

No king without a crown – impact of the nanomaterial-protein corona on nanobiomedicine

Besides the wide use of nanomaterials in technical products, their application spectrum in biotechnology and biomedicine is steadily increasing. Whereas the physico-chemical properties and behavior of nanomaterials can be engineered and characterized accurately under idealized conditions, this is no longer the case in complex physiological environments. In biological fluids, proteins rapidly bind to nanomaterials forming the protein corona, critically affecting the nanomaterials' biological identity. As the corona impacts *in vitro* and/or *in vivo* nanomaterial applications, we here review the concept of the protein corona and its analytical dissection. We comment on how corona signatures may be linked to effects at the nano–bio interface and conclude how such knowledge is offering novel opportunities for improved nanomedicine.

Keywords: bioinformatics • blood system • colloidal chemistry • label-free quantification • mass spectrometry • nanomaterials • nanomedicine • nanotoxicology, proteomics

Nanomaterials, which are defined as materials with at least one external dimension in the size range of approximately 1–100 nm, can be engineered in almost unlimited combinations concerning properties related to their chemistry, shape, size and surface characteristics [1]. Nanoparticles (NPs) are objects with all three external dimensions at the nanoscale, can be generated by various chemical, biotechnological and/or physical procedures, and can be specifically tailored to optimally meet their intended (biomedical) applications [1]. In contrast to these engineered NPs, naturally occurring NPs (e.g., volcanic ash and soot from forest fires) or the incidental by-products of combustion processes (e.g., welding and diesel engines) show usually a high physical and chemical heterogeneity.

Over the last decades, there has been an explosion in the technical applications of nanomaterials and their use in biotechnology is steadily growing [2,3]. Also, in the field of nanomedicine, nanomaterials and NPs are increasingly considered as new promis-

ing tools [4–6]. For example, nano-enabled drug delivery systems are expected to display improved solubility, pharmacokinetics and biodistribution, and thus may be easier to administer with fewer side effects [4,7–9]. Moreover, due to the fact that NPs can be engineered to be small enough to directly interact with the cellular machinery, it is expected that these formulations could overcome hardly accessible biobarriers and reach various target organs, for instance the brain or tumors [10–18].

NPs are typically able to hijack the endocytosis machinery and enter almost any cell type [19]. Also, as NPs are of similar size as many subcellular components, they escape established biological defense mechanisms directed against larger-size particulate matter. Moreover, the ability to manipulate particular nanomaterial/NP features, such as their physical, chemical and biological properties, opens up a huge variety of possibilities in rationally designing nanotools for drug delivery, as imaging agents and/or for diagnostic/theranostic purposes [20–28].

Dominic Docter^{*1}, Sebastian Strieth¹, Dana Westmeier¹, Oliver Hayden², Mingyuan Gao³, Shirley K Knauer⁴ & Roland H Stauber¹

¹Department of Nanobiomedicine, ENT, University Medical Center of Mainz, Langenbeckstr. 1, 55101 Mainz, Germany

²Siemens AG, Corporate Technology, Guenther-Scharowsky Strasse 1, 91058 Erlangen, Germany

³Institute of Chemistry, Chinese Academy of Sciences, Bei Yi Jie 2, Zhong Guan Cun, Beijing 100190, China

⁴Institute for Molecular Biology, CENIDE, University Duisburg-Essen, Universitätsstr. 5, 45117 Essen, Germany

*Author for correspondence: docter@uni-mainz.de

However, one should not neglect that these developments will also lead to an increasing exposure of humans and the environment to nanomaterials. Once inside cells, NPs may cause adverse effects [29] and even permanent cell damage [30,31]. As potential mechanisms, oxidative stress, inflammation, genetic instability and the inhibition of proper cell division have been described, which depending on the (patho)physiological context may contribute to cell death [32–35]. Thus, the discussion about nanosafety aspects and regulations is certainly important and still ongoing [6,36–39].

Fate of NPs in biological environments – friend or foe for medical applications?

When designed for drug delivery and imaging purposes, NP administration often requires intravenous injections [3,8]. The detailed knowledge about physical and chemical aspects associated with the behavior of NPs in physiological systems in general has been recognized as an important factor not only for understanding nanotoxicology [40,41], but also for (re)shaping the future of nanobiomedicine. Numerous physical and chemical interactions define the fate and thus also success or failure of nanobiomedical applications in physiological environments. Here, NPs are exposed not only to relatively high ion concentrations or drastic pH changes [42], but also to a huge variety of complex biomolecules [43]. Body fluids are indeed complex (e.g., blood, lung lining fluid, saliva and intestinal juice, among others) and may in some cases contain more than 2000 different proteins in widely varying concentrations [44,45].

