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Ecotoxicological significance of bio-corona formation on micro/nanoplastics in aquatic organisms

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The unsustainable manufacturing, utilization and inadequate handling of plastics have led to a surge in global plastic pollution. In recent times, there has been increasing concern about the plausible hazards associated with exposure to micro/nanoplastics (M/NPs). As aquatic systems are considered to be the likely sink for M/NPs, it is crucial to comprehend their environmental behavior. The bioavailability, toxicity and fate of M/NPs in the environment are predominantly dictated by their surface characteristics. In the aquatic environment, M/NPs are prone to be internalized by aquatic organisms. This may facilitate their interaction with a diverse array of biomolecules within the organism, resulting in the formation of a biocorona (BC). The development of BC causes modifications in the physicochemical attributes of the M/NPs including changes to their size, stability, surface charge and other properties. This review details the concept of BC formation and its underlying mechanism. It provides insight on the analytical techniques employed for characterizing BC formation and addresses the associated challenges. Further, the eco-toxicological implications of M/NPs and the role of BC in modifying their potential toxicity on aquatic organisms is specified. The impact of BC formation on the fate and transport of M/NPs is discussed. A concise outlook on the future perspectives is also presented.

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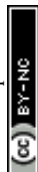
1. Introduction

Plastics are organic polymers characterized by their substantial molecular mass and synthesized through the polymerization of monomers derived from petroleum, natural gas or coal.^{1,2} Plastics find extensive application in daily life owing to their cost-effectiveness, resilience and lightweight nature.^{3,4} In 2019, worldwide plastic production surged to 368 million metric tons (Mt) and it is projected to undergo a twofold increase within a span of 20 years.^{5,6} Current estimates indicate that the accumulation of plastic waste in the environment is projected to surpass 250 million tons by the year 2025.⁷ According to estimates, only 9% of the plastic waste produced globally has undergone recycling, with 12% being incinerated, resulting in a substantial 79% being accumulated in the environment.⁸ The COVID-19 pandemic has exacerbated the generation and mis-handling of plastic waste.^{9–12} Upon introduction into water systems, a substantial proportion of plastic debris undergoes fragmentation or degradation. Consequently, this results in the formation of plastic fragments known as mesoplastics (>5 mm and <25 mm), and further degradation leads to the generation of microplastics (<5 mm) or nanoplastics (<1 mm or 1000

nm).^{6,13} This phenomenon carries inherent environmental risks that have captured global attention.¹⁴ In recent years, there has been a progressive increase in the abundance of micro- and nanoplastics (M/NPs) within aquatic environments.¹⁵ Primary M/NPs refer to the particles deliberately manufactured with multiple sizes, surface charge and incorporated into various products.^{16,17} On the other hand, secondary M/NPs are generated through physical or chemical fragmentation of larger plastic particles within the environment, as well as through biological processes such as digestion by the macro-invertebrates.^{18,19} In addition, M/NPs present in the aquatic ecosystems could interact with a wide range of organic compounds, pollutants and other environmental components through adsorption and desorption processes.^{20,21} These interactions have significant implications on the bioavailability, degradation, toxicity fate and transport of both MPs/NPs and the associated compounds.^{22–24} The interactions between M/NPs and various environmental components have gained substantial attention, and thus, intensive research is carried out in this area. The interactions between M/NPs and environmental components are affected by a range of intrinsic factors, which include the properties of M/NPs and environmental components, and extrinsic factors such as pH, temperature and ionic strength.^{25,26} Besides, the hydrophobic surface and the specific surface area of M/NPs enable them to competitively adsorb organic biomolecules.¹⁴ The term “corona” refers to the substances adsorbed on the fine particulates, and it is

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comprised of both “hard” (hardcorona) and “soft” (soft corona) layer.^{27,28} The hard corona is typically composed of a monolayer of tightly bound molecules that forms within the first few minutes of the particle’s interaction with its surrounding media. The soft corona, on the other hand, consists of loosely bound molecules that cover the hard corona. Unlike the stable hard corona, the components of the soft corona are unstable and can quickly exchange with the surrounding environment.²⁸

Natural organic matter (NOM) can undergo adsorption onto M/NPs and results in the formation of a biomolecular-coated layer referred to as the ecocorona (EC).²⁵ NOM is an intricate blend of organic compounds encompassing humic and fulvic acids, proteins, lipids, carbohydrates, and various other organic substances.²⁹ Similarly, when M/NPs come into contact with biological fluids or biological systems, a layer of biomolecules forms on their surface, which is commonly referred to as the biocorona (BC).³⁰ Typically, BC comprises diverse biomolecules such as proteins, lipids, nucleic acids and carbohydrates. Further, BC formation has been observed to exert notable influence on the transport, uptake, distribution, biotransformation and toxicity of M/NPs.^{28,30,31} Based on our comprehensive literature survey, we found that there is only one review article by Cao *et al.* that extensively examines the formation of BC formation pertaining to M/NPs.²⁸ Despite the profusion of scientific literature discussing corona formation on nanoparticles, there remains a paucity of review articles that specifically addresses BC formation in the context of M/NPs. Thus, we have endeavored to bridge the knowledge gap by elucidating the mechanisms underlying the formation of BC and explicating its importance in the ecotoxicity, fate and transport of M/NPs. Further, a succinct overview of the analytical techniques employed to characterize BC formation on M/NPs is provided, while shedding light on the challenges encountered in implementing these methods.

