe cases, joint replacement surgery may be necessary. Here, implants designed to mimic the movement of natural joints are used to replace damaged areas, and can significantly improve quality of life by restoring mobility and reducing pain. However, they can end up degrading over time due to several reasons, from the body's immune response to the implant to mechanical wear and tear (Fig. 6). To avoid this, much research has been carried out in recent years to find biocompatible[265] and biodegradable[266] implants.

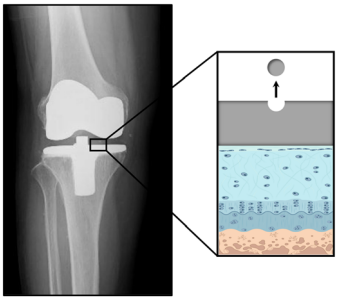

Fig. 6 Scheme of NM release in synovial fluid from knee orthopaedic devices. Representative picture of how NMs are discharged into the synovial fluid from the surface of orthopaedic devices implanted within the knee joint. Adapted from Sato *et al.*[267] with permission from Elsevier, copyright 2024.

Bio–nano interactions

NM presence in joints has become a matter of concern in orthopaedic devices, where particles generated at the contact surface of the implant bearing are the primary cause of aseptic loosening. This is often a limiting factor for patient response to an implant, particularly when metallic residues of the implants have the potential to translocate into the circulatory system. The viscosity and complex biological composition of the synovial fluid could influence the NMs' properties that are released by articular prosthesis since it is the first substance with which they come into contact prior to their eventual translocation to the bloodstream.[246] For instance, Brown and co-authors have reported the effect of synovial fluid on the colloidal properties of two groups of PLGA nanocarriers (with cationic and anionic functional

groups) and cartilage retention *ex vivo*. PLGA is one of the most effective biodegradable polymeric NM, and widely used as a carrier for drug delivery systems.[268,269] As a result, it was stated that the synovial fluid induced aggregation of cationic PLGA NPs after incubation at 37 °C for 30 minutes, probably because of the NPs' interaction with the predominate hyaluronic acid.[270] This was confirmed by an increase in the hydrodynamic diameter, as well as a reverse change in the zeta potential. However, no aggregation was detected in the case of anionic PLGA NPs, *i.e.*, no change in the hydrodynamic diameter and zeta potential. Additionally, cationic PLGA NPs showed higher cartilage retention compared to anionic NPs, which clearly suggests that synovial fluid is a modulator of the NPs' fate in the joint. The effect of synovial fluid on the NMs' properties can be extended to include the corrosion induction of the implantable materials that may end up with adverse human health effects.[71] For instance, in a study conducted on selected patients who had previously undergone hip resurfacing arthroplasty using an alloy of chromium–cobalt metal-on-metal (MoM), it was reported that the release of oxidised cobalt ions (Co^{+2}) from the metal debris that wears away from the MoM hips caused inflammation to the surrounding tissues, accumulation in white blood cells, mitochondrial damage, and abnormal nuclear morphology of macrophages. In addition, the patient's blood levels exhibited extremely high Cr and Co contents, which confirmed the release of NMs from the joint replacement to the bloodstream.[71,271] This raises a serious question about the mechanism of *in vivo* dissolution of Co/Cr/MoM because, on a bulk scale, this MoM alloy is extremely stable/corrosion resistant. A recent study reported by Simoes *et al.* found a much higher release of MoM ions relative to Co and Cr in the presence of bovine serum albumin (BSA), as confirmed by inductively coupled plasma mass spectrometry (ICP-MS).[272] In the same context, the adsorption of synovial proteins can affect the MoM hips' properties, inhibit their function, and thus increase ion dissolution. Accordingly, the MoM hips were revised after 3.5 years due to squeaking, clicking, and painful hip movements, which opens another question about how materials are tested in terms of safety assessment before they are used as implantable materials. To address these issues, plasma coating of hip joints (ball, socket, and shaft) with Si_3N_4 has been studied as a safe-by-design solution to offer potential lifelong *in vivo* use without revision.[273] These new joints were subjected to extensive wear testing by robot flexing under load, encased in simulated synovial fluid (BSA, Lubricin, and PBS). The NPs generated were substantially reduced in concentration compared to MoM joints, confirming higher lubricity. Toxicity was assessed by oxidative stress, cytokine release, TNF-α cytotoxicity, and DNA strand break assays. All 4 toxicity assays yielded non-significant outcomes in contrast to MoM joints. As a result of these findings, two CEN, one ISO, and one ASTM standards have been secured based on the joint testing method and the toxicology assays to ensure the safety-by-design of hip joints.[274]

