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Environmental dimensions of the protein corona

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Abstract

The adsorption of biomolecules to the surface of engineered nanomaterials, known as corona formation, defines their biological identity by altering their surface properties and transforming the physical, chemical, and biological characteristics of the particles. In the first decade since the term protein corona was coined, studies have focused primarily on biomedical applications and human toxicity. The relevance of the environmental dimensions of the protein corona are still emerging. Often referred to as the eco-corona, a biomolecular coating forms upon nanomaterials as they enter the environment and may include proteins, as well as a diverse array of other biomolecules such as metabolites from cellular activity and/or natural organic matter. Proteins remain central in studies of eco-coronas because of the ease of monitoring and structurally characterising proteins, as well as their crucial role in receptor engagement and signaling. The proteins within the eco-corona are optimal targets to establish the biophysicochemical principles of corona formation and transformation, as well as downstream impacts on nanomaterial uptake, distribution, and impacts on the environment. Moreover, proteins appear to impart a biological identity leading to cellular or organismal recognition of nanomaterials, a unique characteristic in comparison to natural organic matter. We contrast insights into protein corona formation from clinical samples with those in environmentally relevant systems. Principles specific to the environment are also explored to gain insights into dynamics of interaction with / exchange by other biomolecules, including changes during trophic transfer and ecotoxicity. With many challenges remaining, we also highlight key opportunities for method development and impactful systems on which to focus the next phase of eco-corona studies. By interrogating these environmental dimensions of the protein corona, we offer a perspective on how mechanistic insights into protein coronas in the environment can lead to more sustainable, environmentally safe nanomaterials, as well as enhance the efficacy of nanomaterials used in remediation and in the agri-sector.

In the past decade, protein corona studies have grown into a vibrant area of bio-nano interaction research. The protein corona is a layer of proteins that spontaneously adsorbs to the surface of engineered nanomaterials (ENM) when exposed to biological systems.[1] These proteins equip ENMs with a biological identity which influences ENM adsorption, distribution, biotransformation, and fate within an organism and within an ecosystem.[2,3] In both humans and the environment, the corona also plays a critical role in mitigating or prompting toxicological responses following ENM exposure.[2,4,5] Characterisation of the corona informs estimates of dissolution kinetics in environmentally relevant matrices,[4] increases accuracy of models for the environmental transport and fate of ENMs,[4,6] and enhances conclusions of ecotoxicity studies.[2]

In parallel to the biomedical grand challenge of designing ENMs that acquire customised protein coronas to increase efficacy of nanopharmaceuticals,[7] characterisation of the environmentally formed corona informs design of ENMs with enhanced control and sustainability in environmental applications, including remediation, biofouling, and agriculture. For example, the protein corona mediates ENM uptake and fate in plants.[6] Citrate coated gold ENMs became randomly distributed within the leaves of the fava bean plant (*Vicia faba*) after application to the leaf surface, while bovine serum albumin (BSA) coated gold ENMs adhered to trichome hairs rather than being internalised.[8] In addition, by selectively coating the ENMs with an antibody with high affinity for a specific chemical moiety in the stomata, localisation was limited to that leaf component.[8] Particles with pre-formed peptide coronas can be used to deliver targeted payloads and tune chloroplast redox function.[9] As insight emerges on the connection between ENM properties, directed corona formation, biodistribution, and fate, more effective and informed design principles for ENMs can be applied to plant nanobiotechnology and other environmental applications.

Traditionally, ENM corona studies have focused on proteins, however, coronas acquired from environmental matrices are comparatively more diverse and complex. Here, we differentiate between the environmental corona that forms independently of living organisms and the eco-corona that forms in the presence of an organism. The environmental corona consists primarily of a diverse set of natural organic matter (NOM) and humic acids.[4] The less studied eco-corona[10] may include portions of the environmental corona, as well as proteins and a broad range of dissolved organic matter (DOM), exopolymeric substances, and metabolites[11,12] from multiple species (**Figure 1a**). The molecular diversity of the eco-corona is reflective of the biodiversity of the microenvironment in which it is formed. Proteins are not always the most abundant constituents in the eco-corona, especially if formed outside of an organism;[4] however, the robust body of literature on proteins eases characterisation and increases accessibility of

biophysical insights into eco-corona formation. Moreover, proteins provide a biological identity to an ENM, initiating organismal or cellular recognition that leads to responses such as uptake or rejection by activation of stress pathways. We build herein on previous reviews of the protein corona[1,13–16] and focus solely on expanding the environmental dimensions. After a brief overview of the diversity of protein corona studies to date, we highlight considerations unique to corona formation in environmental systems. We summarise seminal work defining the implications of the corona for transformation, organismal uptake, and fate of ENMs in the environment, along with emerging studies that assess changes in the corona through ENM transformation and transfer into organisms and up the trophic chain. Finally, we share our perspective on challenges and opportunities in understanding eco-coronas, predicting their role in determining impacts of ENMs within the environment, and controlling their formation and composition for more sustainable, effective environmental applications.

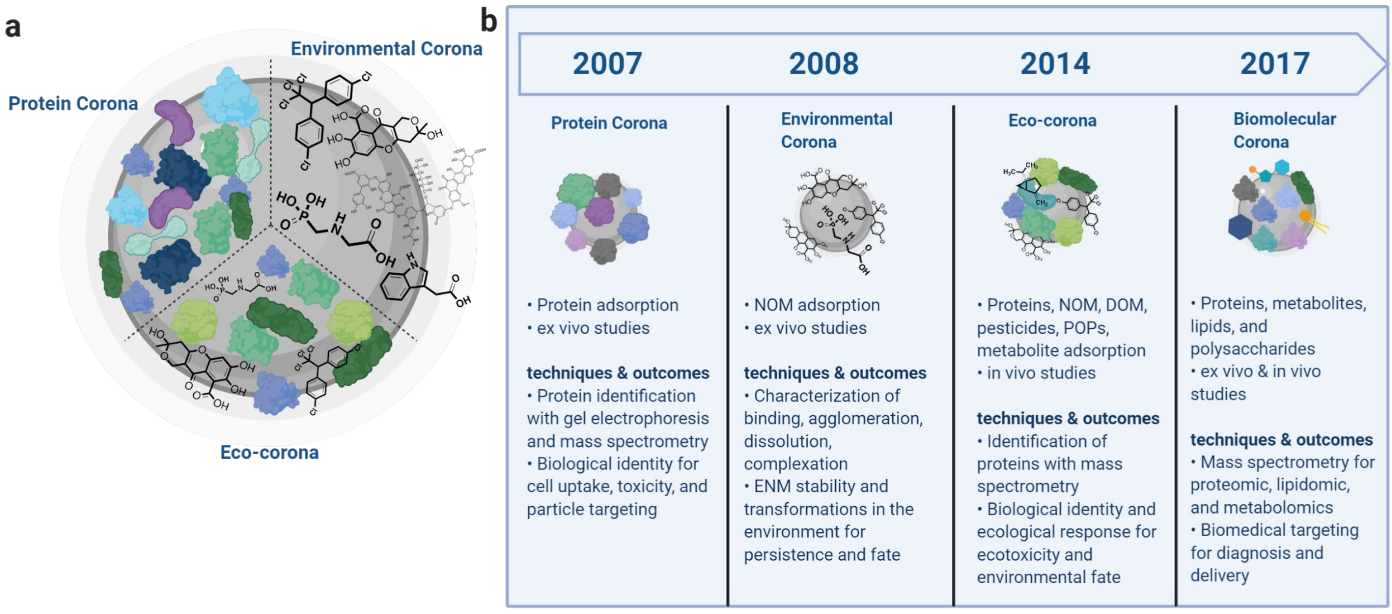

Figure 1. (a) Comparison of the protein, environmental, and eco-coronas. Formed within organisms at locations of high protein content, the term protein corona has been used to describe the binding of proteins to ENM surfaces, but also incorporates lipids, metabolites (typically <1000 Da which are either reactants, intermediaries or products of enzymatic processes), and other biomolecules. To date, the term environmental corona has described a corona formed in aquatic environments with high concentrations of NOM, including humic substances. By contrast, the eco-corona incorporates features of both the protein and environmental coronas, where the balance of proteins and other molecules varies. **(b) The evolution of the protein corona concept,** adapted from Hadjidemetriou and Kostarelos.[17] Studies of protein adsorption to surfaces and particles dates back to at least the 1960s. The term protein corona was first coined in 2007. Protein corona studies developed with mass spectroscopy-based proteomics

to aid identification of the proteins bound at the surface of ENMs and explore the role of surface curvature in altering protein structure and function relative to macroscale surfaces. Protein corona studies evolved in parallel with those on the environmental corona, but the characterisation techniques and goals for each area remained separate, with environmental corona focusing mainly on the dispersion stabilisation provided by NOM. The environmental dimensions of the protein corona began to appear later, as the concept of the eco-corona and its role in (nano)ecotoxicity emerged. Both the eco-corona and biomolecular corona embrace the diversity of molecules in solution with the goal of understanding and controlling downstream biological responses to nano-enabled technologies.