2. Ecotoxicological implications of M/NPs on aquatic organisms

Plastic pollution in freshwater and marine habitats has emerged as a serious matter of concern throughout the world.³² M/NPs present in the aquatic environment have the ability to interact with various organic compounds such as drugs, pesticides and other pollutants by means of adsorption and desorption.³³ Such interactions have a profound impact on the environmental dynamics, bioavailability, degradation, transport and toxicity of M/NPs.^{34–37} The diverse adverse effects such as oxidative damage, retarded growth, organelle damage, early mortality, decreased food uptake, inflammation, immune malfunctions and aberrant behavior are the major detrimental effects of M/NPs on the aquatic biota.^{38–43}

2.1 Freshwater systems

Globally, several studies have documented the occurrence of M/NPs in the freshwater systems such as surface waters, rivers, lakes, rivers sediments, reservoirs and drinking water.^{44–48} This abundance and bioavailability of M/NPs serve as a major threat

to the freshwater biota.^{49,50} For instance, several detrimental effects of M/NPs have been observed in the toxicological investigations on zebrafish (*Danio rerio*).⁵¹ Studies have unveiled that zebrafish exposed to M/NPs exhibited diminished food intake, disruption in the intestinal functioning, villi disintegration, excessive ROS production, inflammatory responses and modification in gills and intestinal mucosa.^{52–56} Recently, Hansjosten *et al.* (2022) reported that polystyrene nanoplastics (PS-NPs) of 31.2 mg L⁻¹ inhibited the hatching of zebrafish embryo.⁵⁷ Exposure of PS-NPs with fathead minnow (*Pimephales promelas*) showed that the PS-NPs affected their innate immune response, enhanced oxidative burst and activated the neutrophil function.⁵⁸ Similarly, PS-NPs of 0.1 μm inhibited the brain acetylcholinesterase (AChE) activity in red tilapia (*Oreochromis niloticus*).⁵⁹ Common goby (*Pomatoschistus microps*) juveniles have exhibited less predatory ability and efficiency on exposure to PE for 24 h.⁶⁰ Similarly, 10 days exposure of polyethylene (PE)-MPs to rice midge (*Chironomus tepperi*) larvae significantly decreased the survival rate and diminished the adult emergence rate. Furthermore, it was reported that the uptake of 1 μm PE MPs triggered the inhibition of locomotion in *Daphnia magna*.⁶¹ Additionally, combination toxicity studies on cadmium MPs revealed that the exposure to MPs has elevated the ROS generation and neurotoxicity in the Asian clam (*Corbicula fluminea*).⁶²

2.2 Marine systems

Freshwater and terrestrial habitats are considered to be the sources and pathways through which M/NPs are transported to the marine systems.^{60,63} Several reports highlighted the presence of M/NPs in oceans, marine culture zones, fish farms, salts from oceans and even in the commercial salts.^{64–67} Studies have shown that the M/NPs are frequently ingested by marine organisms and lead to various detrimental effects. For instance, the interaction of MPs with gilthead seabream (*Sparus aurata*) has significantly enhanced the catalase and glutathione reductase (GRD) activity.⁶⁸ Mediterranean mussel (*Mytilus galloprovincialis*) interaction with PE and PS-MPs resulted in the MPs accumulation in their gills, hemolymph and significant inflammatory responses.⁶⁹ Similarly, blue mussel (*Mytilus edulis*) on exposure to PE-MPs showed a denotable histological change and significant inflammatory responses.³⁷ In addition, it was reported that exposure to PE-MPs (11–13 μm) might lead to a significant change in the acetylcholinesterase activity of *Scrobicularia plana* (clam) that pave the way for neurotoxicity.⁷⁰ It was reported that scallop (*Pecten maximus*) uptakes PS-NPs and get accumulated in its digestive tract and tubules followed by deposition in muscles, kidneys and gonads.⁷¹ The toxicological investigation of carboxylated nanoplastics (COOH-NPs) on the larvae of marine rotifer (*Brachionus plicatilis*) for 48 h showed a significant lethal effect.⁷² However, aminated nanoplastics (NH₂-NPs) showed a negligible lethality as they got aggregated at the microscale level. Studies on the NPs uptake behavior of *Artemia franciscana* reveals the presence of NPs in their gut and on the mandible, tail and appendages.⁷³ The results revealed the fecal release of ingested COOH-NPs (5–100 mg L⁻¹), though, the complete excretion was not observed.⁷⁴



The previous reports clearly demonstrate that the characteristics of microplastics, including their type, size, surface charges, and interactions with other organic and inorganic compounds, play a critical role in determining the extent of toxicity imposed upon freshwater and marine organisms. Notably, the interaction between biological molecules and M/NPs would lead to significant variations to the properties of M/NPs. These insights underscore the imperative of comprehensively understanding the intricate dynamics of M/NPs and their interactions in order to assess the implications they might have on the aquatic biota.

3. Biocorona formation on M/NPs

An ecologically relevant encapsulation of particles like plastics, MPs or NPs or non-plastics by various biomolecules such as polysaccharides, proteins, *etc.* is often referred to as an “environmental- or ecocorona”.^{31,75} M/NPs in the aquatic environment can easily interact with the bio-molecules such as nucleic acid and proteins secreted by the aquatic organisms.^{76,77} The particle surface will undergo substantial changes owing to these interactions and this alters the biological reactivity of the particles altogether.^{78,79} Sometimes, the particles can be internalized by the organisms, and they can interact with the biomolecules in the intracellular environment leading to formation of BC.^{80–82} Following their release into the environment, various bio-organics may compete with one another to adsorb the hydrophobic surface of M/NPs.⁸³ BC is also formed over the M/NPs through EPS from the *exo*-proteome (proteins released as a result of signaling and habitat adaptation) or generated through the metabolic activity of aquatic organisms (DNA, carbohydrate, protein and so on).⁸⁴ The formation of EC essentially involved natural/dissolved organic matter (NOM/

DOM), pesticides, persistent organic pollutants (POPs), metabolite adsorption, and on the other hand, BC is all about proteins, metabolites, lipids and polysaccharides.³⁰ Researchers have documented the EC formation on NPs within the culture medium of *Daphnia magna*. Additionally, they have observed the formation of BC within the organism’s physiological fluid after homogenization.^{85,86}

To form BC (Fig. 1), M/NPs interact with the biomolecules through hydrophobic and electrostatic modes.^{87,88} Due to a variety of nanomaterial (NM) usage, particularly in the field of biomedicine, the formation of BC on the surface of NMs has lately received substantial attention.^{89,90} The BC is dynamic in nature, since, the protein adsorbs and desorbs from NM surfaces with time.^{78,91} As a result, the BC develops two distinct layers: (1) the hard corona, and (2) the soft corona.⁹² On the other hand, hard corona, experiences a significant number of conformational changes, sluggish exchange rates, lengthy retention times and high binding affinities.⁹³ Due to differences in the forces involved and the chemical composition, the proteins in the hard and soft corona layers would interact differently in the aquatic environment.^{94–97}