Dermal and ocular exposure to NM

Despite the permeability of the involved organs being much lower, dermal and ocular exposures to NMs have also been studied in recent years. For instance, the *in vitro* absorption of TiO_2 and ZnO in cosmetic formulations through porcine skin found that these particles do not penetrate the outermost layer of the epidermis. Total recoveries of zinc and titanium were close to the amount applied, indicating no significant absorption into the skin. Therefore, their use in topical products such as sunscreens is not considered a health risk due to the absence of internal exposure.[275] However, studies have demonstrated that particles smaller than 10 nm could passively penetrate the skin through the stratum corneum lipid matrix and hair follicle orifices, reaching deeper skin layers and hair follicles, which can lead to potentially adverse effects.[276] Additionally, researchers highlighted that damaged skin allows for greater penetration of NPs.[277] To evaluate this rather unlikely scenario,[278] *in vitro* and *in vivo* studies have been applied to skin cells. Monteiro-Riviere *et al.* found that AgNPs complexed with different serum proteins significantly influence their uptake by human epidermal keratinocytes (HEK), with proteins generally suppressing citrate-coated AgNP uptake, while having minimal effect on silica-coated AgNPs.[279] The presence of a biomolecular corona actively influences NM behaviour, as researchers have also shown in a study on ZnO NPs, where particle size and protein corona formation affected toxicity and cellular responses in HEK cell lines.[280]

Research on ocular environmental exposure to NMs is less well characterised. Research studies have indicated that metallic NMs can cause eye irritation and inflammation through direct contact, with chronic exposure leading to elevated toxicity.[281,282] For example, the exposure of rabbit eyes to TiO_2 NP induced ocular surface damage.[283] In contrast, when investigating the *in vivo* safety of oral and ocular topical applications of 100 nm SiO_2 NPs in rats, blood biochemical parameters and ophthalmic examinations revealed no abnormal findings in any of the animals after 12 weeks.[284] However, organic NPs like liposomes are becoming a safer alternative for ocular drug delivery nanosystems.[285] Furthermore, researchers showed that coating cationic liposome–DNA complexes with artificial biomolecular coronas made of fibronectin (FBN) and Val-Gly-Asp (VGA) tripeptide significantly enhanced their uptake by corneal epithelial cells, which was being reduced by mucin.[286]

Dose and media impact on NM interactions

Understanding the impact of NM exposure on cells is essential to guarantee their safe use. Apart from the size, which does not seem to drastically alter the corona

composition,[287] surface chemistry is also an essential factor to foresee potential NM toxicity.[288,289] For example, in the case of oxide particles (OxPs) such as SiO_2 NPs, hydroxyls (OH) exposed at the surface can lead to cell toxicity.[290] However, the quantity of NPs and the biological fluid in which they are dispersed are two critical variables, which may also affect NP–cell interactions.

When assessing the nanosafety profile of NMs, the presence or absence of proteins in cell culture medium was shown to affect the NMs' toxicity. This indicated that the cell culture medium has a role in "feeding" the cells, and can indirectly affect viability by protecting them. Early studies by Hu *et al.*[291] with graphene oxide NPs and Lesniak *et al.* with silica NPs[245] showed that the protein corona mitigates NP toxicity by lowering their surface energy and interaction. NMs exposed to serum-free media and without corona lower their binding energy by strongly interacting with cellular membranes, causing rupture and necrosis.[292] While a serum-free environment does not represent a realistic exposure scenario, a high dose of NPs in a cell culture media with low protein might lead to an incomplete coverage of the NPs by the corona, and a possible toxic response caused solely by this phenomenon. Recent studies indicate that the corona can be visualised by Cryo-TEM, representing a realistic picture of the NP *in situ* corona, which could provide useful information of the dosimetry.[293,294]

The nature of the cell culture medium greatly impacts how NMs behave and interact with cells. Citrate-capped AuNPs exposed to Dulbecco modified Eagle's medium (DMEM) and Roswell Park Memorial Institute medium (RPMI) with fetal bovine serum showed significant differences in biomolecular corona composition and cell uptake. Testing 15 nm AuNPs on HeLa and U937 cell lines, the NPs showed higher internalisation and cytotoxicity when incubated with RPMI, compared to DMEM.[295] Furthermore, studies have shown that matching biological media with the cell type favours the uptake of NPs, as if cells preferred to uptake proteins from the same species.[296] It is also crucial to take into account how the amount of NPs has an impact on the biomolecular corona formation. Changing the dosage of NPs can alter the ratio of the NP surface area to accessible proteins, which in turn, can alter the corona structure.[297] This dose-dependent effect can profoundly impact the interaction of NPs with biological systems, including cellular absorption, signalling pathways, and possible toxicological reactions (Fig. 7).

