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Isolation methods for particle protein corona complexes from protein-rich matrices

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Background: Nanoparticles become rapidly encased by a protein layer when they are in contact with biological fluids. This protein shell is called a corona. The composition of the corona has a strong influence on the surface properties of the nanoparticles. It can affect their cellular interactions, uptake and signaling properties. For this reason, protein coronae are investigated frequently as an important part of particle characterization. *Main body of the abstract*: The protein corona can be analyzed by different methods, which have their individual advantages and challenges. The separation techniques to isolate corona-bound particles from the surrounding matrices include centrifugation, magnetism and chromatographic methods. Different organic matrices, such as blood, blood serum, plasma or different complex protein mixtures, are used and the approaches vary in parameters such as time, concentration and temperature. Depending on the investigated particle type, the choice of separation method can be crucial for the subsequent results. In addition, it is important to include suitable controls to avoid misinterpretation and false-positive or false-negative results, thus allowing the achievement of a valuable protein corona analysis result. *Conclusion*: Protein corona studies are an important part of particle characterization in biological matrices. This review gives a comparative overview about separation techniques, experimental parameters and challenges which occur during the investigation of the protein coronae of different particle types.

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Introduction

The impact of micro- and nano-particles on the human body has been the focus of research for almost two decades. In particular, nanoparticles, which are mostly defined by a size between 1 and 100 nanometers in at least one dimension, are of interest to the scientific community.[1,2] Their higher surface-to-volume ratio, as compared to bigger particles, changes their physicochemical properties. Nanoparticles therefore may show higher biological reactivity.[3] Furthermore, their small size may allow them to escape from the clearance mechanisms of the body, and thus they circulate longer in the blood and pass through cell membranes more easily.[4] In combination with their greater reactivity, this increases their biological impact and implies a potential risk for undesired effects such as

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oxidative stress or cellular import of chemical substances adsorbed to the particle surface.[5] Furthermore, the shape of particles is of special interest since it influences their bioavailability. For example, cylindrical nanoparticles have been shown to interact more strongly with cells than spherical ones.[6] Surface modifications also affect nanoparticle reactivity by changing their zeta potential and influencing their binding kinetics and thus their biological interactions.[5,7] Nowadays, most scientists agree that it is insufficient to interpret results from toxicological or biochemical studies without proper characterization of the respective nanoparticles and analysis of their interactions with the test environment.[8] Particle composition, shape, size and size distribution, and ion release, as well as stability of particle dispersions are of major relevance, as are their surface chemistry and coating which are directly involved in building an interface between the particle and its biological environment.[5,7] This biological interface involves physicochemical, thermodynamic and kinetic interactions between the nanomaterial surface and the surfaces of biological components such as proteins, phospholipids, or DNA contained in biological fluids or present in living cells. The interface represents the contact point between a particle and the biological system and is called the "nano-bio interface" or "protein corona", even though the particle corona may also contain components other than proteins.[9] The composition of the protein corona depends on the surrounding matrix, for example blood serum, plasma or the extra- and intra-cellular protein matrix and can change over time and after uptake or transportation into another cellular compartment.[3] The corona can have a major influence on different processes, for example the attachment of particles to cell surfaces, the triggering of cellular signals, the modulation of particle uptake, distribution and excretion, the release of ions or other substances from the particles, or even immunological reactions.[10–12] For example, some proteins make the nanoparticle surface more hydrophilic.[13] The term "nanoparticle protein corona" was introduced by Cedervall *et al.* in 2007 to describe the formation of protein layers on a nanoparticle's surface.[14] The concept of a biological interface for particles, however, had already been studied earlier using the terminology "interface", or being described as protein adsorption to particles and its role in overall biological interactions.[15–19] For a more detailed description of the history of protein corona research please see below. Over time, experimental set-ups and especially read-outs became more and more sophisticated, ranging from simple gel electrophoresis in the 1960s to "omics" approaches in recent years as schematically shown in Fig. 1.

The number of publications on nanoparticle corona analysis has massively increased during the last decade (Fig. 2). Moreover, already about 100 reviews have been published that deal with the nanoparticle protein corona and the relationships between nanoparticles, their attached proteins and different downstream effects. Some discuss the evolution, composition

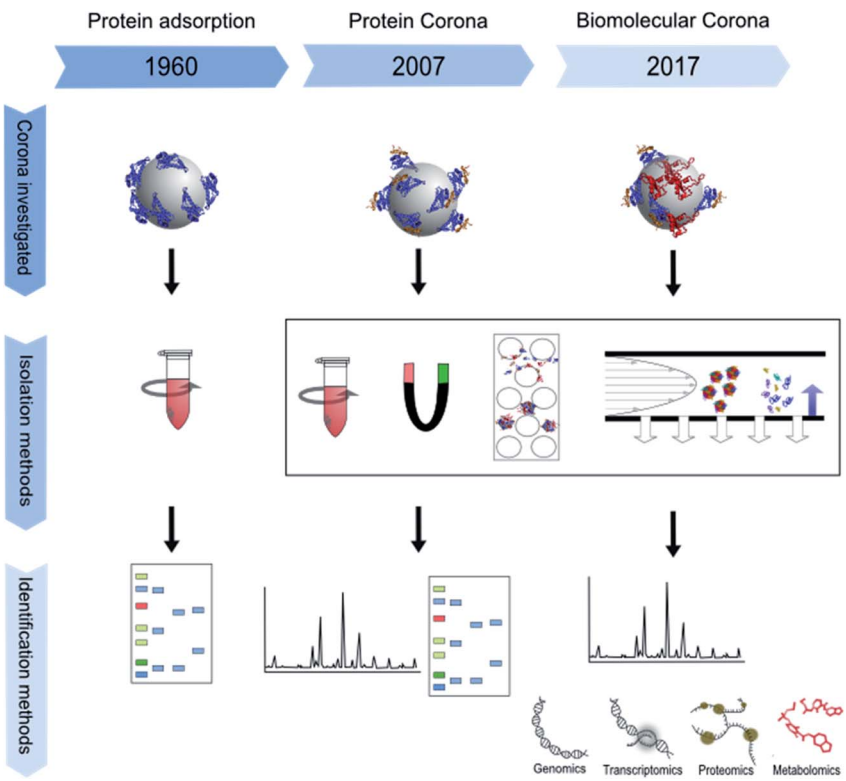

Fig. 1 Development of protein corona research over time. Qualitative protein adsorption studies were performed in the 1960s to explore pharmaceutical nano-applications, followed by molecular identification of the protein corona in the 2000s with a focus on nanoparticle toxicology. The latest results obtained by means of novel analytical and "omics" approaches suggest a "biomolecular corona" comprising different types of biomolecules.