Bio-membranes like vesicles, engaged in various physiological functions such as storage of carbon, protection of tissues and resistance to external chemicals, must have lipids as the necessary component.⁹⁸ The biological role and structure of lipid are presumably less well understood than proteins. In biological systems, there are several lipid derivatives that differ in their hydrophobic tails and hydrophilic heads, including waxes, triacylglycerides, phospholipids, sphingolipids and glycolipids.⁹⁹ Lipids, particularly unsaturated lipids, may be oxidized during their interaction with M/NPs, leading to the production of $-O-$, $-OH$, $-OOH$, and hypochlorite moieties on hydrophobic tails of lipid molecules. These modified lipids

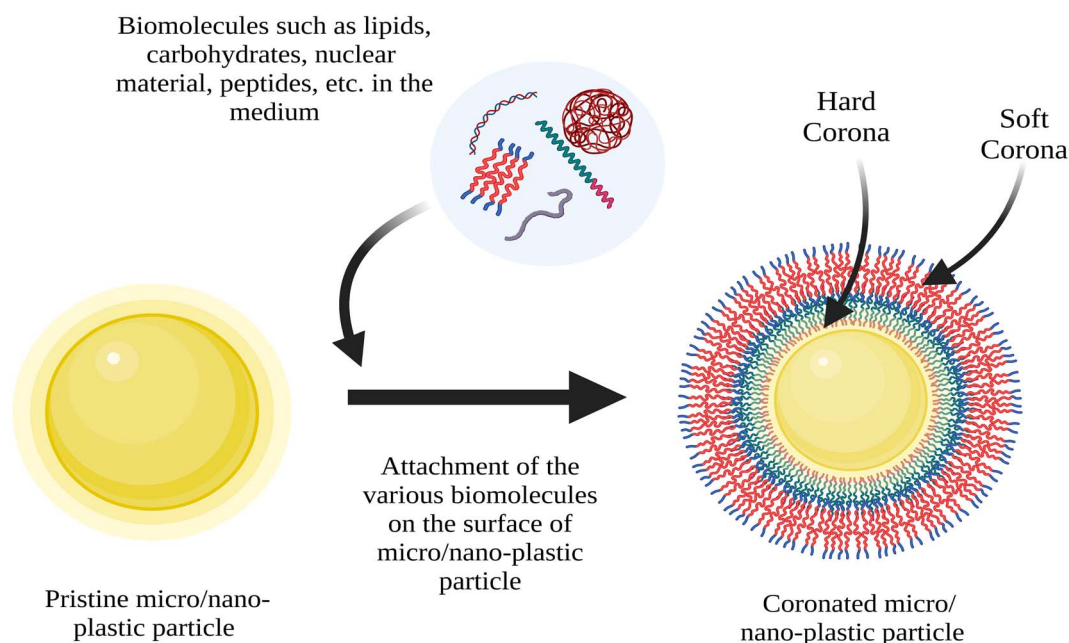


Fig. 1 Formation of biocorona and coronated micro/nanoplastics (M/NPs).



frequently participate in a variety of biological activities. For instance, iron-dependent programmed cell death has recently been linked to ferroptosis, which is activated by lipid peroxides as an essential danger signal.^{100,101}

The surface properties of the pristine or as formed M/NPs, including surface charge and functionalization, play a determinant role in their biological interactions.¹⁰² Presently, reactive functional groups such as sulfonic, carboxylic, phenoxy hydroxyl, alcoholic hydroxyl, carbonyl, hydroxide and amine groups¹⁰³ are noted in the environmental polymers. In addition, multiple chemical groups might attach on the surface of M/NPs during the migration and degradation in the environment. The presence of various surface chemical groups largely dictates the fate and toxicity of M/NPs in the environment. In biological systems, corona formation on M/NPs surface might result in three types of adverse effects, *i.e.*, antagonism, synergism and independent action.¹⁰⁴

Various intermolecular interactions (Fig. 2) that influences the adsorption of biomolecule on the surface of M/NPs may include ionic (electrostatic) interaction (attractive and repulsive), van der Waals forces, hydrogen bonding, hydrophobic interactions, hydration forces and acid–base interactions. The key driving forces such as hydrophobic and ionic interactions, combined with an entropy gain originate from the release of small molecules (*e.g.*, water and counter-ions), from the interface between the biomolecules and the surface. Hence, the size of the biomolecules will largely influence its ability to adsorb onto surfaces.¹⁰⁵

From the preceding discussion it is evident that the interaction(s) between various biomolecules and M/NPs is influenced by properties of biomolecules, and physico-chemical characteristics of the M/NPs. This will in turn determine the

types of forces involved in the interactions in forming multiple layers of surface bound biomolecules. This will critically influence the fate and biological reactivity of the M/NPs.

4. Ecological implications of BC

The contamination of M/NPs poses a significant environmental crisis on a global scale.^{6,106} The adherence of various biomolecules to the surface of M/NPs results in the formation of BC, which alters the surface properties of the M/NPs.⁶ The formation of BC is closely tied to the fate and toxicological consequences of M/NPs.^{43,107} The aquatic ecosystem is highly vulnerable to the risks associated with M/NPs pollution, with BC formation playing a crucial role in determining the toxicological effects on aquatic organisms (Fig. 3). In our study, we have emphasized the toxicological implications of M/NPs on aquatic organisms, specifically zoobenthos, zooplankton and nekton. The biomolecules present in these organisms are instrumental in the formation of BC on M/NPs.