Evolution of corona studies

Although study of the protein corona has produced over a decade of insights, progress in understanding eco-coronas lags the biomedically relevant protein corona. This mirrors similar trends in toxicology, where a 10-15 year lag between clinical toxicology and ecotoxicology studies is common. For example, the first nanotoxicology papers were published in the 1990s, with ecotoxicology papers emerging roughly fifteen years later.[18] Consistent with this, the environmental dimensions of the protein corona were characterised only after a significant focus on formation of the corona in human blood products. The term eco-corona appeared in 2014 with increasing focus since (**Figure 1b**).

Protein corona studies typically fall into two categories. The first provides a molecular level analysis of one protein interacting with one ENM type, while the second identifies all proteins to assess the complexity of the entire corona population. The former often provides atomic level structural insight into key proteins interacting with the ENM and provides well-controlled, model systems for evaluation of protein structural changes and ENM transformations. Single protein model systems have elucidated the role of charged patches on a protein surface[19] and highlighted how ENM surface coating, structure, and curvature alter protein orientation and conformation on ENMs.[20] While many biomedical studies focus on BSA or other blood proteins, they can provide physicochemical insights applicable to environmental systems.[21–23] By contrast, studies focused upon identifying and quantifying the complete population of proteins within the corona make atomic level insights challenging, but better mirror the complexity of a living system and the environment and facilitate exploration of corona dynamics and evolution as NMs move within (or between) organisms. Environmentally focused protein coronas vary widely in biological matrix and species, and

provide insight into the complexity of the ENM surface, ENM transformations, organismal response, and environmental fate.

Formation of the protein corona in the environment

Features mediating eco-corona formation. The body of data on protein coronas has paved the way for an expansion into environmental systems. Reviews on protein corona formation in biomedically relevant systems establish the chemistry of interaction at the ENM surface, as well as protein–protein competition and displacement steps (the Vroman effect).[1,18] Because corona formation is governed by the energetics of interaction, and kinetics and thermodynamics of proteins at the surface, the fundamental principles apply equally to environmental systems.

Proteins interact at the surface of ENMs via an array of noncovalent interactions and, in the case of metal ENMs, may even chelate oxidised metals at the surface (**Figure 2a**). Upon interaction, the proteins may unfold and/or displace or carry other proteins and biomolecules with them to the surface. In turn, these interactions may catalyse ENM dissolution, or mediate agglomeration. As yet, understanding of protein corona formation is far from comprehensive. Many biophysical properties of proteins can be uniformly derived from the protein sequence, independent of species; however, post-translational modifications (PTMs), which have a demonstrated impact on corona formation and cellular uptake,[24,25] can vary significantly across species. Thus, additional protein properties need to be incorporated into proteomic identification and characterisation of corona proteins.[26,27] Although these variables are not uniquely relevant to eco-corona formation, they are of particular interest and merit additional study, especially in multispecies systems.

The chemical and physical properties of ENM, matrix, and biomolecules all mediate the concentration, population, orientation, and structure of proteins within the corona. ENM properties such as surface coating,[28–30] size,[29,30] and core composition can be optimised; however, solution conditions which impact corona formation (e.g. pH,[31] temperature,[32,33] UV exposure,[34] concentration of solutes,[35] flow[36]) and protein properties cannot generally be engineered by researchers, but are determined by the biological system or solution conditions (**Figure 2b**). As ENMs enter the complex solution of an ecosystem, they can agglomerate, dissolve, or undergo other chemical transformations to alter these designed properties and subsequently impact eco-corona formation. Solution conditions are homeostatically controlled within an organism, but can be variable in the environment, altering protein corona populations. This highlights the need for additional study of corona dynamics and the impact of

environmental features, such as UV radiation and pressure, on corona formation. Moreover, the impact of biomolecular diversity and dynamics is poorly understood, including the role of dissolved / natural organic matter (DOM/NOM),[4] PTMs, and persistent organic pollutants (POPs) in protein corona formation and ENM transformations.

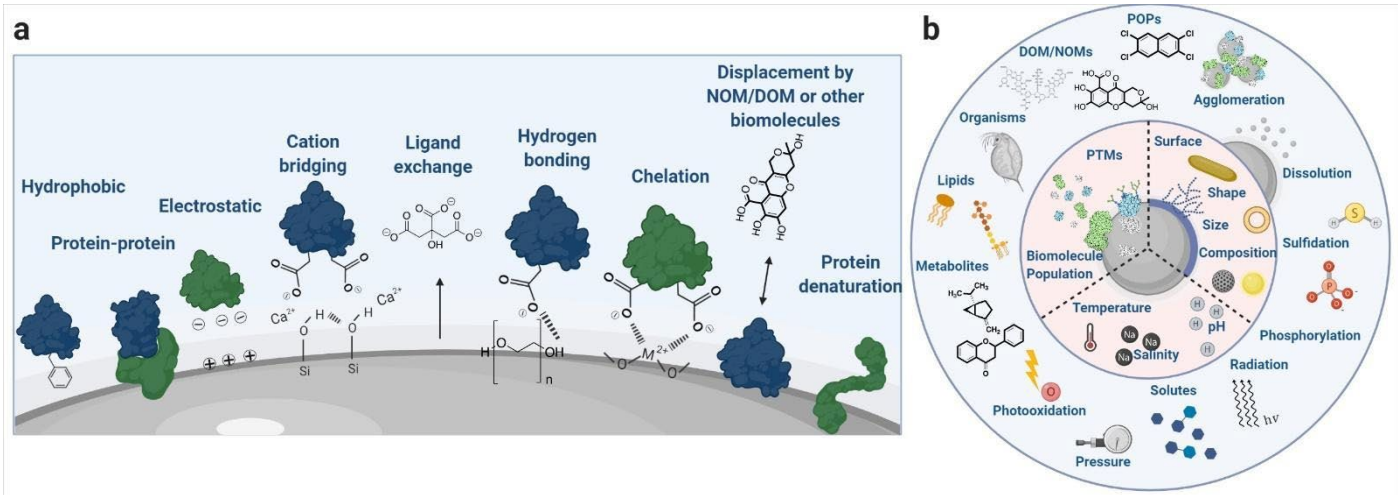

Figure 2. Properties that control protein corona formation. (a) Physicochemical interactions that mediate eco-corona formation, modified from Markiewicz et al.[4] with permission from the Royal Society of Chemistry. The mechanisms of interaction between proteins and ENMs include hydrophobic interactions involving nonpolar amino acids, protein-protein interactions, electrostatic attraction or repulsion, cation bridging, ligand exchange, hydrogen bonding, chelation, and displacement of proteins by other biomolecules. Such interactions may lead to denaturation of adsorbed proteins on the ENM surface. **(b). Examples of ENM (top right of circle), solution (bottom of circle), and biomolecular (top left of circle) properties that mediate corona formation.** The properties better understood because of their relevance to human health are included in the inner circle (tan) and those of priority for the environmental dimensions of the protein corona, but less explored, are included in the outer circle (pale blue).

Nanomaterial and protein transformations. Coronas transform ENMs, by driving dissolution, agglomeration, sulfidation, and reprecipitation. Although beyond the scope of this piece, recent reviews on environmental coronas provide detailed insight into ENM transformations upon interaction with NOM and DOM.[4,37] Bio-transformations of ENMs in soil,[38] wastewater,[6,39] and aquatic environments[10,40–43] impact subsequent protein corona formation, and are essential considerations in assessing downstream (nano)ecotoxicology and nanosafety.