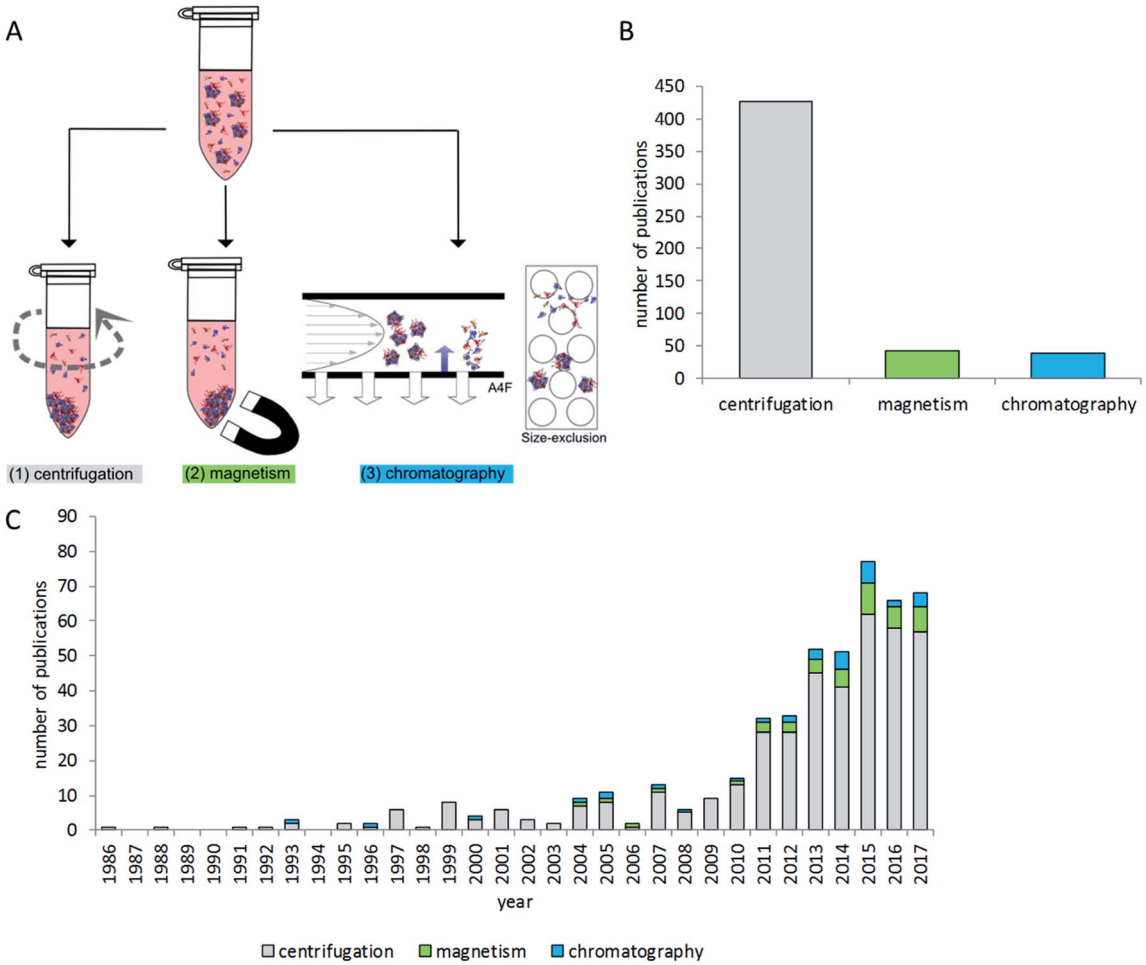

Fig. 2 Usage of different separation methods for corona analytics. (A) Schematic overview of the different methods that are used to separate particles with their protein corona from unbound proteins in solution. Centrifugation, magnetism (for magnetic particles only), and chromatography-related methods are used. (B) Total number of publications that report centrifugation, magnetism, or different chromatographic methods as separation steps to isolate particles with their protein corona from the surrounding matrix. (C) Time-resolved overview of the publications shown in (B), demonstrating a strong increase in protein corona-related studies after 2010 and the increasing use of non-centrifugation methods.

and kinetics of the protein corona,[20–23] some deal with implications for cellular uptake[24–26] and some are focused on the relationship between the nanoparticle corona and the immune system.[27,28] The authors of the aforementioned articles agree that information about the corona is essential for assessing and understanding the impact of particles on the human body, as through its (protein) composition the corona determines the biological identity of a particle which is recognized by the cell. All in all, it became clear that the corona composition of different particles is not only dependent on the particle material, its surface chemistry, and the composition of the surrounding media, but is also subject to time-dependent changes. Therefore, corona analytics does not only include the determination of a complex protein composition, but should also consider the complex kinetics of corona formation.

It has to be kept in mind that choosing a method for isolation of particles with their corona proteins from a protein-rich matrix, such as a cell culture medium containing bovine serum as a supplement or blood samples, is a critical step in the workflow. Isolation methods for the protein coronae of nanoparticles are not standardized. From many publications in the field it remains unclear whether the most appropriate isolation method for the particular scenario investigated has been chosen based on thorough pre-testing and method optimization. The separation step has a major influence on the results and their interpretation, as it decides whether proteins can be identified as part of the corona or not. False positive or false negative results may be obtained due to association or dissociation of proteins from the corona resulting from physical or chemical forces during the isolation and purification process. As an additional aspect in corona analytics it should be noted that most model matrices, *e.g.* cell culture medium, reflect the *in vivo* situation only to a limited degree.[14,29–32] Model media can be used to simulate the behavior of nanoparticles, *e.g.* after direct injection into the bloodstream or when focusing on the cellular environment. They do not reproduce the fact that

nanoparticles *in vivo* may be transferred through several body compartments with different compositions of body fluids, for example through the oral cavity, stomach and intestinal lumen, and then into enterocytes, and further *via* the blood stream into other organs.[33] All these aspects determine how the protein corona is composed, and therefore how it influences cellular uptake and downstream effects.[13,34]

In the context described above, this review aims to give an overview of the different methods that have been developed to isolate nanoparticles with their protein corona from different environments, in order to enable the subsequent analysis of the protein corona composition. The main focus will be on the isolation methods for corona analytics in the context of specific research aims. To this end, particle sizes, surface modifications and materials are considered in relation to the respective isolation method used, and the results are compared among these different characteristics. This work thus provides a broad overview on the aspects that need to be considered in nanoparticle protein corona analytics.