4.1 Effect of BC formation on the eco-toxicity of M/NPs

4.1.1 Freshwater systems. The secretion of biomolecules is a widely recognized behavior between predators and prey in aquatic systems, raising the question of whether M/NPs interact with these secreted proteins and other biomolecules. Investigating such interactions is crucial for gaining a comprehensive understanding of eco-toxicity. *Daphnia magna*, a keystone species and a bio-indicator for toxicity studies was used to condition the dispersion media.¹⁰⁸ This conditioning led to a significant release of proteins, which quickly bound to the NH_2 -NPs and COOH -NPs, resulting in the formation of

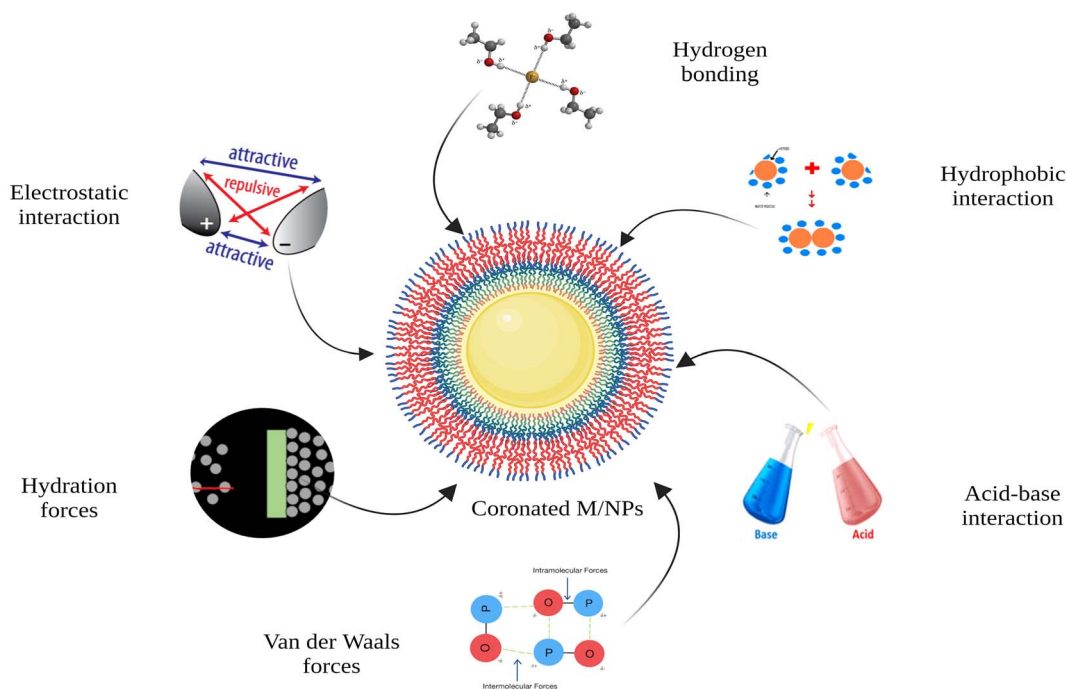


Fig. 2 Forces involved in biocorona formation.



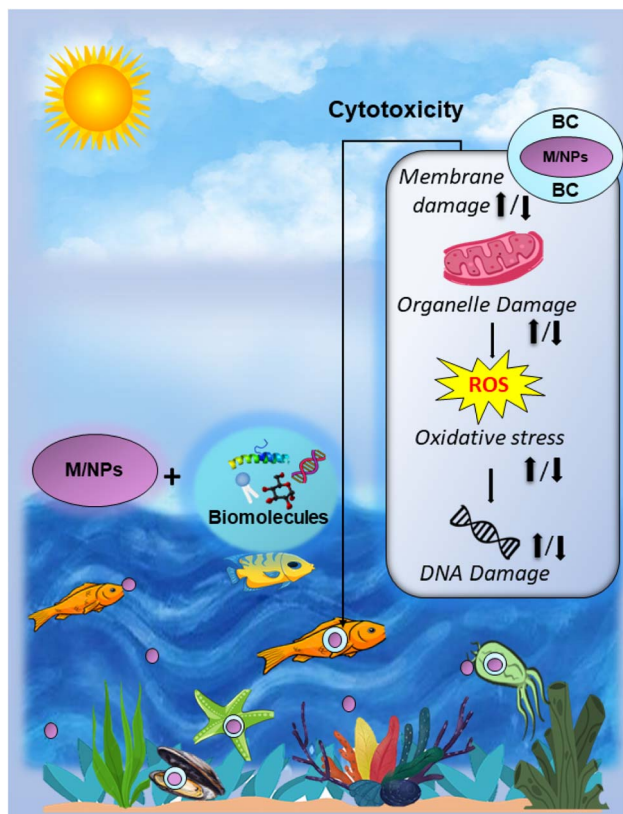


Fig. 3 Toxicological impact of biocorona formation on aquatic organism.

a corona. Interestingly, NPs with bound coronas exhibited a lower EC50 value compared to unconditioned NPs and were less efficiently removed from *D. magna* than uncoated NPs. The presence of NPs in the intestines hindered the organism's ability to feed on algae for the subsequent 6 hour period.⁹⁰ Luo *et al.* conducted a recent study examining the impact of PS-MPs and the complex formed by the corona of bovine serum albumin (BSA) on the intestines of zebrafish. The findings demonstrated that the formation of the BC enhances the toxicity and aggregation of PS-MPs. This, in turn, hampers food intake and triggers the generation of ROS in the zebrafish gut. Further, it was observed that the Keap1-Nrf2-ARE signaling pathway in the gut was compromised by the presence of PS-MPs combined with BSA, indicating a disruption in the cellular defense mechanisms.¹⁰⁹

4.1.2 Marine systems. The toxicity of NH₂-NPs in the hemocytes of *Mytilus galloprovincialis*, a marine bivalve, was investigated in a study.¹¹⁰ Hemocytes were exposed to three different concentrations (1, 5 and 50 g mL⁻¹) of NH₂-NPs. Lower concentrations of NH₂-NPs triggered the production of hydrolytic enzymes and ROS, indicating an initial inflammatory response. However, higher concentrations of NH₂-NPs led to a decrease in phagocytosis and severe disruption of lysosomes (LYZ), indicating damage to lysosomal integrity and immunocompetence. The findings suggest that the formation of protein corona on NH₂-NPs contributed to the observed lysosomal damage.^{110,111} In another study, the effects of NH₂-NPs and