While some proteins appear to increase the stability of ENMs,[44] others catalyse agglomeration. Moreover, proteins can alter the ENM through chemical reactions. Proteins with exposed metal binding residues drive dissolution and reprecipitation of metal and metal oxide ENMs.[45] In turn, ENMs can react with proteins through photooxidation or reduction,[46] or via metal binding.[47] Adsorption to ENMs can induce protein conformational changes, altering function and cellular recognition. In a simple example, polystyrene ENMs with different coatings caused BSA to denature to different degrees; upon greater unfolding, the BSA was no longer recognised by the native receptor, decreasing cell uptake.[54] Protein conformational changes or unfolding at an ENM surface can lead to protein fibrillation.[33,48]

While a corona of just one protein does not represent the complexity of the eco-corona, such studies have established their importance in interrogating the features that control the interactions and transformations that mediate ENM fate and behaviour. Cellulase, a ubiquitous extracellular enzyme secreted by soil microorganisms, decreases deposition rates of polyethylene glycol coated titanium dioxide (TiO_2) ENMs due to strong electrostatic repulsion of the negatively coated corona-ENM and the negatively charged soil particles.[49] The results varied, however, in different solutions depending on ionic strength. Such biomolecular-mediated interactions of ENMs are important for the ENM environmental fate, and for the efficacy and stability of any ENM-based application, including the growing interest in ENM-based agricultural sensors and pesticide delivery.

ENM transformations can also occur as a result of eco-corona formation within an organism. Aquatic invertebrates are mainly osmoconformers, where the ionic strength of their biofluids reflect that of the environmental media. Thus, in marine species, the eco-corona forms at NaCl concentrations near 500 mM, which is over 3 times higher than the typical concentration in human plasma. These species also have significantly higher concentrations of di-/tri-valent ions, which are notable for their effect on ENM transformations.[50] Because of these dramatically different conditions and the wider spectrum of protein populations, TiO_2 ENMs have very different agglomeration behaviour in culture medium than in the hemolymph serum of the marine bivalve *Mytilus galloprovincialis.*[43,51–53] Given the importance of mussels and other filter feeders and invertebrates in aquatic ecosystems, the eco-coronas acquired by ENMs upon interaction with these key species are important in elucidating fate and transport of ENMs in aquatic systems.

Corona composition reflects location and history

When ENMs enter the environment, corona formation can occur both outside and inside of an organism. If formed outside an organism, the eco-corona consists of a different mixture of biomolecules than if formed once internalised (**Figure 3a**). Extracellular fluids generally have a lower protein content with limited protein characterisation to date. Once internalised by an organism, the eco-corona evolves to more closely resemble a protein corona or biocorona as described in biomedical literature. An unexplored area is whether ENMs excreted from organisms with their eco-corona are subsequently internalised by other organisms of the same species or by other species, and the potential impacts of this for proteostasis.

Corona formation outside of an organism. Even when formed outside of an organism, some aspects of the eco-corona result from organismal response to ENMs. Proteins from the organisms excretions combine with natural colloids to create a microenvironment that subsequently alters ENM uptake and toxicity.[43,51,54–56] Proteins within the eco-corona are identifiable and linked to biochemical pathways to provide a unique window into biological response. Since ENMs trigger a change in biomolecular secretion when entering an organism's microenvironment, characterisation of eco-corona proteins illuminates stimulation of organismal pathways by ENMs and provides insight into the ENM fate and toxicity[56,57] (**Figure 3**). Given the combined importance of ENM transformations and organismal response, the study of eco-coronas highlights the importance of *in situ* characterisation.[43]

Some of the best studied examples of exogenous eco-coronas are in aquatic systems with *Daphnia magna,*[10,51,57] *M. galloprovincialis,*[43,58] and *Paracentrotus lividus*.[5,59] In the presence of polystyrene ENMs, *D. magna* secretes proteins indicative of heightened stress response and environmental sensing.[51,57] The extracellular protein concentrations increase over time, contributing to ENM instability and agglomeration. Moreover, the eco-corona enhanced uptake of polystyrene ENMs, increasing toxicity, and lowered feeding because they were more slowly removed from the gut.[51]

In a terrestrial example, the earthworm *Eisenia foetida* secretes a coelomic fluid to maintain moisture in the body and help physiological activities. The eco-corona formed in the presence of *E. foetida* was dependent on ENM core composition. The silver ENM eco-corona mainly consisted of lysenin, an immune related protein, while the silica ENM eco-corona contained distinctly different proteins.[55] These coronal variations reflect differences in organismal response to the presence of, and interaction with, the ENMs. Silver ENM induced lysenin secretion, which, in turn, increased ENM uptake by immune cells to protect other cells and tissues from silver ENM interaction. Further temporal transcriptional analysis revealed that the initial up-regulation of lysenin was eventually suppressed,

followed by induction of genes involved in metal stress and immune signalling.[60] Combined with insights from the aquatic studies, results highlight the interplay between ENMs and organisms, which forms the eco-corona, transforms ENMs, and alters toxicity.

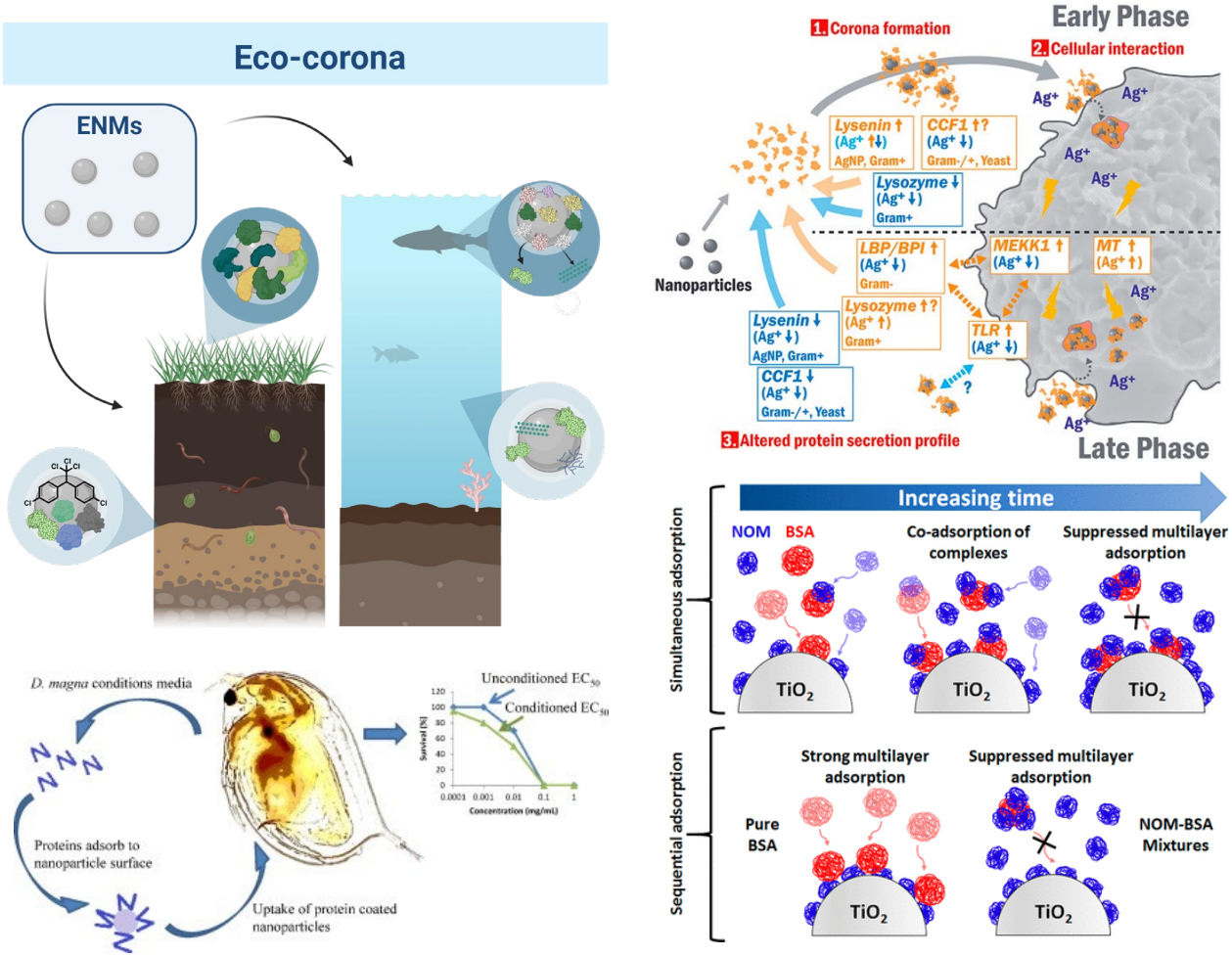

Figure 3. Eco-coronas can form outside or inside organisms. (a) In both terrestrial (left) and aquatic (right) systems the eco-corona can form outside of an organism (top dark blue circles) or within an organism (bottom light grey circles), resulting in very different corona compositions and features influencing corona formation. When formed within an organism, the eco-corona predominantly consists of proteins with minor contributions from other molecules. Characterisation of the corona of internalised ENMs elucidates ENM transport and metabolic response. When formed outside, but near an organism, the eco-corona includes exoproteins and other molecules such as NOM and exopolysaccharides. These secreted molecules in the eco-corona provide insight into biological response to ENMs in the environment. **(b)** *D. magna* proteins in the eco-corona indicate sensing and heightened stress response. The eco-corona increases ENM uptake leading to lowered feeding. Reprinted from reference,[51] with permission from Elsevier. **(c)** A similar story in *E. foetida* shows secreted proteins increase ENM uptake and induce stress and immune signaling. Reprinted from reference[60] by permission of the publisher (Taylor & Francis Ltd.). **(d)** Competitive