Terminology

Even though the interest in protein corona analytics has increased in the context of nanotoxicology and nanomedicine in recent years, the adsorption of proteins to surfaces is not a new discovery. Protein films on surfaces were already reported in the 1920s.[35] Research on protein adsorption and its role in biological interactions with surfaces and materials began in the 1950s by Bangham and Vroman.[16,18] Subsequent studies on protein adsorption were focused on prolonging the blood circulation half-life of particles by reducing protein adsorption, in order to prevent their opsonization and recognition by cells.[15] Compared to the history of research on protein adsorption, the term protein corona has been introduced rather recently. It was proposed in 2007 to describe spontaneous self-assembly and layering of proteins on nanoparticle surfaces.[14] Other researchers describe it as the biomolecular coating of a particle that is actually in contact with a biological system (*e.g.* a cell or an organ), which interacts with a nanoparticle dispersed in a biological medium.[29] The interest in investigating the protein corona increased since it was proposed that the protein corona determines the interaction with the surrounding biological matter.[36,37] Aggregation and formation of a protein corona in the extracellular environment will alter nanoparticle size, shape, and surface properties, providing the particle with a "biological identity" that is distinct from its initial "synthetic identity".[38] The term corona is an appropriate description for proteins adsorbed to a particle surface as it comes from the Latin word for circle or wreath. However, "corona" is also used in other contexts in the nano field, like in "core-corona nanoparticles" as an alternative term to describe core–shell nanoparticles that are composed of different materials in concentric layers.[39–41] In addition, the term corona is used for stabilized polymers in some publications.[42] It also has to be kept in mind that "nanoparticle" is a rather new word for a nanoscale structure. Other terms used for particles in the same or comparable size range are, for example, colloid, submicron particle, dust, fine particulate matter, and others. While reviewing the literature, the above terminology has to be taken into account to prevent exclusion of relevant literature.

The corona has become an important parameter within the characterization of nanoparticles and their interaction with biological systems. When a particle surface comes into contact with a protein-rich environment for the first time, it gets rapidly covered with proteins. At this moment, the protein corona composition is mainly driven by the abundance of the proteins in the surrounding medium. Over time, however, the specific affinity of individual proteins to the particle surface gains importance. Vroman and coworkers have postulated in the 1980s that during the adsorption of blood proteins to surfaces, small proteins cover the surface faster, due to their higher mobility, whereas bigger proteins with higher affinity are adsorbed later but are exchanged more slowly, and therefore get enriched over time.[43,44] This was later termed the Vroman effect. The adsorption of proteins to surfaces is in a dynamic state and therefore proteins with long residence times and slow exchange rates become enriched on the particle surface and gradually replace more abundant proteins with lower affinities.

The protein corona is loosely subdivided by some researchers into the so-called "hard corona" and the "soft corona". Sometimes, the soft and hard coronae are defined by their spatial arrangement, with the inner coating of particles (*i.e.* the proteins in direct contact with the particle surface) called the hard corona and the outer layer called the soft corona.[45,46] In contrast, hard and soft coronae may also be defined based on protein affinities, with the hard corona consisting of proteins with a high affinity to the particle and having evolved over a longer period, while the soft corona is accordingly described as the (outer) layer of the protein corona with lower protein affinity to the particle surface, but high abundance in the biological fluid and a high rate of ad- and desorption.[5,23,29,47–49]

Another nano-specific term appeared in the literature about 10 years ago: the term "nanoparticle/biological interface" or for short the "nano-bio interface" was used by Nel *et al.* in their review discussing the interactions of nanoparticles with not only proteins but also other biological structures like membranes, cells, DNA or organelles.[9] An issue of JACS Select was entitled 'Chemistry at the Nano-Bio Interface' and presented many studies about modification and manipulation of the interaction of nanoparticles with biological systems for medical and bio-sensing purposes.[50] However, the composition of the protein corona was not the focus of most of these studies. Approaching the issue from the reverse point of view, the term cell "vision" has been established.[51–53] It describes a complementary concept to the protein corona and emphasizes the role of the cell when it comes to a cellular reaction to nanoparticles. During the first contact of nanoparticles with cells, the structure of the cell membrane with its phospholipid composition, surface proteins and glycocalyx defines how the cell comes into contact with a nanoparticle and its protein corona. Depending on the cell type, the "vision" of cells is different and so is the cellular reaction to a specific nanoparticle.[53,54]

Due to the diversity of terminology, literature mining aiming to provide a broad overview of the critical steps of separating particles with their protein corona from the surrounding protein-rich matrix is a difficult task. Therefore the NCBI PubMed database was searched using multiple search terms ("nanoparticle corona", "particle corona", "protein corona", "nano corona" and "particle protein adsorption"). The resulting 4427 hits were further screened manually for contents related to centrifugation and chromatographic and magnetic separation methods related to the separation of particles with proteins on their surface from protein-rich matrices. Studies dealing with fibers and larger surfaces were excluded. Studies which were only investigating binding affinities of single proteins to particles or beads were excluded as well. The remaining 412 studies were then evaluated to give an overview on the state of the art of protein corona isolation methods, as presented in the following sections.

Application of separation methods in corona analytics

Research on nanoparticle-matrix interactions is very diverse and reflects the variety of nanoparticle applications. A brief overview of investigated particle types and experimental conditions is given in Fig. 2. Literature analysis revealed that the most widely used method of choice for nanoparticle–protein corona isolation is centrifugation. This technique is rather easy to implement with standard laboratory equipment, and it is also considered time- and cost-effective. In addition, magnetic separation is commonly used for particles with magnetic properties, for example iron oxides. Size exclusion chromatography is used rarely, mostly with polymers and silver nanoparticles.

Most of the available studies focus on proteins which are generally assumed to play the most decisive roles in nanoparticle corona formation.[5,23,49,55] A few studies also analyze other biomolecules interacting with nanoparticles, *e.g.* lipids or nucleic acids.[3,56–58] Even though most studies have the analysis of particle–protein interactions in common, very different aspects related to protein adsorption and corona formation are investigated. For example, some studies focus on the relationship between particle properties like surface chemistry and protein corona formation[14,23,59–65] and others analyze the protein corona as particle properties (like size or shape) needed to interpret cellular or biological effects.[26,38,66–72] Further aspects include improving particle properties to change protein corona composition and biological response;[73–75] targeting or characterizing of particles for usage as drug carriers or for other medical purposes;[76–81] or the comparison of different particles.[60,68,82] Some studies report on the topic of biological function by single-protein measurement or functionality analysis. Consequently, several studies investigated the binding properties of, for example, fibrinogen,[83,84] lysozyme[85,86] and others[86–88] using colorimetric protein quantification or ELISA. Kondo *et al.* combined protein binding studies with enzymatic activity assays of catalase, trypsin and peroxidase.[64] Jayaram *et al.* investigated cellular oxidative stress by the measurement of oxidized corona proteins.[89] Li *et al.* focused on corona-dependent particle uptake and enzymatic activity in inflammatory processes.[90] Taken together, drawing conclusions on biological effects in relation to protein corona composition remains challenging and a major focus of many research projects.[91]