COOH-NPs on the oyster (*Crassostrea gigas*) gametes were examined. The results demonstrated that the interaction of COOH-NPs with oyster spermatozoa resulted in cell expansion, ROS generation and increased complexity. However, the exposure of oocytes to both types of NPs had a lesser impact. These variations in the NPs effects are largely attributed to the interaction between NPs and seminal biomolecules (corona formation), the type of gamete and the characteristics of cell membranes.¹¹² Marques-Santos *et al.* (2018) conducted a study on the impact of protein corona (PC) formation with NH₂-NPs on the immune cells of a sea urchin species, *Paracentrotus lividus*. The researchers observed the formation of PC when NH₂-NPs were incubated with celomic fluid, both with and without EDTA. When the phagocytes were exposed to NH₂-NPs in the celomic fluid, they exhibited nuclear alterations similar to apoptosis and a decrease in the lysosomal membrane stability. However, in the presence of EDTA, these effects were eliminated, indicating the involvement of PC in facilitating the interaction between NH₂-NPs and coelomocytes, as well as their toxic effects. These findings suggest that the PC enables the identification of NPs by the cell membrane, leading to their uptake and subsequent toxicity.¹¹³

It is apparent from the preceding studies that the formation of the BC on M/NPs can elicit either a decrease or an increase in the toxic potential of these particles. The extent of such alterations is primarily determined by the specific biomolecules involved in the corona formation, in addition to the inherent physiochemical properties of the M/NPs. This observation highlights the critical influence of both biomolecular interactions and the intrinsic characteristics of MPs in shaping their overall toxicological impact.

4.2 Impact of BC on fate and transport of M/NPs

The fate and transport of M/NPs in the aquatic systems are highly influenced by the corona formation on M/NPs. For instance, the impact of proteins (BSA and LYZ) on the transportation and retention of NPs was studied in order to gain insight into the corona formation on NPs. The electrostatic charge of proteins, along with salinity levels and the size of the NPs, play a significant role in NP transportation. BSA effectively dispersed NPs of sizes 200 nm and 500 nm by enhancing steric repulsion forces, thereby, greatly improving their transportability. Conversely, the formation of a LYZ corona resulted in a substantial NP aggregation and hindered the transport of 200 nm NPs. Interestingly, LYZ exhibited a minimal binding affinity for the surfaces of 500 nm and 1000 nm NPs, suggesting a limited impact on the transportation of larger-sized NPs. However, when seawater salinity decreased from 35 to 3.5 PSU, LYZ was found to induce aggregation of 500 nm NPs, thereby, impeding their transportability.¹¹⁴ The impact of BC on the transportation and deposition of microplastics (MPs) in quartz sand was investigated under NaCl solutions with ionic strengths of 5 mM and 25 mM. COOH-MPs and NH₂-MPs were utilized as MPs with negative and positive surface charges respectively. BSA and trypsin (TRY) were selected as proteins, each possessing distinct electrical charges. The presence of these two



proteins had contrasting effects on the transport of COOH-MPs in quartz sand. It was found that negatively charged BSA facilitated the transport of COOH-MPs in quartz sand, while positively charged trypsin hindered COOH-MPs transport. Interestingly, both types of proteins enhanced the transport of NH₂-MPs in quartz sand, regardless of their opposite effects on COOH-MPs. The improved transport of COOH-MPs in suspensions containing BSA was attributed to the steric interaction caused by the adsorption of BSA corona onto the surface of COOH-MPs as well as the repulsive effects, resulting from the presence of BSA in the solutions. On the other hand, the reduced movement of COOH-MPs in suspensions with trypsin was due to the larger size and decreased electrostatic repulsion of COOH-MPs resulting from trypsin adsorption onto their surface as well as the additional deposition sites, created by trypsin adsorption onto the quartz sand. The enhanced electrostatic repulsion resulting from BSA adsorption onto NH₂-MPs surfaces led to increased transport of NH₂-MPs when accompanied by BSA in the solutions.¹⁴⁵ In a recent study conducted by Li *et al.* (2021), it was observed that BSA exhibited diverse effects on the aggregation kinetics of different surface-coated NH₂-NPs under environmental conditions. The surface functional groups of PS-NPs played a crucial role in determining the amount of protein adsorption and the electrostatic destabilization or steric stabilization of PS-NPs. Due to the distinct surface characteristics of PS-NPs and the complex interactions between PS-NPs and BSA, a low concentration of BSA promoted the aggregation of negatively charged PS-NPs and PS-bare aggregates, while retarding the aggregation of positively charged NH₂-NPs. The formation of a protein corona provided significant stability to the bare and negatively charged PS-NPs, indicating their high mobility in surface waters.⁹⁶ Similarly, the effects of BSA (negatively charged) and bovine trypsin (TRY) (positively charged) on the aggregation of PS-NPs were investigated using time-resolved dynamic light scattering. The PS-NPs remained stable in the presence of BSA at moderately high protein concentrations (>10 mg L⁻¹) but exhibited rapid aggregation in the presence of TRY. These findings clearly demonstrate the significant influence of electrostatic charge of proteins and their concentration on the aggregation behavior of NPs.

The aggregation kinetics of PS-NPs in the aquatic environment were examined using five different proteins, namely BSA, bovine hemoglobin (BH), human serum albumin (HSA), bovine casein (BS) and collagen type I (Col I). The experiments were conducted under natural water conditions, with variations in pH, protein concentration and ionic strength to mimic real-world scenarios. The findings revealed that the structure of the proteins, their electrostatic characteristics, and the chemical composition of the solution played significant roles in PSNP-protein interactions, leading to distinct aggregation patterns in NaCl and CaCl₂ solutions. In the presence of BSA, BS and Col I, PS-NPs remained stable in NaCl solution due to steric hindrance and electrostatic repulsion. However, at a concentration of 300 mM NaCl, BH destabilized PS-NPs, resulting in an aggregation rate of 1.71 nm s⁻¹. On the other hand, in CaCl₂ solutions with a concentration below 20 mM, HSA, BH and Col I

destabilized PS-NPs through mechanisms such as steric hindrance, cation bridging and compression of the double layer. In contrast, BS stabilized PS-NPs by precipitating Ca²⁺ ions reduced the screening effect of charges. The quantity of proteins and the pH of the solution influenced the formation of the PC, protein structure and surface charge, and all that impacted the stability of PS-NPs.¹⁴⁶