Engineered NPs are usually stabilized by electrostatic repulsion, steric hindrance, or depletion forces [46]. Steric stabilization is realized by macromolecules attached to the NP surface [46]. Charged functional groups or surfactant molecules on the NP surface produce a Coulomb potential and, thus, give rise to electrostatic repulsion between individual particles carrying charges of the same polarity. Short-ranged,

attractive, van-der-Waals-type forces dominate at small interparticle distances and cause the pronounced tendency of NPs to aggregate [46]. Aggregation is prevented by the longer-ranging Coulomb forces between charge-stabilized NPs. Both van-der-Waals-type and electrostatic forces are however quantitatively affected by the ionic strength of the surrounding medium ([46] and references therein), which is happening in physiological environments.

Hence, one need to keep in mind that whereas the rational design of NP behaviors can be achieved in a controlled and stable environment during synthesis, the situation changes dramatically when such nanodevices are introduced into complex environments. Here, NPs adsorb various (bio)molecules due to their high surface energy [46–48]. Hence, besides the changes in ionic strength, physical and chemical interactions with proteins and/or other biomolecules (e.g., phospholipids, sugars and nucleic acids, among others) will in most cases significantly affect the NPs' behavior and fate.

By enshrouding the particle, the protein adsorption layer defines the NP surface and mediates further interactions between the NPs and the biological environment [49–52]. Moreover, the protein coating does not only directly mark the biological identity of NPs, but can also indirectly cause its 'transformation' by drastically altering the NPs' colloidal stability. Here, the protein corona can either have either a stabilizing effect by inducing steric stabilization [53] or a destabilization impact, caused by protein-mediated bridging, charge compensation and/or by introduction of charge inhomogeneity onto the NP surface.

Upon aggregation, multiple interactions may result in stronger affinities compared with proteins binding to single NPs, which is more likely to occur in a biological solution in which particles are highly diluted. Moreover, there could even be a trapping of (abundant) proteins in such aggregates with low or no affinity for single NPs. Depending on the NP,

Table 1. Factors influencing composition and evolution of the protein corona.

Factors	Ref.
Exposure temperature	[74]
Exposure time	[47,58,73,75–79]
Nanoparticle hydrophobicity	[43,50,80–84]
Nanoparticle size/surface curvature	[50,52,55,58,66,69,83,85–87]
Nanoparticle surface charge	[47,69,79,80,84,88,89]
Nanoparticle surface functionalization	[50,55,80,88]
Relative ratio physiological media/nanoparticle concentration	[43,72]
Topology	[90]