Method development and validation for corona or protein adsorption analysis constitutes another important aspect.[92–96] In addition, the investigation of conformational and structural changes of particle-adsorbed proteins;[97–99] analyses of differences between model particles and the real-life environment;[100,101] the search for *in vitro* methods to predict the *in vivo* haemocompatibility of nanoparticles;[83] investigation of the relationships between the particle surface, protein adsorption and immune response;[102] interaction analysis of proteins with particles for analytical purposes;[103] surface functionalization of particles with proteins;[104] kinetic analysis;[105] enzymatic degradation of the protein corona;[106] and the characterization of gold particles from immunocolloidal methods play a role.[88]

Particles used for corona studies

Literature evaluation was used to determine which type of particles has been studied more or less extensively for protein corona formation and composition. Fig. 3 shows that silica nanoparticles constitute the largest fraction of particles analyzed for their protein corona, which is not surprising since they are considered promising materials for diverse biomedical or industrial applications[107–110] and are also frequently analyzed in toxicological studies.[111] The big group of polymers contains very different materials. Most of them are of interest for research because they are being developed for a variety of diagnostic and therapeutic applications.[14,60,112–119] These can be polystyrene (PS) with and without surface modifications, polyethylene glycol (PEG), polylactide-derivatives (PLGA), resins, crosslinked cellulose, carbon beads or polylactic acid (PLA). The same holds true for the separately listed group of chitosan particles.[120,121] By contrast, the main reason for the high interest in polystyrene particles is their use as model particles that are available in many different sizes and narrow size distributions, labeled with different fluorophores and with different surface modifications.[122–124] In addition, their potential use in pharmaceutical industries[98,125,126] and also the growing concern about the environmental fate of plastic particles appear to underlie the interest in their protein corona.[127,128] Gold nanoparticles are, in most cases, of interest due to their potential for drug delivery applications.[129–133] In addition, gold is very applicable in analytical investigations due to its low reactivity, low solubility and high electron density. The same holds true for iron oxide nanoparticles that are used in high-sensitivity biomolecular magnetic resonance imaging (MRI) and tumor targeting.[31,134–138] Additionally, iron oxide particles are also used for analytical purposes due to their magnetic properties.[139,140] As shown in Fig. 3, mainly iron oxide nanoparticles are separated by magnetic methods. However, some particles from other materials have also been separated by magnetism-based

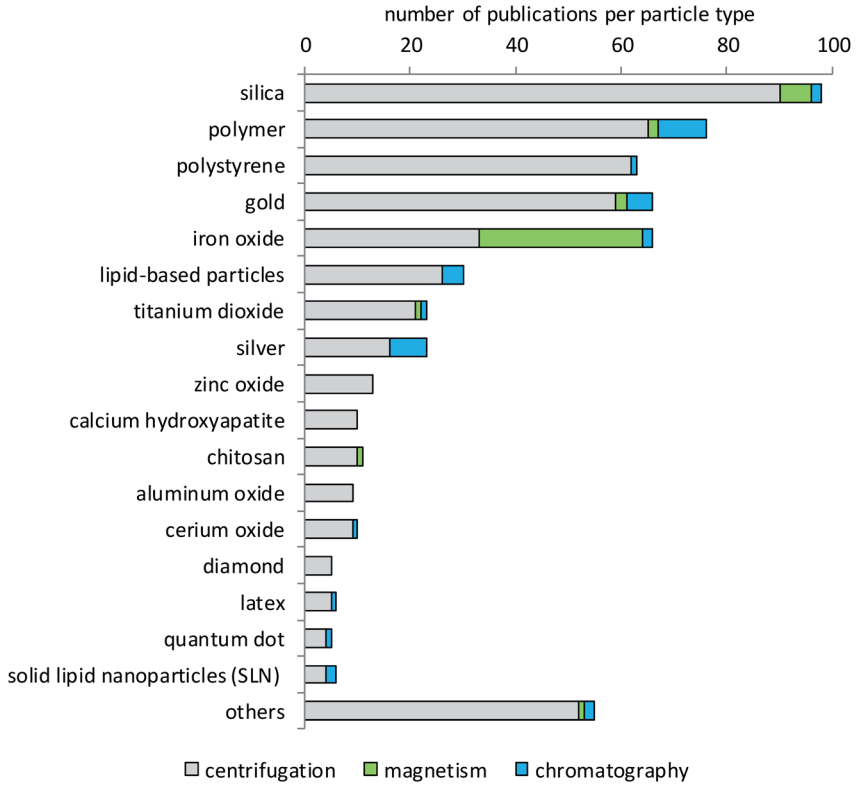

Fig. 3 Number of publications using different types of particle material in combination with the separation techniques centrifugation, magnetism and chromatography. When particles consisted of two or more materials (*e.g.* an iron oxide core covered by a second material), the outer layer was used for material categorization, because it is considered the relevant surface for nanoparticle–biomolecule interactions. The polymer category includes all polymers except polystyrene, chitosan and latex, which are listed separately.

techniques. This applies to cases where iron oxide nanoparticle cores are embedded in other materials to combine the magnetic properties of the iron oxide with the desired properties of the other materials. Such magnetic nanoparticles can be functionalized and used in different fields like biology, medicine, physics and materials science.[104] Most particles that are subsumed in the group of lipid-based particles are of interest as drug targeting agents.[141–145] These can be in the form of liposomes, lipoplexes or solid-lipid-nanoparticles, which show different properties in surface reactivity, protein binding and drug delivery. Titanium dioxide has a lot of applications, for example as a pigment or photocatalyst, in sunscreens, in food colorants, for electrical purposes or in the biomedical field, *e.g.* in dental and orthopedic reconstructive surgery.[146–151] Titanium dioxide is also used as a comparator in studies dealing with airborne particulate matter.[152] Zinc oxide is used in cosmetics, especially sunscreen products as a UV-protective agent, in the agricultural and food industries, and in packaging. Moreover, its use in food fortification is discussed, due to the essential role of zinc in many metabolic and enzymatic processes.[153–155] Other particles used are, for example, aluminum,[156] calcium compounds,[157] copper oxide and rare elements such as cerium, bismuth, cobalt, gadolinium or tin oxide.[82] Very recent studies investigated, for example, the corona formation on quantum dots,[158] colloidal silica nanoparticles[159] and BSA-stabilized gold nanoparticles.[160]

Biological matrices used for corona studies

The most studied biological fluid in corona analytics is blood, either whole blood or, in the majority of cases, plasma or serum (Fig. 4). In particular, human blood has been of interest, congruent with the intended use of many particles for medical applications. In addition, animal blood from common test animal species such as rat or mouse is also frequently used for corona analysis in studies dealing with animal trials, or as a comparator to human blood.[161,162] Bovine blood components, such as serum or plasma, are also of interest for corona studies, due to the fact that bovine serum is often used as a supplement in cell culture studies. Other, not blood-derived matrices include buffers with individual or mixed proteins, *e.g.* aimed at investigating the specific binding behavior of a certain protein.[163,164] Few studies have been conducted dealing with fluids of the gastrointestinal tract, food components, urine, or the lymphatic system.[165–167] Several studies provide a comparison of the coronae formed after incubation with different matrices. Some groups compared blood, blood plasma and serum as matrices.[168,169] Other groups compared bovine and human serum.[165,170] Jiang *et al.* compared bovine serum and a cell culture medium.[171] Lundqvist *et al.* made a comparison between extracellular fluids (blood plasma) and the intracellular matrix (cytosolic fluid) to simulate corona changes