adsorption results in a dynamic intermolecular interaction when NOM and BSA form the eco-corona on TiO_2 ENMs. When NOM and BSA co-adsorb (top), complexation of NOM to BSA prior to adsorption hinders any subsequent multilayer adsorption after the ENM surface is saturated. By comparison, sequential adsorption of NOM and BSA leads to different results, whereby BSA readily overcoats NOM, but multilayer adsorption is suppressed upon complexation of NOM to BSA. Reprinted with permission from reference [22] (copyright 2018 American Chemical Society).

Corona formation upon nanomaterial internalization. Most protein corona studies have been performed with endogenous biofluids to provide insights into the determination of ENM fate, transport, and toxicity within the organism. The protein coronas from fish or fish cells have been characterised, including rainbow trout gill cells,[42] zebrafish,[61] and plasma of smallmouth bass.[62] In all three, the protein corona analysis revealed mechanisms of toxicity or cellular / organismal stress. In a particularly creative study with intact rainbow trout gill cells,[42] subcellular fractionation and subsequent recovery of silver ENMs from intact subcellular compartments enabled characterisation of the protein corona at varying stages of ENM processing and storage. This evolving fingerprint marked the trail of silver ENM processing by cells and identified mechanisms of metabolic stress response through identification of specific stress response proteins.

Not all protein coronas are easily characterised due to incomplete proteome databases, a high dynamic range of protein concentrations, and high salt concentrations, such as those present in *M. galloprovincialis* hemolymph. Despite these challenges, corona characterisation can still provide important insights into biological response to ENMs. As filter feeders, *M. galloprovincialis* are a keystone species in aquatic ecosystems and have been the focus of several protein corona studies, including the *M. galloprovincialis* hemolymph protein coronas acquired by polystyrene and cerium oxide ENMs.[43,50] The cerium oxide ENM coronas contained the enzyme Cu, Zn-superoxide dismutase, an antioxidant that converts superoxide radicals to molecular oxygen and hydrogen peroxide. The identification of superoxide dismutase is consistent with other observations of oxidative stress and inflammation in response to contaminants, including ENMs.[63,64]

Interplay of proteins with other molecules in the corona. Coronas formed around ENMs as they enter an ecosystem, or organism, incorporate molecules beyond proteins. This heterogeneous molecular mixture includes other components, such as organic matter or small molecule metabolites, that influence the structure of proteins interacting at the surface and the packing of molecules within the corona.

Prior to interaction with a living system, ENMs encounter natural inorganic (e.g. ions, minerals) and organic (e.g. humic and fulvic acids) colloids to form an environmental corona. Although creative approaches are under development to characterise other small organic compounds within the corona,[11,12,65,66] most NOM and DOM is too diverse to identify, monitor, and characterise in as much detail as proteins. Yet, initial, controlled studies of the protein corona formed within the context of organic matter have revealed the importance of considering these macromolecules when defining mechanisms of corona formation (**Figure 3d**). When two types of food grade TiO_2 ENMs were compared, the phosphate groups at the surface of TiO_2 ENM prevented BSA unfolding. Yet, on TiO_2 ENMs pre-coated with oxalate, a model for dissolved organic carbon, BSA unfolded on both ENM surfaces, albeit through different unfolding pathways.[21] In a second study investigating TiO_2 ENMs and BSA,[22] mixtures of NOM and protein co-adsorbed to the ENM surface in a monolayer. However, sequential exposure to the NOM and protein resulted in multilayer formation. This corroborates a more recent study of corona formation on nanoplastics, where different types of NOM led to varying protein adsorption.[10]

As the ENM nears an organism, an eco-corona forms, including a complex mixture of polysaccharides, proteins, lipids, nucleic acids and other biological molecules excreted/secreted by the organism.[10,50,51,56] This eco-corona is most likely composed predominantly of proteins, although the complete corona will also include lipids and metabolites.[11] Similar to recent work in the biomedical corona arena, expansion to characterisation of other biomolecules within the corona is key, including lipids,[67] polysaccharides,[65] and metabolites,[11,12,52] since they appear to also influence the ENM biological identity.[11] Furthermore, DNA and RNA have also been found to be incorporated into the corona from serum,[68,69] thus potentially acting as a method of genetic material transfer between species.

Future studies that broaden the diversity of proteins, ENMs, and heterogeneous molecules within the corona will enable a more complete mechanistic insight into corona formation and evolution. Both controlled (single type of protein and organic matter) and complex studies (full ecosystem) will be necessary to build molecular level insights into mechanisms of heterogeneous corona formation and evolution.

Corona mediation of nanomaterial transfer between organisms. As ENMs transfer between environmental domains and organisms, the eco-corona evolves. Like all biological systems, the eco-corona is dynamic, changing with time, evolving protein concentrations, and metabolic conditions. For example, some proteins have a high affinity for the ENM surface or exist within layers upon the ENM, while others readily exchange.[3,30,31] Changes in mixing or flow alter the populations within the protein corona[32–34] and may reveal a cryptic epitope that triggers

inappropriate cell signaling.[70] In one of the few *in vivo* studies, the protein corona formed within 10 min of injection into blood circulation continued to fluctuate with time in terms of the abundance of each protein within the population,[31] reflective of the highly dynamic protein binding kinetics in living systems. In the eco-corona, photooxidation causes a breakdown of humic acids, highlighting the role of weather and seasonal conditions on the stability and composition of the eco-corona.[4] Thus, each corona characterisation must be understood as providing just a snapshot in time, and a complete understanding of the system is only available through a series of comparative analyses.

Protein corona studies are motivated by the hypothesis that the corona composition reflects the biological identity of the ENMs in a specific exposure scenario. Protein coronas that confer a "self" identity to an ENM elicit less of an immune response, and often increase uptake of an ENM.[51,55,61] By contrast, protein coronas with biomolecules from other organisms or species alter the ENM uptake / toxicity.[43,51,54,55] For example, in the earthworm *Eisenia fetida*, the native protein corona increased silver ENM recognition and uptake, as compared to ENMs with a pre-formed non-native fetal bovine serum (FBS) corona (**Figure 4**).[55] The biological identity of the protein corona can also be sex-specific.[61,62,71] *Danio rerio* blood cells preferentially accumulated ENM with female blood coronas over male or FBS coronas.[61] In *Micropterus dolomieu*, egg-specific proteins were identified only in coronas formed using female plasma, suggesting a mechanism for reproductive toxicity of silver ENMs by promoting accumulation of these proteins in developing oocytes.[62] The composition of the eco-corona is thus reflective of the microenvironment of the ENM throughout its lifetime, with proteins exchanging as ENMs are sorbed to, or internalised by, an organism or undergo trophic transfer.[72–74]

As ENMs pass from one system to another, the corona evolves, but some corona proteins persist,[14,35,75,76] reminiscent of an ENM history or memory.[77] On a short timescale, the corona reaches equilibrium as highly abundant proteins with higher dissociation rates are readily displaced by those less abundant, but with lower dissociation rates.[14] As solution conditions and biomolecular concentrations change, additional exchange ensues. In a study of silver ENMs in blood plasma, significant changes to pH and temperature resulted in retention of roughly 40 - 50% of the proteins within the corona.[76] In ENMs passed from plasma to cytosolic fluid, the protein corona evolved significantly, but retained a fingerprint from plasma indicative of the original ENM exposure.[77] In the aforementioned *E. fetida* study, ENMs pre-coated with an FBS corona undergo corona exchange to acquire excreted *E. fetida* proteins.[55] This new hybrid protein corona increased cell-ENM response. This exchange within the protein corona

reflects the exchange that occurs upon trophic transfer of an ENM. Study of persistent proteins, or coronal memory, in situations of trophic transfer involves proteins from different species, which is easier to follow experimentally than in a single species. In that sense, exploration of memory and exchange within ENM eco-coronas are likely to progress faster than the biomedical protein corona field, and modelling using molecular dynamics[16,78] or machine learning[79,80] approaches will likely play a pivotal role in predicting eco-corona exchange and its impacts on higher level organisms.