The aforementioned studies provide valuable insights into how BC formation and water chemistry affect the fate and transport of M/NPs in aquatic systems. One of the critical factors in the fate of bio-coronated M/NPs is the surface charge of the attached biomolecules. The surface properties of these biomolecules influence the overall charge of the M/NPs, affecting its stability, aggregation, and interaction with other particles or surfaces. For instance, if the biomolecules confer a positive charge to the M/NPs, they may have a higher tendency to repel each other, leading to reduced aggregation and improved dispersion in the water column. Apart from the impact of the surface charge of the attached biomolecules, the surface functionalization of M/NPs also plays a significant role in their fate and transport. Similarly, particle size is another crucial aspect influencing the fate and transport of bio-coronated M/NPs. Smaller nanoparticles generally have a larger surface area relative to their volume, leading to increased interactions with biomolecules and the surrounding environment. The nature of the surrounding medium, including pH, chemical composition of the water and the presence of other dissolved substances, also has a profound impact on the behavior of bio-coronated M/NPs. Understanding the fate and transport of bio-coronated M/NPs is a complex and multifaceted process due to the intricate interplay of all these factors. A comprehensive grasp of these process is required for evaluating the environmental implications of BC.

5. Potential methods to characterize biocorona formation

The accurate quantification and identification of the composition of BC are crucial for comprehending the ecological implications of M/NPs. This knowledge is pivotal as the composition and quantity of the BC directly influences the properties of M/NPs. Various analytical techniques have been employed to characterize the composition of BC formed on diverse nanomaterials. In many instances, a combination of multiple detection methods has proven to be the most efficacious approach. Generally, two primary approaches, namely *ex situ* and *in situ* techniques/methods, are utilized for the analysis of BC formation on M/NPs.

5.1 *In situ* techniques/approaches

In situ approach assists in assessing the BC while the nanomaterial is disseminated in the physiological environment.¹⁴⁷ Numerous researchers have engaged in *in situ* measurements to gain a more comprehensive understanding of the formation of the soft corona and the protein corona.¹⁴⁸ These investigations have enabled them to obtain reliable information on the



interactions between NM–protein and protein–protein within the chosen medium, without the need for purification.¹¹⁹ Initially, *in situ* approaches were proposed for characterizing corona formation, which has been supported by material characterization techniques. Such techniques include Fourier transform infrared spectroscopy (FTIS), Raman spectroscopy (RS), time of flight secondary ion mass spectrometry (TOF-SIMS), ultraviolet-visible spectroscopy (UV-vis), atomic force microscopy (AFM), *etc.*,^{28,120–122} However, the majority of these methodologies are inadequate for identifying all the diverse molecular constituents present in coronas. The dispersion of M/NPs is also a critical factor in determining the composition of the adsorbed corona. For instance, the formation of aggregates is a common occurrence among M/NPs, particularly, under high salinity conditions.^{112,123} The formation of corona on these agglomerated M/NPs will vary from the widely dispersed M/NPs.¹²³ Hence, efforts directed at comprehending the process of BC formation on M/NPs should involve a comprehensive assessment of M/NPs, encompassing an in-depth analysis of their colloidal dynamics.

5.1.1 Characterization methods. The hydrodynamic dimensions of M/NPs undergo modifications upon corona formation, and this phenomenon could be assessed through the application of dynamic light scattering (DLS).¹²⁴ To probe the interaction between proteins and particles, UV-vis spectroscopy could be employed to analyze the variations in absorption peaks before and after binding.^{125,126} Fluorescent labeling frequently facilitates the dynamic assessment of corona formation on M/NPs in real-time.²⁸ The interplay between the coronas and M/NPs can be evaluated through the application of microscopic methodologies such as transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM).^{117,127} These advanced techniques enable the examination of morphological transformations of the materials during their interaction with coronas, yielding high-resolution images.^{126,128,129} TEM is commonly employed in situations where the hydrodynamic diameter of M/NPs greatly surpasses the geometric diameter, indicating a low dispersion state and heightened aggregation of M/NPs.^{130,131} SEM offers the advantage of generating superior-quality imagery of plastics, facilitating their differentiation from organic substances.^{132–134} In contrast to SEM, AFM enables more precise characterization of particles, and the preparation of samples is comparatively straightforward.¹³⁵ FTIR and RS could be employed for the identification of organic constituents within the corona.^{83,134,136,137} FTIR is particularly effective in detecting the structural modifications in proteins, as it utilizes the absorption of amide bonds to discern their secondary conformations.^{138,139} On the other hand, RS analyzes the vibrational modes of molecules, offering supplementary insights.¹⁴⁰ In the case of proteins, RS reveals characteristic bands associated with the peptide backbone, sulfur-containing side chains or aromatic side chains.¹⁴¹ According to reports, the presence of lipids, sodium dodecyl sulfate and folic acid in PS-NPs was successfully determined by utilizing surface-enhanced Raman scattering.¹⁴² AFM coupled with RS or FT-IR, could identify the elemental composition of M/NPs and generate images and IR

absorption spectra with a spatial resolution ranging from 50 to 100 nm.^{31,128} In terms of capabilities, RS offers higher resolution, broader spectrum coverage and less susceptibility to water interference compared to FTIR.¹⁴ To assess the metal elements within the coronas, energy-dispersive X-ray spectroscopy (EDS) could be combined with SEM and TEM techniques.¹⁴³ Recent advancements, such as hyperspectral dark-field microscopy and the integration of AI and machine learning, support the characterization of adlayers present on MPs.^{28,144}

5.2 *Ex situ* techniques/approaches

The *ex situ* approach proves valuable in evaluating the BC by isolating the nanomaterial–corona complex from its physiological milieu.^{117,145} The nanomaterial could be separated through exposure to extreme temperatures, high salinity, enzymatic treatment and detergents. Isolation can be complete or partial, depending on the strength of the interaction between macromolecules and the nanomaterial, followed by, subsequent analyses.¹⁴⁵ This strategy yields highly effective results for quantitative corona structure analysis. While the *ex situ* approach is commonly employed for protein corona (PC) analysis and has provided valuable insights into this phenomenon, it does not provide definitive information regarding the protein adsorption process or precise details about the soft corona.^{117,119}