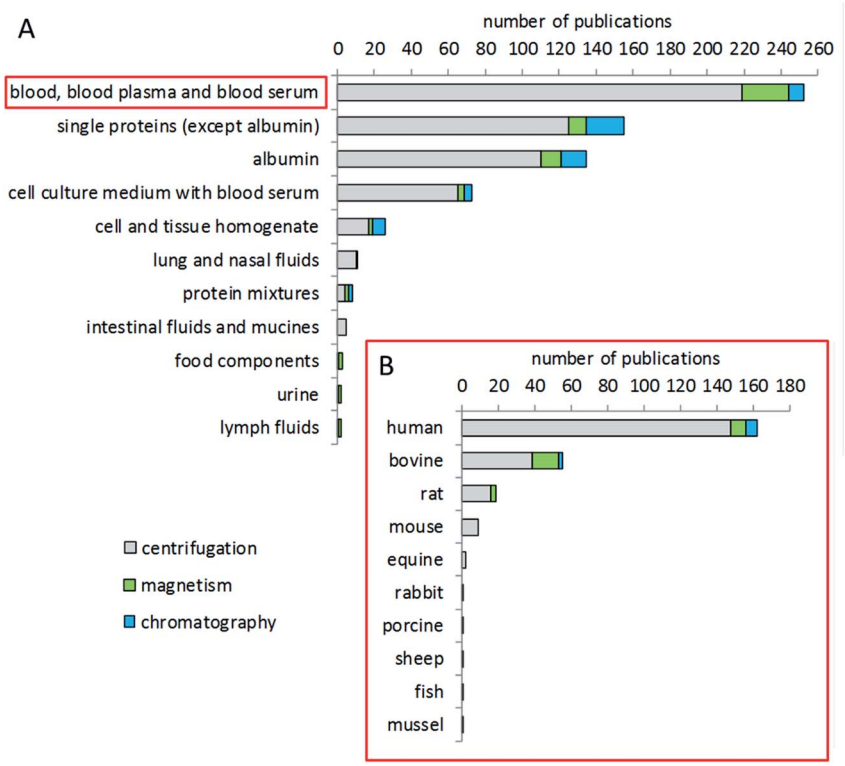

Fig. 4 Biological matrices used in corona analytics. (A) Number of publications using different matrices. (B) Papers analyzing the protein corona in blood or related matrices, itemized by the species of origin.

after cellular uptake of nanoparticles.[21] Some groups investigated the corona from blood components from humans and different animal species such as mice,[172] rats, sheep and rabbits.[173]

Choice of temperature and time for corona formation

Research in the field of nanoparticle-bio interfaces focuses mainly on humans or other mammalian systems, and therefore the incubation temperature is often set to 37 °C,[82,174] as shown in Fig. 5A. Nonetheless, incubation at room temperature has also been performed,[140,148] and sometimes lower temperatures down to 4 °C have also been used.[175–177] The latter ensure structural stability of proteins, while at the same time physiological relevance might be questioned. The incubation time for corona formation at the particle surface is often set to 60 minutes, assuming this to be an appropriate period to establish equilibrium.[30,74,153,178,179] However, short-term and long-term studies have also been performed, with incubation times ranging from 1 minute to several days.[110,180–183] Only a few studies investigated protein corona formation *in vivo* in mice,[184,185] for which, in our view, the biggest challenge is the particle recovery and purification after *in vivo* administration.

The process of corona formation does not only depend on biophysical parameters, but is also rather dependent on biological processes such as cellular uptake or intracellular distribution into different compartments. So, the extracellular corona differs strongly from the corona after uptake or, for example, vesicular delivery. This topic has been profoundly reviewed by Monopoli *et al.*[3] Integrative approaches of bioinformatic simulations and corona formation experiments are performed to study protein corona kinetics.[19] In particular, Tenzer and colleagues have demonstrated the early development of the corona, revealing that corona composition is established within the first few minutes of protein-particle contact, while the protein amount changes over time.[183] Mortensen and co-workers monitored changes of the protein corona from 1 to 48 hours of incubation.[110] In this context, corona formation is described as a very dynamic process based on the "Vroman effect". In particular, very mobile, small and highly abundant proteins tend to form the corona in early kinetics, while bigger, less mobile and higher affinity proteins can replace them in later stages and form a thermodynamically stable protein corona. Mortensen *et al.* further stated that the presence and absence of specific proteins at a certain time point can influence interactions between nanoparticles and cells. This knowledge reinforces the importance of dynamic investigations on the kinetic process of corona formation. Nevertheless, based on our literature evaluation, the choice of isolation method appears to not be correlated with the incubation time or temperature used for corona formation.

Separation techniques – centrifugation

Proper interpretation and comparison of corona data from different particles and studies require the use of reproducible methods to isolate particles with their corona out of complex,

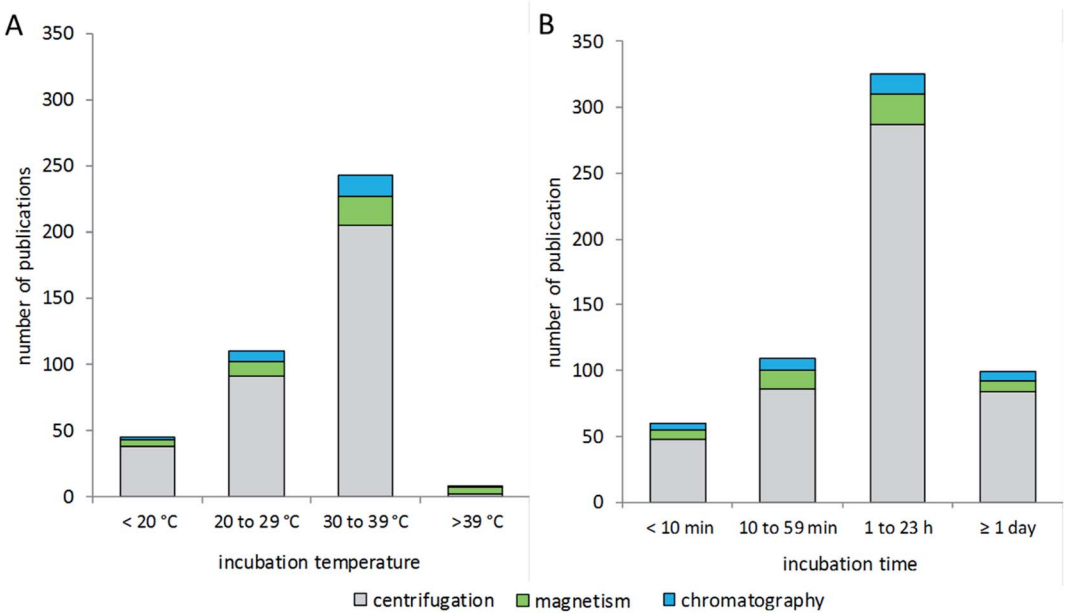

Fig. 5 Use of different incubation temperatures (A) and times (B) in corona analytics. Papers containing vague information such as "room temperature" were counted in the category 20 to 29 °C; "overnight" was categorized as ≥1 day. For more detailed information about the choice of incubation time and temperature please refer to the supporting information.