Differences between protein coronas may not solely lie in the protein identity. Biomarkers such as PTMs are often overlooked, but are a key to biological identification. For example, FBS and human serum contain proteins similar in quantity and homology. Yet, silica ENM toxicity increased nearly four-fold when ENMs were pre-coated with fetal bovine (vs human) serum proteins.[81] Among other differences, organismal identifiers such as PTMs on proteins in bovine and human serum mediate identification, uptake, and toxicity. PTMs have a demonstrated role in mediating corona formation[24] and cellular response. Just as PTMs can play a role in disease specific design of ENMs for biomedical applications,[26,82,83] they also deserve attention in ecotoxicity and design of ENMs for agricultural applications.

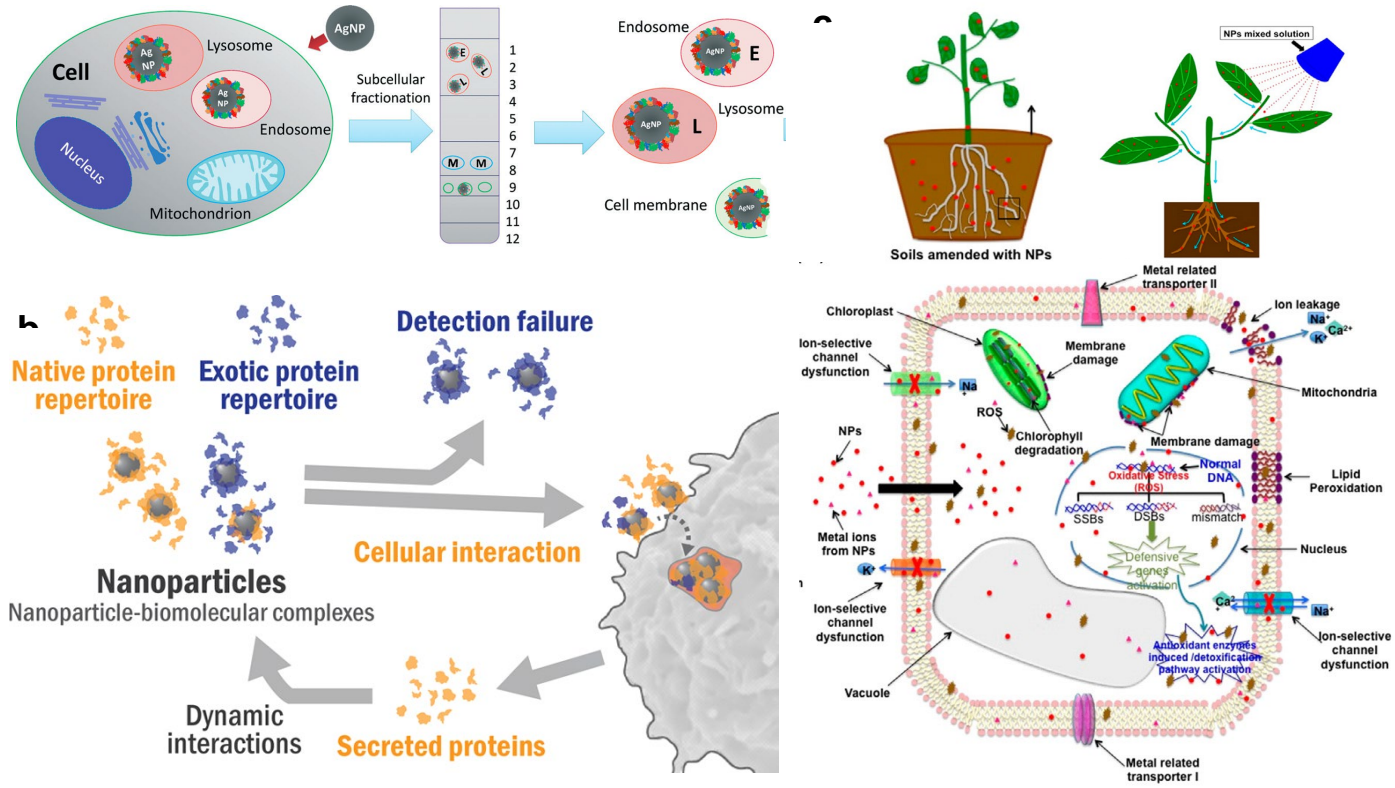

Figure 4. Protein coronas are formed upon ENM internalisation by an organism. (a) Studies of the corona provide insight into cellular transport of ENMs. Reproduced from reference[42] with permission from the Royal Society of

Chemistry. **(b) The evolving biological identity of the ENM provides a snapshot in time.** Reprinted with permission from reference.[55] Copyright 2018 American Chemical Society. **(c) The corona mediates organismal transport and cellular response.** Reproduced from reference[84] by permission of the publisher (Taylor & Francis Ltd).

Challenges and opportunities

Protein corona studies thus far have used broad strokes to outline key features mediating corona formation, transformation, and impact on ENM environmental fate. To achieve the ultimate goals of (i) controlling corona composition in order to direct ENM fate and effects, (ii) to enabling ENM fate prediction, and (iii) facilitating ENM design for safe environmental applications, we recommend moving toward coordinated, systematic evaluations of corona formation (**Figure 5A**). In parallel, evaluation of the ENM corona within key organisms, or across model mesocosms, will provide focus and drive the advances in experimental techniques necessary to establish deep knowledge in key systems.

Expanding the toolset

As design principles for using the corona to target ENMs emerge from the biomedical field,[16,85–87] the application of these paradigms is challenged in environmental systems by the current lack of biodiversity of reported coronas, the enormous variations in biological matrices,[88] and environmental transformation of ENMs. To leverage the full potential of the experimental work, comparative studies and emerging computational methods will be required. In silico predictions of protein-ENM binding interactions are established, but with limited domains of applicability currently.[16] Machine learning[79,80,89] also holds promise to expand beyond the experimentally accessible variables and assess features most influential to corona formation. Although machine learning approaches have demonstrated strength, the databases upon which they are built are limited. A shared, publicly available database for experimental characterizations of the corona would strengthen modeling. With ontologies and reporting practices established, formation of such a database is straightforward. As emerging experimental techniques lessen the cost of corona characterization and increase throughput,[16,90] the breadth of proteome and secretome characterisations across organisms, ENMs, and environmental conditions will advance comparative analyses and predictive insights into corona formation. Such computational approaches can also be applied to coronal characterisation for prediction of cellular interaction and uptake.[80,129]

As techniques expand for ENM tracking in the environment and monitoring in situ in complex environmental samples, corona mediation of organismal response, ecosystem transport including up the food chain, and utility in ENM targeting can stretch beyond a few model systems. The push toward more *in vivo* studies parallels that of protein corona studies in the biomedical community,[91] but similar care must be taken to design reproducible and quality analyses that ensure, for example, that the corona characterised is indeed the one present in situ in the organism or subcellular location.[27,92] While the environmental field is well aware of the importance of in situ studies to further understanding of biological response, experimental limitations include a lack of techniques to recover ENMs after exposure and protocols for sample processing that minimize coronal disturbance, guaranteeing that the characterized corona is equivalent to the one formed in the environment.