5.2.1 Pre-treatment. The pre-concentration of M/NPs is often achieved through various techniques such as ultracentrifugation, ultrafiltration, continuous flow centrifugation and cloud point extraction. Subsequent separation is typically accomplished using methods such as membrane filtration, size-exclusion chromatography (SEC) and field flow fractionation.⁹¹ Among these methods, centrifugation or ultracentrifugation is commonly employed to separate nanomaterials and their BC from the surrounding medium.^{146,147} Recent studies have successfully employed ultracentrifugation to separate plastic particles from the sediments and aqueous matrices.¹⁴⁸ Prior to separation, the digestion of the matrix with acid or alkali is often considered crucial for removing non-bonded organic materials and enhancing the separation efficiency in ultracentrifugation.^{28,146} However, there is a risk of losing the BC during this process. Centrifugation remains a simple and rapid isolation approach, as the quality of identification is directly influenced by the quantity of available material. The extraction of coronas from M/NPs could be accomplished through careful planning of pre-treatment methodologies that consider the chemical properties of the specific compounds utilized in the process. The solvent extraction techniques such as H₂O₂ treatment, alkaline digestion with KOH and NaOH, and the use of Fenton's reagent, could effectively dissociate absorbed small organic substances from the M/NPs.¹⁴⁹ The pH of the extraction solvent could be adjusted to modify the charge state of the coronas or facilitate the breaking of bonds between the coronas and M/NPs.²⁸ PC could be isolated from M/NPs using a buffer containing sodium dodecyl sulfate and other ionic detergents capable of solubilizing proteins.¹¹⁹ The quantification of total protein in the extraction buffer could be achieved through methods such as the bicinchoninic acid (BCA) assay, Bradford



assay or UV-vis spectroscopy.^{150,151} Additionally, SEC, gel electrophoresis and western blotting could be employed to separate the proteins for further analysis.^{132,152} Following pre-concentration, microscopic and spectroscopic techniques are often integrated to extensively evaluate the corona formation.

5.2.2 Spectroscopic analysis. In recent times, an array of mass spectrometry (MS)-based methodologies, including inductively coupled plasma-MS (ICP-MS), gas chromatography-MS (GC-MS), liquid chromatography-MS (LC-MS), time-of-flight secondary ion-MS (ToF-SIMS) and capillary electrophoresis-mass spectrometry (CE-MS) have been employed to investigate the corona formation on M/NPs.^{31,86,111,153} These highly sensitive techniques exhibit exceptional accuracy in examining the structural aspects of the corona. MS could also be combined with other separation techniques such as LC, GC and CE to yield comprehensive insights into the complex coronas. LC-MS is utilized to analyze thermally labile components of coronas, including lipids, proteins, and peptides.¹⁴ It provides qualitative and quantitative information about intricate mixtures of coronas, making it the method of choice for protein corona research.¹⁵⁴ LC-MS requires a relatively small sample volume, which is advantageous when dealing with limited sample availability. Moreover, it employs a label-free quantification

approach, eliminating the need for an indicator molecule.¹⁵⁴ GC-MS, on the other hand, is particularly effective in tracking highly hydrophobic and volatile organic substances, making it widely employed for monitoring plastic additives and organic pollutants adsorbed on MPs.¹⁵⁵ ICP-MS has gained widespread use in the analysis of M/NP coronas for the detection of inorganic elements, particularly, trace metal contamination.¹⁵⁶ When investigating the hard protein corona, ICP-MS could be employed to examine the overall protein layers.¹⁵⁷ CE-MS offers a novel approach for analyzing protein and metabolite coronas. On the other hand, TOF-SIMS is particularly well-suited for characterizing organic compounds compared to ICP-MS. By detecting the precise mass and intensity of secondary ions and ion clusters, TOF-SIMS could utilize the SIMS peak to identify the elemental and molecular components. Unlike SEM/EDS, TOF-SIMS allows for scanning depths of less than 2 nm, making it more suitable for analyzing extremely thin layers and nanomaterials. Moreover, TOF-SIMS could generate distinct 3D profiles with excellent mass and spatial resolution, making it suitable for imaging organic components within coronas.²⁸ However, achieving a comprehensive understanding of M/NP coronas remains a significant challenge that may require a logical integration of different analytical approaches. For

Table 1 Recent analytical approaches in the characterization of biocorona^a

Type of M/NPs	Size	Method of BC isolation and measurement	BC composition	Refs.
PS M/NPs	55–1000 nm	Centrifugation, ELISA, CLSM, FS, NDS and PQ kit	Proteins, DNA and carbohydrates	159
PS-NPs	300 nm	Centrifugation, TEM, SDS-PAGE, nano-HPLC-Q exactive MS, BCA assay, and western blotting	Proteins	160
PS-NPs (NH ₂ and COOH)	90–110 nm	Centrifugation, SDS PAGE, SA and MS	Proteins	161
PS-NPs (COOH)	60 nm	Centrifugation, SEM and TEM	Proteins and carbohydrates	162
PS-NPs	23 nm	Centrifugation, SEC, FM, DLS and PQ kit	Uronic acid, and proteins	31
PS-NPs (NH ₂)	100–120 nm	Centrifugation, bradford assay, SDS-PAGE, gel staining, nano-LC-MS/MS, ELISA and LSCM	Proteins	163
PS-NPs (NH ₂)	50 nm	Centrifugation, SDS PAGE and nano-HPLC-ESI-MS/MS	Proteins (32 kDa)	164
PS-NPs (NH ₂ COOH and plain)	50–150 nm	SEM, FS, CD spectra, UV-vis spectra FT-IR and EDS	Proteins	96
PS M/NPs	50–500 nm	Centrifugation, CLSM and FS	Glycoprotein biopolymer	165
PS-NPs (COOH)	60 nm	Centrifugation, TEM, SDS-PAGE, FS, and SEC	Proteins	162
PS-NPs	20/100 nm	DLS, FS, and CD spectra	Proteins (BSA and TRY)	166
PS-NPs (NH ₂)	50 nm	DLS, TEM, centrifugation, SDS-PAGE gel staining and nano-HPLC-ESI-MS/MS	Proteins	113