protein-rich matrices. Particles should be isolated without losing the attached proteins, while at the same time false positives should also be omitted, making separation methodology a complex analytical challenge. Currently, a plethora of different experimental approaches is used, hampering direct comparisons between many studies. In addition, many papers lack information about the controls included in the experiment to exclude false positive or negative results. In the following, we summarize and compare current methods for nanoparticle–protein corona isolation, divided into three main categories: centrifugation, magnetism and chromatography.

The most common method for separating particles and their protein corona from a matrix is centrifugation or ultracentrifugation. Here, the different densities of particles and proteins in the matrix are used for separation. Using centrifugation-based approaches, various aspects of particle–protein interactions have been studied.[5,87,183] The major issue with this method is the risk of false positives: proteins, protein complexes which originally did not bind to the particle, or proteins that bind to particle-attached proteins but not to the particle itself, may sediment during the centrifugation process together with the particles and their corona. On the other hand, false negatives may occur due to dissociation of proteins from the nanoparticle–corona complex due to centrifugation forces. It is therefore very important to determine the number of washing steps and the centrifugation time which is appropriate for separating a specific type of nanoparticle–corona complex from a certain protein-rich medium. It appears, however, that this optimization is often not performed, or at least not described in the respective publications.

The speed and duration of centrifugation must be optimized to the respective particle species, to assure complete separation of nanoparticles with their corona from the surrounding media, while at the same time preventing protein aggregates ending up in the pellet. Konduru *et al.* noted that the density of the particles (which has a significant influence on the sedimentation properties) cannot always be directly estimated from the density of the material the particles are made of, due to agglomeration and media inclusion effects.[186] Therefore, suitable controls are necessary to check for remaining nanoparticles in the supernatant and for unintentionally precipitated protein aggregates in the pellet.

While considering appropriate centrifugation conditions, different properties of the nanoparticles and the experimental set-up have to be taken into account. In general, one harsh centrifugation step is often not sufficient for complete isolation of particle-corona complexes without false positive results. These may, for example, result from protein complexes that have a higher density than the investigated particles or from proteins trapped in cavities of particle agglomerates.[30] On the other hand, insufficient centrifugation speed may leave particles in the supernatant.[187] Similar considerations are to be made for the washing procedure. Here, washing time, centrifugation speed and the number of repetitions need to be balanced. Furthermore, the choice of the washing buffer may influence the binding of proteins to the nanoparticles due to changing pH values, salt concentrations, and temperature.[188,189] Fig. 6 summarizes the aspects to consider when setting up a centrifugation-based separation method. Unfortunately, only a comparably low number of publications contain experimental details about the centrifugation procedure and its optimization. Here, it would be desirable to establish standards of reporting in order to allow the reader to judge the applied method.

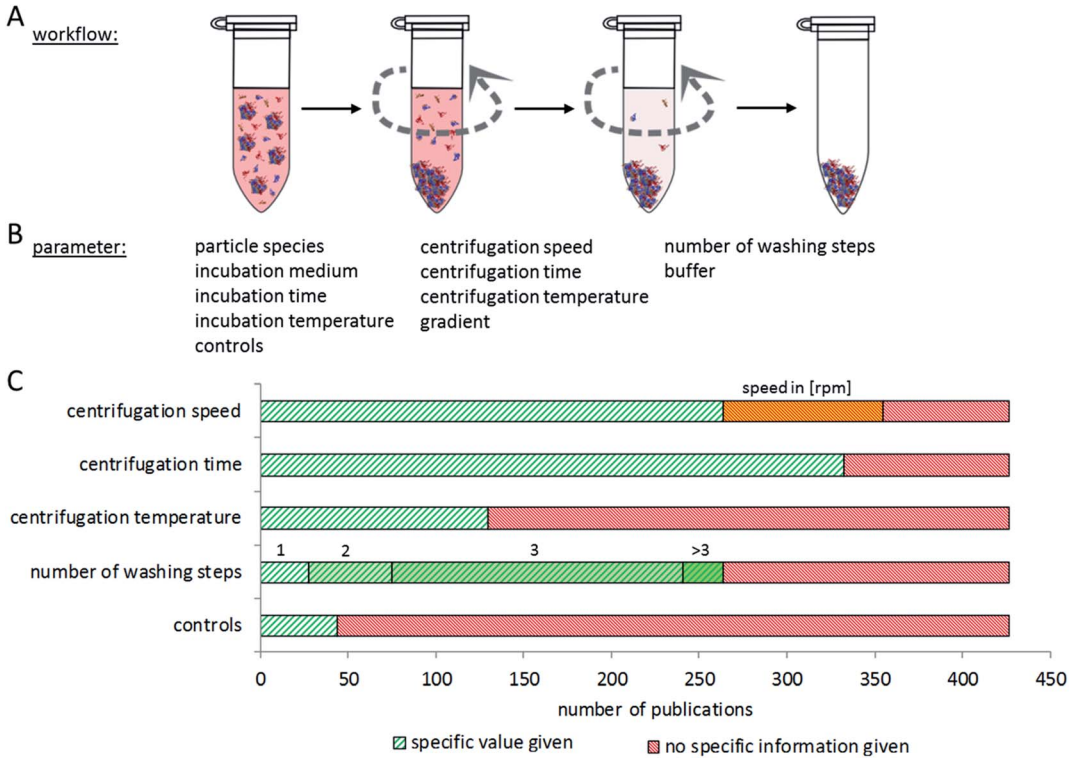

Fig. 6 Overview of the centrifugation-based particle separation methodology. (A) General workflow comprising repeated washing and centrifugation steps. (B) Selection of parameters to be considered when conducting centrifugation experiments for protein corona analysis. (C) Synopsis of the published literature: the number of publications containing information about centrifugation speed, time, temperature, washing steps and controls is presented. Green: specific information contained in the publication; red: no specific information contained; orange: only rpm values are given in the paper, but not the exact centrifugation force.