Overcoming the challenge of biodiversity. One of the fundamental challenges facing corona studies is the incredible diversity of biomolecules in the environment.[55] Recent estimates puts the global number of prokaryote species at 8.7 million,[93] and while many proteins are conserved between closely related species there are an array of unique proteins in each species and domain of life. To date, proteomic libraries are limited to a small but growing

number of species, with 19147 reference proteomes in Uniprot in February 2021.[94] Proteomic databases are limited, but include important model organisms and keystone species, such as *D. magna,*[51,66] *Saccharomyces cerevisiae*,[35] and *E. coli*.[95] When reference proteomes are unavailable, expanded biodiversity can be obtained with creative approaches such as that taken by Gao *et al.* to examine the protein corona formed on silver ENMs in *M. dolomieu* plasma.[57] By focusing on common plasma proteins, they expanded their database search to well conserved homologs in the phyla. This expanded biological breadth must be balanced with accuracy and confidence in protein identification, which is eased through clear and open reporting[27] to enable reanalysis if a reference proteome becomes available later. Despite the seemingly small differences between plasma proteins across species, a recent study demonstrated that the species of origin of plasma proteins influenced both the proteins in the corona, as well as ENM stability and agglomeration.[96]

ENMs predominantly enter the ecosystem via soils and landfill sites, and to a lesser extent from wastewater treatment plants (WWTP) and airbourne release (**Figure 5B**).[97,98] In the case of soils, ENMs are either directly applied as nano-fertilisers and nano-pesticides, or inadvertently distributed through WWTP sludge used in agriculture as a fertiliser.[99,100] Soils contain a rich source of biomolecules, from plant debris to humics. The rhizosphere in the root systems of plants also contains a diverse array of microorganisms and secretions from plants,[101] contributing a complex mixture of biomolecules for eco-corona formation. Given the rising interest in nano-enabled agricultural products, the relative lack of protein corona studies in plants is surprising. Crops such as maize, rice, and soybeans, for example, have reference proteomes in Uniprot and are of interest for their different root structures and associated rhizospheres while representing major staple crops for a significant proportion of the human population. The corona formed in leaves, soils, and from rhizospheres are currently unexplored, but elucidation of their composition will enable prediction of ENM uptake, development of plant sensors, and toxic response within major agricultural crops to help maintain food security with the increasing prevalence of ENMs in agricultural soils.[9,102–104] Moreover, just as biomedical studies have identified proteins that target ENMs to key organs, so corona studies in plants can enhance efficacy of biopesticides through targeting and control of the corona. Further analysis of the role of the corona in ENM trophic transfer may also lead to guided reactivity of ENMs within specific plants or symbiotic microbes.

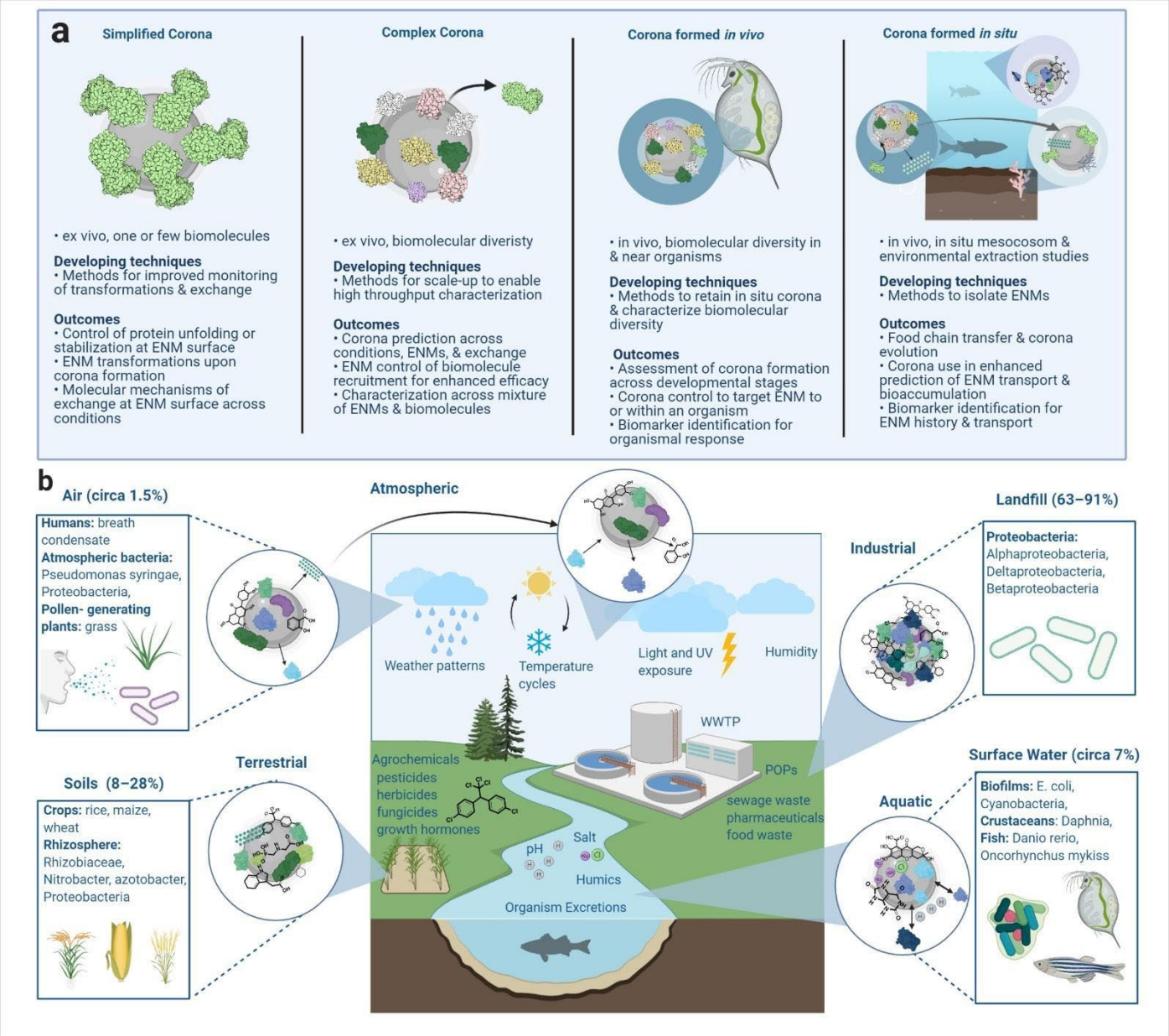

Figure 5. (A) Unlocking the opportunities of the environmental ENM corona requires studies that span size scales and levels of complexity. From left to right, corona studies evaluate individual ENMs and proteins, biomolecular diversity in the corona, *in vivo* response to ENMs by individual organisms, and corona exchange with mesoscale transport. As techniques are developed at the molecular scale, they can be applied to enhance insights at the organismal and mesocosm scale, where studies are just emerging. As studies tackle greater complexity, the community will elucidate further applications for the corona in the field. **(B) Eco-coronas form and transform as ENMs enter and move between industrial, aquatic, terrestrial, and atmospheric environments.** Within each environment, the ENM eco-coronas will consist of different concentrations of proteins, subsets of other relevant biomolecules, and conditions. Suggested matrices are provided in boxes for environmental and eco-corona evaluation based on models of environmental ENM sinks. A few example organisms of interest, that have a

reference Uniprot proteome database, are also provided within the boxes. The percent (%) of industrially produced ENMs ending up in each environment is shown in parenthesis above the boxes.[98]

As ENMs enter various ecosystems, the eco-corona formed will incorporate very different molecular components. This diversity presents unique challenges to characterisation and results in differing downstream effects. Much of the early work presented here on eco-coronas has focused on aquatic systems, where researchers built upon the body of literature from environmental coronas and protein coronas to explore ENM transformations in natural waters.[10,105,106] This work sets the precedent as the field expands to other environmental areas. In landfill sites, for example, a diverse array of biomolecules from food stuff, bacterial growth, and scavenging animals such as rodents are likely to be prevalent,[107] along with chemicals from household waste such as plasticisers and flame retardants. Rainfall may transport ENMs from landfill to soil or surface waters. Although the molecular diversity of these ecosystems present challenges for characterisation, this scenario may best enable the "Trojan horse" effect, whereby ENMs concentrate and transport toxins into organisms and the environment. An unexplored question to date is whether naturally occuring nanoscale particles present in the environment, such as silica or titania, display coronas that differ from those acquired by ENMs.