Overall, the corona is derived from biomolecules embedded in biological fluid, which may undergo post-translational modifications (PTMs), such as phosphorylation, ubiquitination, acetylation, and glycosylation.[298] These PTMs can have a direct effect on the biomolecular conformation, and possibly on the binding to the NPs' surface.[299] Proteins in the blood are highly glycosylated, a common PTM where glycans – structures of chained and branched monosaccharides – are covalently linked to proteins during their synthesis.[300] While glycans were believed to be inert biomolecules, it is now well established that they affect protein folding and stability, controlling a wide range of biological processes as well. Glycan abundance in proteins raises the question of whether they could be an active component of the biomolecular corona. Circulating plasma proteins are highly abundant in the N-glycan form, and depending on the protein, the glycan structures and abundance change.[301]

By interacting with glycan-binding proteins (or lectins), glycans can trigger specific biological pathways.[301] Glycans are recognised by lectins, which are ubiquitously expressed receptors in nature and involved in cell adhesion and immune response.[302] For example, endothelial cells express E-selectin, an adhesion lectin that recruits leukocytes.[303] In contrast, macrophages express lectins such as the mannose receptor to recognise microbial ligands and promote innate immunity.[304] In particular, glycan structures that carry a terminal neuraminic acid (also known as sialic acid) can interact with sialic acid immunoglobulin-like lectin (SIGLEC) receptors and modulate the recognition of the "self", attenuating inflammation and promoting phagocytosis evasion.[305–308] Additionally, loss of sialic acid glycan can lead to the exposure of the galactose residue,[309] which is recognised by asialoglycoprotein receptors, such as the

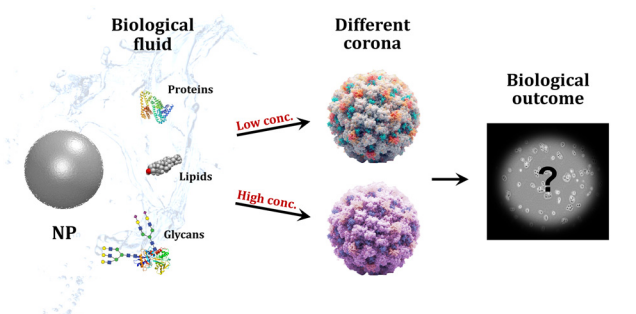

Fig. 7 Differences in the fluid/NP ratio change the corona with possible biological impacts. When particles are exposed to biological fluids, the concentration of their biomolecules and the NP dose can produce different corona compositions, which at the same time may alter the biological outcome.

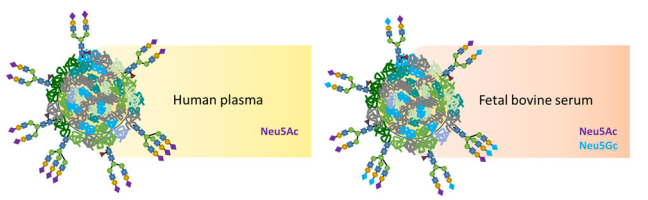

Fig. 8 The origin of biological fluid impacts corona composition. Matching the biological fluid with the testing model can affect the outcomes of the experiments, especially considering the glycan component of the biomolecular corona. For instance, glycans in the human serum contain only the terminal monosaccharide sialic acid in the form of Neu5Ac, while other mammalian species, such as bovine, contain multiple sialic acids, including the non-human form Neu5Gc.

Ashwell-Morell receptor in hepatocytes,[310,311] leading to protein uptake and turnover.[309]

There are noticeable differences in glycans and monosaccharides between species. For instance, Lin *et al.* found significant divergences in glycosylation among human, bovine, and recombinant fetuin.[312] Differences are also relevant with sialic acids, where humans lack the CMP-*N*-acetylneuraminic acid hydroxylase enzyme that generates the *N*-glycolylneuraminic acid (Neu5Gc), common in mammals (such as bovines), starting from the acetylated form *N*-acetylneuraminic acid (Neu5Ac).[313,314] Studies have shown that the different nature of the monosaccharide (Neu5Ac or Neu5Gc) affects the binding of the glycan with the specific SIGLEC receptor, leading to different modulation of the immunological system.[315–317]

Since blood plasma is highly glycosylated,[301] the biomolecular corona is expected to carry glycans at the bio–nano interface. Wan *et al.* have first shown that loss of glycosylation in the biomolecular corona leads to higher cellular uptake in M1 and M2 macrophages.[318] Moreover, Clemente *et al.* identified the glycan structures as part of the AuNPs' biomolecular corona, confirming that they are biologically accessible and available for binding towards specific lectins.[24] These findings open the doors to new potential applications of NMs, exploiting the biomolecular corona and its glycosylation. Trinh *et al.* demonstrated that NP's exposure to biological fluids derived from patients with lung cancer carries glycan features specific to the chronic disease,[25] giving a new angle to the existing paradigm of the personalised corona previously described.[319,320] Because the same NPs can carry different glycosylation structures as reflected in the diverse terminal monosaccharide residues, this may suggest that the glycan components of the biomolecular corona can influence the NP's biological interactions when binding to lectins in organisms (Fig. 8).[321]