Different variations and advancements of classic centrifugation methods have been introduced in nanoparticle corona research: sucrose cushions are used to reduce the interaction time of nanoparticles and media. Unbound proteins are separated from the nanoparticles by the sucrose cushion and agglomerates also tend to float above the cushion. This way, snapshots of nanoparticle–protein interactions can be analyzed with high resolution[30,183] The sucrose cushion may be used either as a homogeneous sucrose solution with a defined density or as a gradient. The latter enables the harvesting of different fractions of nanoparticle-corona complexes to investigate the fate of nanoparticles, their protein corona and unbound proteins.[30,190,191] In particular, for low-density particles, ultracentrifugation is the method of choice, sometimes in combination with a sucrose cushion.[192] Ultracentrifugation is mostly referred to as centrifugation at >100 000×g, and can be subdivided into analytical and preparative ultracentrifugation. Analytical ultracentrifugation allows monitoring of the concentration of an analyte in the sample in real time, for example by analyzing fluorescent nanoparticles. This way, information about sedimentation properties and size distribution can be obtained.[193] However, a high centrifugation speed may lead to aggregation of proteins, thereby increasing the risk of obtaining false positive results by agglomeration. Sequential centrifugation has been used to achieve neat separation of particle-corona complexes.[194,195] This technique can be very useful, while at the same time multiple purification steps bear the risk of disruption of the corona equilibrium.

Separation techniques – magnetism

The second way to isolate particles with their corona is the use of magnetic force. Provided that the particle species is suitable for use with magnetic separation methods, magnetism is used, for example, as an easier and faster approach for separation.[196] In general, the magnetic properties of the nanoparticle are provided by iron oxide (Fig. 3). Iron oxide nanoparticles are promising tools in targeted cancer diagnostics, and they are also available in versions coated with other materials such as silica to form hybrid particles that combine the different properties of the two materials.[197] Nevertheless, centrifugation methods are also used for iron oxide nanoparticles, sometimes even for comparison. Bonvin *et al.* used both magnetic separation and centrifugation methods on the same nanoparticles.[196] Different experimental setups are available: Luborsky and Drummond described an experimental setup with magnetic columns and magnetic gradients which has been adapted to nanoparticle-related experiments.[198] Using magnetic forces for separation is thought to have less of an impact on the structure of the nanoparticle–protein corona complex than centrifugation.[196] Nevertheless, it has to be noted that the risk of agglomeration increases with particle size. Because of this it is

not recommended to use magnetic separation for nanoparticles with diameters greater than 10 nm. Thus, for larger particles, multi-step purification with increasing centrifugation intensities could be more appropriate.[196] A big advantage of magnetic separation is the reduction of false-positive proteins due to aggregation under centrifugal forces. It also reduces the loss of protein after multiple washing steps. Nevertheless, washing steps are needed after magnetic separation too, but the loss of particles at each step might be lower. The limited number of applicable particle species as well as the possibility of interactions between magnetic particles and other required analytical methods are disadvantages of this separation technique.

Separation techniques – chromatography

Chromatographic approaches are used less frequently than the above methods, probably because they are in general more time-consuming and cost-intensive and allow only a relatively low throughput of samples. In addition, they are not suitable for a variety of particle types with bigger sizes, for polydisperse particles, and for particles which adhere to the column material. However, they provide a possibility for investigating association/dissociation rates and affinity of individual proteins bound to nanoparticles, and they also allow collection of different fractions of a sample with less perturbation to particle–protein complexes.[14] In general, two main methods are used, size exclusion chromatography and flow-field-flow-fractionation methods, such as asymmetric flow-field-flow-fractionation (A4F).[199]

Described for the first time in 1950, the principle of size exclusion chromatography is separation by the hydrodynamic volume of the analyte.[200] Smaller particles interact more with the stationary phase (mostly composed of porous particles) and therefore need more time to pass through the column while bigger particles elute faster. This principle helps in determining association and dissociation rates: complexes with a stable corona have an increased hydrodynamic diameter compared to blank particles, since proteins remain attached to the particle. In contrast, if particle–protein affinity is weak, proteins tend to dissociate quickly, causing no measurable increase in the hydrodynamic diameter. Therefore, the latter will elute at the same time as their non-incubated counterparts.[14,201] Also, particle–protein complexes can be collected in fractions relative to their size and used for further analysis.[201] However, interactions with the stationary phase may occur and lead to a change in the protein corona. Not only due to competing interactions between the particle, corona and stationary phase, but also due to shearing forces, this may result in a loss of protein–particle interaction.

A4F is a method to separate analytes in a wide size range (1 nm to 100 μm).[202] It also helps to reduce potential non-specific interactions.[87] A4F uses a liquid cross flow that is established in a channel with a nonporous and a porous wall. Particles are thus exposed to a laminar flow that pushes the particles along the tube, and a cross flow that forces them to the bottom of the channel. Due to their Brownian motion (which is more for smaller particles), the analytes can also float back into the channel, thereby avoiding passage through the porous membrane.[203,204] A4F is especially used for analysis of complex samples and stable protein coronae.[7,87,199] Fractions can be collected without much perturbation of particle–protein complexes. However, method establishment is usually very time-consuming due to the variety of parameters to be optimized.[205]

Ashby and colleagues combined the chromatographic method of flow field-flow fractionation with ultracentrifugation to obtain information about the composition of hard and soft coronae.[87] However, ultracentrifugation tends to yield false positives or negatives due to dissociation of proteins owing to centrifugal forces or due to down-centrifugation of non-corona proteins. These often unconsidered issues aggravate the identification especially of the soft corona proteins, as already mentioned at an early stage of corona analytics by Lundqvist in 2008.[5]

Comparability of different methods

A few studies are available comparing the results obtained with different corona isolation methods. Bonvin *et al.* compared magnetic separation and a new multi-step centrifugation method for their iron oxide nanoparticles in human blood and lymph serum.[166] The results showed that the hard corona obtained by magnetic separation differed from that obtained by the new centrifugation method, as only about half of the identified corona proteins were commonly detected using both methods. The parallel use of the two different methods was thus helpful to verify the presumably true positive results, but it was not possible to firmly distinguish whether the other proteins identified by only one single method represent false or true positives. Similarly, Pisani *et al.* compared magnetic separation with centrifugation and also concluded that the results differ substantially.[93] By contrast, other analyses have revealed greater similarity of coronae obtained by using different separation methods: Monopoli *et al.* isolated nanoparticles by centrifugation, size exclusion chromatography, and magnetic separation. The protein composition of isolated hard coronae in the latter analyses was very similar between the different methods.[206] Bekdemir *et al.* compared their centrifugation results using mercapto-undecanoic acid (MUA) gold nanoparticles with the results of Rocker *et al.*, 2009 which were obtained using iron–platinum (FePt) nanoparticles of a similar size and with the same surface modification (carboxylated).[193,207] The authors concluded that protein dissociation behavior mainly depends on the nanoparticle surface modification and size, and not on its core material. However, their centrifugation based method requires the adsorption of proteins to be reversible. In contrast to their finding that diluting the sample led to a lower number of proteins adsorbed to the particle, Milani *et al.* showed no reversibility of the protein corona after transfer into more diluted media and thus no Hill coefficient but material dependency was observed. The different results may also be due to the nature of the surface modifications since different materials