A small percentage of ENMs enter the atmosphere from sources such as car exhaust.[97,98,108] In the case of plastics, this may be higher as microplastics have been found several kilometers up in the atmosphere.[109] With regards to human health, the protein corona of atmospheric (e.g combustion and pollen) particles are extensively studied through characterisation in bronchial lavage fluid.[108,110] Prior to interacting with humans, nothing is known about the eco-corona of these particles. The prevalence of airborne microbes have been extensively characterised,[111,112] all of which produce proteins and present them in cell membranes or even secrete them.[113] Importantly for eco-corona studies, these organisms are proteomically well-characterised, making them surprisingly accessible for future studies of atmospheric eco-corona formation, role in cloud nucleation, and potential downstream impact on the protein corona formed if the ENMs reach our lungs. Furthermore, the co-adsorption of airborne contaminants such as volatile hydrocarbons to ENMs has not been investigated to date as a potential route of exposure for humans.

Dynamic environmental conditions. Since protein, or eco-coronas, can form both endogenously and exogenously, they encounter a wide range of environmental conditions with significant impacts upon ENM stability

and evolution of the corona composition. Until experimental approaches to isolate ENMs improve and corona characterisation becomes more cost effective, studies of corona evolution and transport across organisms will require high ENM concentrations, well above those expected from accumulation. Analysis of depurated ENMs and their associated coronas may provide a first approach reflecting organismal responses to ENMs.

To date, no studies have thoroughly investigated the aging of the corona using realistic environmental conditions. Given that ENMs are exposed to extremes of temperatures, including freeze-thaw cycles on ENM, the eco-corona may offer insights into the longevity of both the particles and their coronas; similarly, UV exposure from sunlight, which is already known to affect ENMs, may affect corona stability although no corona studies under varying UV radiation have been performed as yet. A major route of environmental release of ENMs is via waste water treatment plant;[98] studies have begun to investigate how these process impact upon the ENM[114] and to model ENM transport in water ways.[115,116] The abundance of biomolecules passing through WWTPs, and in waterways themselves, offers a significant source of biomolecules to form the corona and a source of mechanical modification to ENMs as they flow along river beds with varied salinity, pressure, temperature, light penetration, DOM concentrations, and oxygenation from rivers to oceans. All of these influence both the formation and stability of the ENM corona. Finally, the passage of ENMs through organisms with their acquired coronas may lead to increased biomolecule diversity and further evolution of corona composition. It is unknown how these changing conditions affect the longevity or biodistribution of ENMs released via WWTP or other means into the world's waterways, offering an interesting future direction for eco-corona research.

Expanding choice of nanomaterials. As the study of environmentally relevant protein coronas emerges, there are clear trends in the types of ENMs chosen for environmental versus biomedical corona studies (**Figure 6a**). Not surprisingly, the spectrum of ENMs overlaps with silica, silver, and plastics in the top five ENMs studied under both categories. Yet, the ENMs evaluated for biomedical applications are more diverse, including proportionally many more studies of gold, iron, and lipid particles, while environmental corona research has addressed ENMs considered to be highly toxic (e.g. quantum dots), widely released (e.g. CeO_2) and biocidal materials (e.g. Ag / Cu). Clear gaps exist in the dataset for corona studies on iron or iron oxide particles and graphene and other carbon-based materials, despite their widespread application in environmental remediation. Biocidal ENMs and others under development for agricultural applications should be characterised. Comparison of engineered particles to natural or anthropogenic particles could shed light on the role of “fresh surfaces” and structural features such as crystal phase

in mediating corona formation and downstream reactivity.[117,118] Moreover, micro and nano-plastics are now ubiquitous in the environment[119] and have been identified in a wide range of organisms from marine and terrestrial environments.[120–122] Despite the current lack of corona characterisations on plastic particulates in marine and freshwater environments, the corona also plays a significant role in the adsorption, distribution, biotransformation, and fate within organisms upon exposure to plastic particles.[51,123] As reservoirs for organic pollutants, plastic particles and their coronas require additional study to evaluate the corona influence on bioaccumulation and toxicity of organic pollutants such as phenanthrene,[124] BFRs,[125] PAHs, PCBs and PBDEs.[126]

Future eco-corona studies require improved decision making on the choice of ENM investigated to ensure environmental relevance and enable them to more precisely inform regulatory guidance and safe-by-design approaches to ENM manufacture and use. The release of ENMs into the environment from textiles, paints, pigments and cosmetics has been researched previously [97,98,127] (**Figure 6b**) and highlights a different suite of ENMs compared to those shown in **Figure 6a**, many of which will be deposited in a heterogeneous mixture. Of greatest environmental concern are TiO_2 ENMs, which enter the environment from paints, pigments, cosmetics and nanofertilizers. TiO_2 is also studied extensively as an environmental risk due to its longevity in the environment.[97] Other ENMs such as ZnO, Al_2O_3 and CeO_2 are also under-represented in the general corona literature and especially in the environmental setting. Only SiO_2 is already extensively characterised, but most studies are approached from a biomedical perspective, using human or bovine blood products. Microplastics and nanoscale plastic debris are a growing concern, deserving additional focus. These particles and fibres are found ubiquitously in the environment and the organisms that inhabit it. Moving forward, a basic science approach is needed to prepare the field for consideration of the next generation of materials, such as the metal oxide hybrid materials used in batteries,[128] or to consider lower concentrations and heterogeneous mixtures of ENMs that better replicate environmental exposure scenarios.[103]

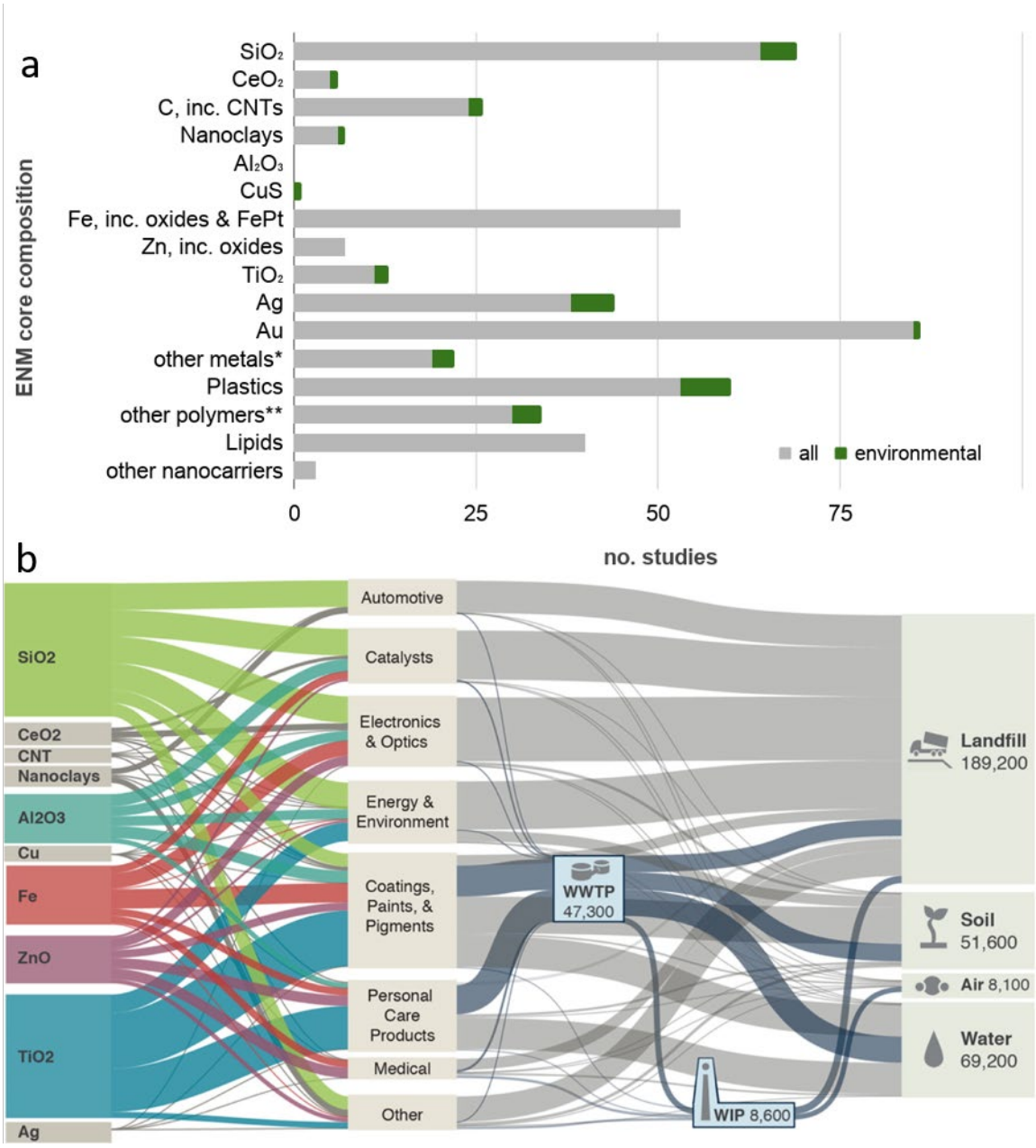

Figure 6. Analysis of the diversity of ENMs used in protein corona studies from 2007-2019. (a) Studies focused on environmental systems are highlighted in green, including any study with protein sources that are not human, bovine, equine, porcine, rat, monkey, or mouse. Manuscripts were searched using SciFinder using the search terms protein corona and nanoparticles. CNTs are carbon nanotubes. Other metals(*) include Co, Gd2O3, quantum dots, apatite, and Pt. Other polymers(**) includes biopolymers. **(b)** Estimated global mass flow of ENMs (in metric tons per year) from production to disposal or release, considering high production and release estimates from 2010. Reprinted with permission from reference.[127] Copyright 2014 American Chemical Society.