^a Acronyms: CD: circular dichroism; CLSM: confocal laser scanning microscopy; ELISA: enzyme-linked immunosorbent assay; FM: fluorescence microscope; FS: fluorescent spectroscopy; LSCM: laser scanning confocal microscope; MS: mass spectrometry; nano-HPLC-ESI-MS/MS: nano-high performance liquid chromatography-electrospray ionisation tandem mass spectrometry; NDS: nanodrop spectroscopy; PQ kit: protein quantification kit.



instance, the quantification of PENPs (24 to 52 nm diameter) was successfully achieved using a combination of SEM, FT-IR and X-ray photoelectron spectroscopy (XPS).¹⁵⁸ Recent approaches for the BC analysis and its composition are depicted in Table 1.

5.3 Limitations and challenges in characterizations

Although various analytical techniques are employed in BC analysis, several challenges are associated with these processes. Centrifugation, a crucial pre-treatment process, presents several obstacles in corona isolation. One major challenge is the potential for false positives. During centrifugation, proteins or protein complexes that did not originally bind to the nanomaterial as well as proteins, and that only bind to the proteins attached to the nanomaterial but not the nanomaterial itself, may sediment along with the nanomaterial and its corona, leading to false positive results. Additionally, false negatives could occur due to protein dissociation from the nanomaterial–corona complex induced by the centrifugal forces.¹⁴⁶ Moreover, the centrifugation approach fails to separate the soft corona because the protein–protein interaction in this structure are too weak to withstand the centrifugal forces.¹⁴⁷ In the case of SEC, selectivity decreases when applied to analytes with extremely high molar mass, and there is a possibility for analytes to interact with the stationary phase.¹⁴⁶ DLS, UV-vis, and fluorescent microscopy are essential methods for evaluating the interaction between biomolecules and M/NPs.¹⁶⁷ However, these techniques have limitations in terms of qualification and sensitivity. While DLS has traditionally been employed to study protein–nanomaterial interactions, a critical limitation arises from the presence of unbound proteins that could interfere with the scattering signal.¹¹⁹ Consequently, sample purification is necessary, which results in the loss of the soft corona that cannot be detected. In the case of fluorescent microscopy, a significant challenge is the need for fluorescence labeling that adds complexity to the experimental processes. TEM, SEM and AFM are indispensable microscopic techniques in the study of coronas. However, these approaches have limitations in terms of low particle population, qualification and quantification. TEM requires sample preparation that could potentially affect the morphology of M/NP–corona complexes. Additionally, the counterstaining becomes necessary due to the inadequate contrast resulting from the smaller size of the nanomaterial and thin protein layer.¹⁶⁸ AFM allows for 3D imaging but suffers from poor efficiency.¹⁶⁹ On the other hand, energy dispersive X-ray (EDX) analysis exhibits high efficiency but is not suitable for detecting organic compounds. RS provides the advantage of analyzing both organic and inorganic compounds but it has limitation in terms of low spatial resolution.¹⁶⁴ FTIR has limitations in detecting the inorganic compounds and lacks sensitivity and accuracy.¹⁴⁶

In contrast, MS-based approaches are highly effective analytical techniques due to their high sensitivity, resolution, reproducibility, accuracy, quantification and selectivity.^{170–172} However, TOF-SIMS is not suitable for analyzing unknown materials and performs poorly in quantification assessments.¹⁷³

GC-MS excels in the analysis and identification of trace amounts of organic molecules with high sensitivity and repeatability, but it cannot analyze thermally labile compounds.²⁸ LC-MS offers better throughput, high sensitivity, greater precision compared to PAGE and reduced user bias.¹⁴⁵ However, this approach involves multiple steps in the sample pre-treatment process. While ICP-MS analysis could detect low levels of metal elements (0.3 ppb), they would only represent the average metal amounts of the overall particle population.²⁸ Moreover, it falls short in characterizing the molecular structure of organic coronas. CE-MS has limitations in terms of loading capacity and the direct analysis of high molecular weight proteins.¹⁷⁴

6. Conclusion and future prospects

This review provides a concise overview of scientific knowledge pertaining to the formation of BC on the M/NPs, analytical techniques for characterizing BC, the eco-toxicity of M/NPs and the ecological implications of BC formation in the aquatic systems. The abundant presence of M/NPs in aquatic environments enables their interaction with various biomolecules leading to the BC formation. The resulting BC alters the size, stability, surface charge, and other physical and chemical properties of M/NPs, which in turn, affect their bioavailability, eco-toxicity and fate, ultimately resulting in diverse ecological effects. The scientific evidence clearly indicates that the BC formation can have both non-toxic and toxic impacts on the aquatic organisms.

Characterizing the composition of BC poses challenges, as it requires a combination of multiple analytical techniques and the development of new characterization technologies. In particular, detecting BC formation in the biological system *in situ* remains a hurdle. The utilization of radiological or fluorescent labeling strategies could aid in the detection of BC in the biological systems. The formation of BC is significantly influenced by environmental factors such as pH, ionic strength, temperature and conductivity. Therefore, scientific investigations are needed to understand the contributions of these factors to the BC formation. Most of the research conducted on BC has utilized commercial M/NPs, model proteins or other biomaterials in the laboratory settings. To gain a comprehensive understanding of the interaction between BC and M/NPs in the aquatic environments, field research is highly recommended. The toxicological experiments involving bio-crowned M/NPs and aquatic organisms should be conducted under ecologically relevant conditions, as recommended by Organisation for Economic Cooperation and Development (OECD) guidelines. While protein corona studies have been predominant in aquatic biota, further investigations on other types of coronas such as lipids, nucleic acids and other types of BC would enhance our knowledge of their ecological impacts.

Conflicts of interest

There are no conflicts to declare.



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