Table 1 Summary and overview of the different particle–protein corona isolation methods and their advantages and disadvantages as discussed in the text

Method	Advantages	Disadvantages
Centrifugation	- Separation according to density and size	- Long centrifugation times can lead to false particle–protein interactions
	- Most frequently used technique	- Several purification steps needed; modifications in the protein–corona system can occur
	- Widely used and easy to use[216]	- Magnetic nanoparticles can agglomerate[196] false-positive highly abundant proteins due to insufficient washing
	- High throughput[216]	- Changing of centrifugation tubes is necessary to exclude carryover of proteins adsorbed to the tube walls
	- Centrifugal speeds and times can be optimized according to the nanoparticle material and media	- Outcome affected by centrifugation force, washing duration, washing solution and solution volumes; must be adjusted for each particle type
	- Tuning experimental conditions makes the method available for a wide range of nanoparticles	- The smaller and less dense the particles, the higher the centrifugation speeds chosen; thus aggregation[30] occurs
	- High resolution results[30]	- Not suited for very small (5–20 nm) or low density nanoparticles (1 g cm^{-3}), because unbound proteins and protein corona complexes cannot be separated effectively[30,217]
	- Possibility of separating different populations co-existing *in situ*[30]	- Not preparative, so no populations can be recovered for further studies[23,29,206]
Size exclusion chromatography (SEC)	- Flexible technique, many stationary/mobile phases	- Interaction between analytes and the stationary phase can occur[218]
	- can be used with standard lab equipment	- SEC selectivity decreases when applied to analytes with a very high molar mass such as nanoparticles[218]
	- Analyte resolution and recovery in SEC is generally superior to A4F[218]	- Low throughput
	- Has been developed into a systematic methodology[14]	- No full recovery of hard corona complexes for further studies[30]
	- Less perturbing than centrifugation[14]	
Asymmetric flow-field-flow fractionation (A4F)	- Complex, heterogeneous and polydisperse dispersions can be investigated without extensive sample preparation[205]	- Long establishment process
	- Reduced to no destruction or alteration of the protein corona	- Must be adjusted for every particle type[205]
	- Prior fractionation by AF4 allows size investigation of complex heterogeneous and polydisperse mixtures[205]	- Low throughput[205]
	- Several detection techniques can be coupled to AF4 (online and offline)[205]	- Expensive
	- Possible automation[205]	- Not routinely available in many analytical laboratories
	- Short measurement time[205]	- Separation of particles from a very polydisperse sample leads to peak broadening and loss of resolution; must thus be divided into several experiments
	- Easy collection of fractions[205]	- Sample loss due to adsorption on the membrane can occur, affecting retention and disturbing quantification of single fractions[205]
	- Absence of a packaging material or a stationary phase[205]	- No full recovery of fraction for further experiments[30]
	- The potential of AF4 increases with increasing molar mass[205]	
	- Once established, AF4 is a multifunctional technique for separation and characterization of nearly all nano-sized[205]particles	
Magnetism	- Low impact on the structure	- Only practicable for small (~10 nm), magnetic nanoparticles[196]
	- High throughput	- Degree of separation decreases with decreasing magnetism[196]

may lead to different ligand packing densities with little change in the hydrodynamic diameter.[193,208]

Approaches using two independent methods allow for a better assessment of the protein corona, but difficulties remain, as highlighted above. Often, false positives or false negatives result from the isolation/separation method. The variation between different isolation methods might, to a large extent, arise from different forces acting on NPs during the isolation process. In addition, the obtained results might be transferred to only a small selection of closely related nanoparticles, thus hampering more general conclusions and making it difficult to compare results from different corona studies.[93,166,196]

Methods for identification of nanoparticle corona proteins

In general, the isolation of nanoparticle–protein corona complexes from biological media is considered to be the most critical point in corona research. However, interpretation of the data also depends on the method of "stripping" the particle for analyzing the composition of the corona by protein identification methods. Different approaches have been used for this purpose, depending on the experimental question addressed. Some methods quantify the total protein content, such as the Bradford and BCA assays[209–211] and UV/vis absorption measurements.[212] The protein amount can be measured by the determination of bound proteins or as a difference in the supernatant. Other methods separate proteins, for example electrophoresis or chromatographic techniques, followed by visualization methods such as Coomassie brilliant blue or silver staining.[60,213] More detailed information about the corona, and thus possibly also insights into the biochemistry of particle–cell interactions *via* the corona, can be obtained using methods for protein identification, for example mass spectrometry. A standard protein identification technique is 2-dimensional gel electrophoresis followed by tryptic digestion and mass spectrometry. Therefore, protein identification by MALDI-TOF is usually used.[91,156] Unfortunately, traditional mass spectrometric methods allow just the identification of a rather small number of highly abundant proteins and is very time-consuming. Advanced mass spectrometry methods, such as LC-MS, orbitrap- or triple-quad-MS can replace the gel electrophoresis steps and lead to a significantly increased sensitivity with lower detection limits. Up to now, using high-resolution mass spectrometry to identify proteins has been the recommended state-of-the-art method, even though this requires bioinformatics data evaluation of big data sets. For a more in-depth review of this topic, we recommend the recent review articles by Carrillo-Carrion and Pederzoli (Table 1).[214,215]

Conclusion

Individual nanomaterials exhibit very specific characteristics. Therefore, the choice of separation method for the isolation of protein–corona complexes depends strongly on the particle and its physicochemical parameters and also on the surrounding matrix, possible unintended interactions and the desired fate of the corona. Centrifugation as the most widely used technique may be the first choice for many particles but may, for example, not be feasible for low-density analytes. Centrifugation is easy to use, but care should be taken for proper controls to exclude false positive and negative results in protein identification. In addition, loss of particle fractions during repeated washing and centrifugation steps is an issue. However, this might be managed by analyzing all fractions for their particle contents to exclude the loss of particle fractions. Additionally, control samples with the particle-free matrix of interest, including stabilizing agents and ions that might be released from the particles, can help to prevent false positives. Nanoparticle corona analysis is a very diverse field of current research, investigating a dynamic process that depends on a plethora of different parameters. To enable comparative and reproducible results across different studies and laboratories, it would be desirable to have harmonized experimental setups for the scientific community, and detailed descriptions of experimental procedures including the use of controls.

Conflicts of interest

There are no conflicts of interest to declare.

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Author's contributions

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