Conclusion and perspectives

As we expand protein corona studies into environmental matrices, we've begun to establish the molecular mechanisms of ENM and protein transformations that mediate ENM biotransformation and biochemistry. Clear differences in composition are described in terms of the eco-coronas formed outside (exogenously) versus inside (endogenously) organisms, but both styles of studies provide insight into organismal response to ENMs and can inform ecotoxicity. To inform broader insights, it is necessary to tackle the challenges of biodiversity and evolving local exposure conditions, while expanding the range of ENMs studied to include those most relevant to the environment. The continuously evolving nature of the environment and eco-coronas offer exciting opportunities to investigate the role and impact of coronal memory and the potential for disruption of proteostasis resulting from release of non-native proteins during corona evolution inside an organism. Here, multi-species studies enable easy parsing of proteins during exchange, allowing the eco-corona field to lead the way in elucidating corona exchange and memory and its impacts for ENM health and safety.

Understanding the dynamics of the eco-corona has implications for ENM adsorption, distribution, biotransformation, and fate within an organism or ecosystem. Moreover, it offers an intriguing possibility to track the transport of ENMs through the environment and the food chain to aid modeling of ENM transport and distribution. Expanding our knowledge of eco-corona formation, stability, and interactions across species and locales will enable development of more reliable and precise models of ENM transport and ecotoxicity, while better informing regulatory bodies to implement more robust and relevant guidance on the safe design, use, and disposal of ENMs, including for environmental applications such as remediation and agriculture.

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Authors declare no competing interests.

Figure legends

Figure 1. (a) Comparison of the protein, environmental, and eco-coronas. Formed within organisms at locations of high protein content, the term protein corona has been used to describe the binding of proteins to ENM surfaces, but also incorporates lipids, metabolites (typically <1000 Da which are either reactants, intermediaries or products of enzymatic processes), and other biomolecules. To date, the term environmental corona has described a corona formed in aquatic environments with high concentrations of NOM, including humic substances. By contrast, the eco-corona incorporates features of both the protein and environmental coronas, where the balance of proteins and other molecules varies. **(b) The evolution of the protein corona concept,** adapted from Hadjidemetriou and Kostarelos.[17] Studies of protein adsorption to surfaces and particles dates back to at least the 1960s. The term protein corona was first coined in 2007. Protein corona studies developed with mass spectroscopy based proteomics to aid identification of the proteins bound at the surface of ENMs and explore the role of surface curvature in altering protein structure and function relative to macroscale surfaces. Protein corona studies evolved in parallel with those on the environmental corona, but the characterisation techniques and goals for each area remained separate, with environmental corona focusing mainly on the dispersion stabilisation provided by NOM. The environmental dimensions of the protein corona began to appear later, as the concept of the eco-corona and its role in (nano)ecotoxicity emerged. Both the eco-corona and biomolecular corona embrace the diversity of molecules in solution with the goal of understanding and controlling downstream biological responses to nano-enabled technologies.

Figure 2. Properties that control protein corona formation. (a) Physicochemical interactions that mediate eco-corona formation, modified from Markiewicz et al.[4] with permission from the Royal Society of Chemistry. The mechanisms of interaction between proteins and ENMs include hydrophobic interactions involving nonpolar amino acids, protein-protein interactions, electrostatic attraction or repulsion, cation bridging, ligand exchange, hydrogen bonding, chelation, and displacement of proteins by other biomolecules. Such interactions may lead to denaturation of adsorbed proteins on the ENM surface. **(b). Examples of ENM (top right of circle), solution (bottom of circle), and biomolecular (top left of circle) properties that mediate corona formation.** The properties better understood because of their relevance to human health are included in the inner circle (tan) and those of priority for the environmental dimensions of the protein corona, but less explored, are included in the outer circle (pale blue).

Figure 3. Eco-coronas can form outside or inside organisms. (a) In both terrestrial (left) and aquatic (right) systems the eco-corona can form outside of an organism (top dark blue circles) or within an organism (bottom light grey circles), resulting in very different corona compositions and features influencing corona formation. When formed within an organism, the eco-corona predominantly consists of proteins with minor contributions from other molecules. Characterisation of the corona of internalised ENMs elucidates ENM transport and metabolic response. When formed outside, but near an organism, the eco-corona includes exoproteins and other molecules such as NOM and exopolysaccharides. These secreted molecules in the eco-corona provide insight into biological response to ENMs in the environment. **(b)** *D. magna* proteins in the eco-corona indicate sensing and heightened stress response. The eco-corona increases ENM uptake leading to lowered feeding. Reprinted from reference,[51] with permission from Elsevier. **(c)** A similar story in *E. foetida* shows secreted proteins increase ENM uptake and induce stress and immune signaling. Reprinted from reference[60] by permission of the publisher (Taylor & Francis Ltd.). **(d)** Competitive adsorption results in a dynamic intermolecular interaction when NOM and BSA form the eco-corona on TiO_2 ENMs. When NOM and BSA co-adsorb (top), complexation of NOM to BSA prior to adsorption hinders any subsequent multilayer adsorption after the ENM surface is saturated. By comparison, sequential adsorption of NOM and BSA leads to different results, whereby BSA readily overcoats NOM, but multilayer adsorption is suppressed upon complexation of NOM to BSA. Reprinted with permission from reference [22] (copyright 2018 American Chemical Society).

Figure 4. Protein coronas are formed upon ENM internalisation by an organism. (a) Studies of the corona provide insight into cellular transport of ENMs. Reproduced from reference[62] with permission from the Royal Society of Chemistry. **(b) The evolving biological identity of the ENM provides a snapshot in time.** Reprinted with permission from reference.[55] Copyright 2018 American Chemical Society. **(c) The corona mediates organismal transport and cellular response.** Reproduced from reference[84] by permission of the publisher (Taylor & Francis Ltd).

Figure 5. (A) Unlocking the opportunities of the environmental ENM corona requires studies that span size scales and levels of complexity. From left to right, corona studies evaluate individual ENMs and proteins, biomolecular diversity in the corona, *in vivo* response to ENMs by individual organisms, and corona exchange with mesoscale transport. As techniques are developed at the molecular scale, they can be applied to enhance insights at the organismal and mesocosm scale, where studies are just emerging. As studies tackle greater complexity, the community will elucidate further applications for the corona in the field. **(B) Eco-coronas form and transform as ENMs enter and move between industrial, aquatic, terrestrial, and atmospheric environments.** Within each environment, the ENM eco-coronas will consist of different concentrations of proteins, subsets of other relevant biomolecules, and conditions. Suggested matrices are provided in boxes for environmental and eco-corona evaluation based on models of environmental ENM sinks. A few example organisms of interest, that have a reference Uniprot proteome database, are also provided within the boxes. The percent (%) of industrially produced ENMs ending up in each environment is shown in parenthesis above the boxes.[98]

Figure 6. Analysis of the diversity of ENMs used in protein corona studies from 2007-2019. (a) Studies focused on environmental systems are highlighted in green, including any study with protein sources that are not human, bovine, equine, porcine, rat, monkey, or mouse. **(b)** Estimated global mass flow of ENMs (in metric tons per year) from production to disposal or release, considering high production and release estimates from 2010. Reprinted with permission from reference.[127] Copyright 2014 American Chemical Society.