Recent studies have also studied whether protein's glycation, a non-enzymatic PTM where the presence of an excess of monosaccharides leads to a covalent linkage to biomolecules (typically occurring under disease conditions such as diabetes), affects the protein corona formation and toxicity of AgNPs. Interestingly, protein glycation resulted in subtle changes in the protein corona secondary structure and corona formation, and both glycated and non-glycated proteins resulted in a decrease in the NPs' dissolution-mediated toxicity.[322]

Summary and perspective

In this manuscript, we reviewed the human exposure to NMs and their impact on health. Central to this narrative is the pivotal role played by the biomolecular corona – the dynamic layer of biomolecules that cover NMs upon contact with biological fluids. Common routes of exposure include inhalation (lungs) and ingestion (gut), with the potential to cause a distinct biodistribution and organ translocation, as well as varying levels of toxicity. We have outlined the significance of the biomolecular corona not only for toxicity studies, but also as a key factor to consider in terms of NM colloidal stability and material transformation.

Inhalation is a main route of exposure for humans, potentially leading to both local and systemic adverse health effects, as particles suspended in the air can potentially enter the body through the lungs. The ability of NMs to reach deep lung tissues and possible translocation to the blood raises concerns not only about respiratory health, but also about systemic toxicity. This is an important reason for further research to evaluate the NM interaction with lung fluids. On the other hand, the ingestion exposure pathway presents a different set of considerations. Ingested NMs have the potential to interact with the gastrointestinal system with the possibility of potential absorption into the bloodstream. Although more research is being done nowadays to understand NM interactions with the digestive system and its organs, there is a need for a comprehensive understanding of NM degradability and changes in the corona composition along the digestive pathway. It is also important to evaluate the direct exposure of NMs to the bloodstream, a scenario that can occur intentionally for medical purposes, or unintentionally due to accidental entry or after translocation from other exposure routes. The immediate distribution of NPs throughout the body, facilitated by direct contact with the bloodstream, carries both therapeutic promise and potential toxicity concerns. NMs can be also released from orthopaedic implants into synovial fluid, raising concerns about potential health risks and the stability of the materials used. The current research focus is to create safer and biocompatible implants to avoid wear and corrosion, especially in cases involving metallic joint replacements. Lastly, dermal and ocular exposures represent other routes of exposure to diverse NMs, although translocation is less likely as the permeability of the involved organs is much lower.

Understanding the biological role and possible cell toxicity of NMs requires a complex understanding of the interaction between NMs and biological media, accounting for the surface chemistry, size, dosage, and the biological fluid environment. For instance, aligning the *in vitro* model's species with the relevant biological fluid ensures a more accurate representation of real-world conditions. Overall, the biological fluids of different exposure routes can impact the biochemical and physicochemical properties of NMs. Therefore, they can be studied for monitoring potential hazards upon exposure. Recent advancements in understanding the biomolecular corona in fluids other than blood have been made in recent years. The differences in the corona composition when embedded in different biological fluids might be a potential tool for biomarker discovery. Equally important is an in-depth characterisation of the NMs' biomolecular corona. In this sense, it is still difficult to link the biomolecular corona to adverse or beneficial

(nanomedicine) effects. Using a standardised framework to ensure consistency and comparability would help to minimise artefacts and common errors,[323] such as biomolecular carryover in the background, potential aggregation induced by separation processes, and unrealistic exposure conditions. For example, there are no specific OECD guidelines dedicated to the evaluation of the biomolecular corona of NMs. However, it is firstly necessary to establish a strong link between the biomolecular corona to biological processes that can be linked to regulatory relevant endpoints. For this to happen, it would be pivotal that standardized methods for the qualitative and quantitative characterisation of the corona are developed and endorsed by international regulatory bodies, such as ISO. Furthermore, it would be beneficial to know the details on the NM characteristics, the dosage (hence the surface area), and the properties of the biological fluid being used, in order to facilitate proper testing and research with them. Emphasis is placed on the need for further research on NMs' interactions with lung and gastric fluids, as these environments are most relevant to possible human exposure. More data and tools are needed to model the biomolecular corona formation for a better prediction of the *in vivo* data. We believe that through targeted corona characterisation, a more accurate assessment of NM behaviour, and potential toxicity, this goal will be achieved.

Data availability

No primary research results, software or code has been included and no new data were generated or analysed as part of this review.

Author contributions

M. P. M., I. F., T. S., T. W., M. D., G. A., A. M.-S. and M. G. S. critically assessed the literature and discussed the content of the review. M. G. S. and A. M.-S. drafted the review. All co-authors contributed to the review and its revision.

Conflicts of interest

There are no conflicts to declare.

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