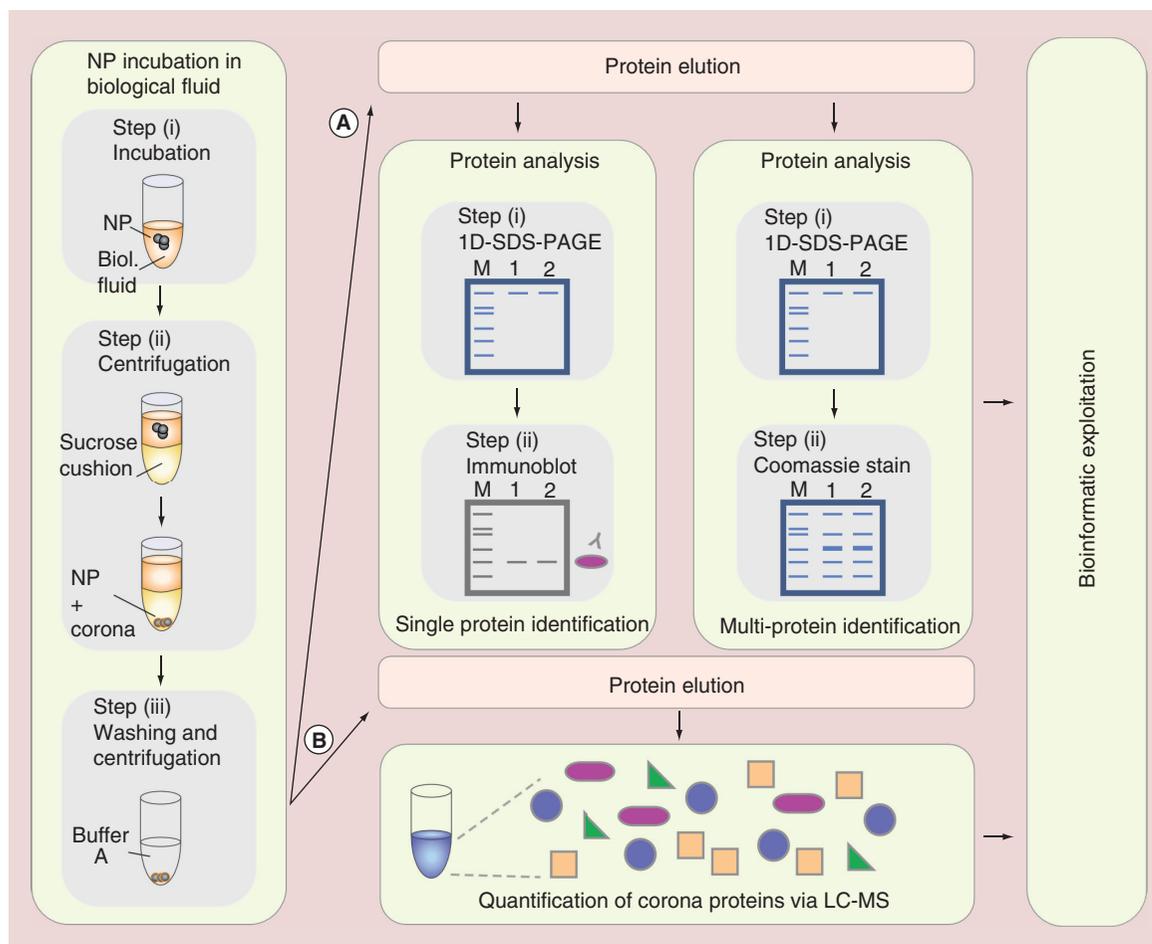


Figure 1. Schematic overview of a workflow to obtain quantitative and qualitative (plasma) protein corona signatures. Following NP incubation in plasma or other protein containing liquids, NP–protein complexes are rapidly separated from unbound proteins by sedimentation through a sucrose cushion, and washed. Corona component analysis can subsequently be achieved by different methods. **(A)** Protein elution and separation via 1D SDS-PAGE allows to directly visualize and compare stained protein patterns (right). Immunoblot analysis identifies ([semi]quantify) the presence of specific corona components (left). **(B)** Protein elution and analysis via label-free quantitative LC-MS allow obtaining qualitative as well as quantitative comprehensive corona protein signatures. Further bioinformatic analysis and exploitation of the data facilitates a rational *in vitro/in vivo* analysis of the potential impact of corona proteins in physiological systems. NP: Nanoparticle. For color figures, see online at www.futuremedicine.com/doi/full/10.2217/NNM.14.184

such aggregation may also require a certain time and thus, additional aggregates may impact the kinetics of corona formation. Whereas subfractions of aggregates by centrifugation techniques are possible during NP synthesis, these effects are hard to control and predict *in vivo*. Collectively, aggregation of NPs will add an additional level of complexity, which has to be considered in the description and application of NPs within physiological systems [47,48,54].

Albeit somehow loosely defined, the term ‘hard corona’ is often used to describe the long-lived equilibrium state, representing a protein signature of an NP in a certain environment [47,48,55,56]. Some models further suggest that on top of this ‘hard corona’ a ‘soft corona’ may exist, consisting of a more loosely associated and

rapidly exchanging layer of biomolecules [48,50,56–58]. However, since this ‘soft corona’ desorbs during current purification processes, its existence and biological relevance remain to be confirmed. Unless stated otherwise, we herein avoid the confusing discrimination between the ‘hard corona’ versus ‘soft corona’ and refer to (patho)biologically relevant NP–protein complexes as the ‘protein corona’.

Clearly, it is the biomolecular corona that primarily interacts with biological systems and thereby constitutes a major element of the NPs’ biological identity affecting multiple molecular-scale interactions [47,48,56,59,60]. The biophysical properties of such a corona-covered NPs often differ significantly from those of the formulated pristine particle during good

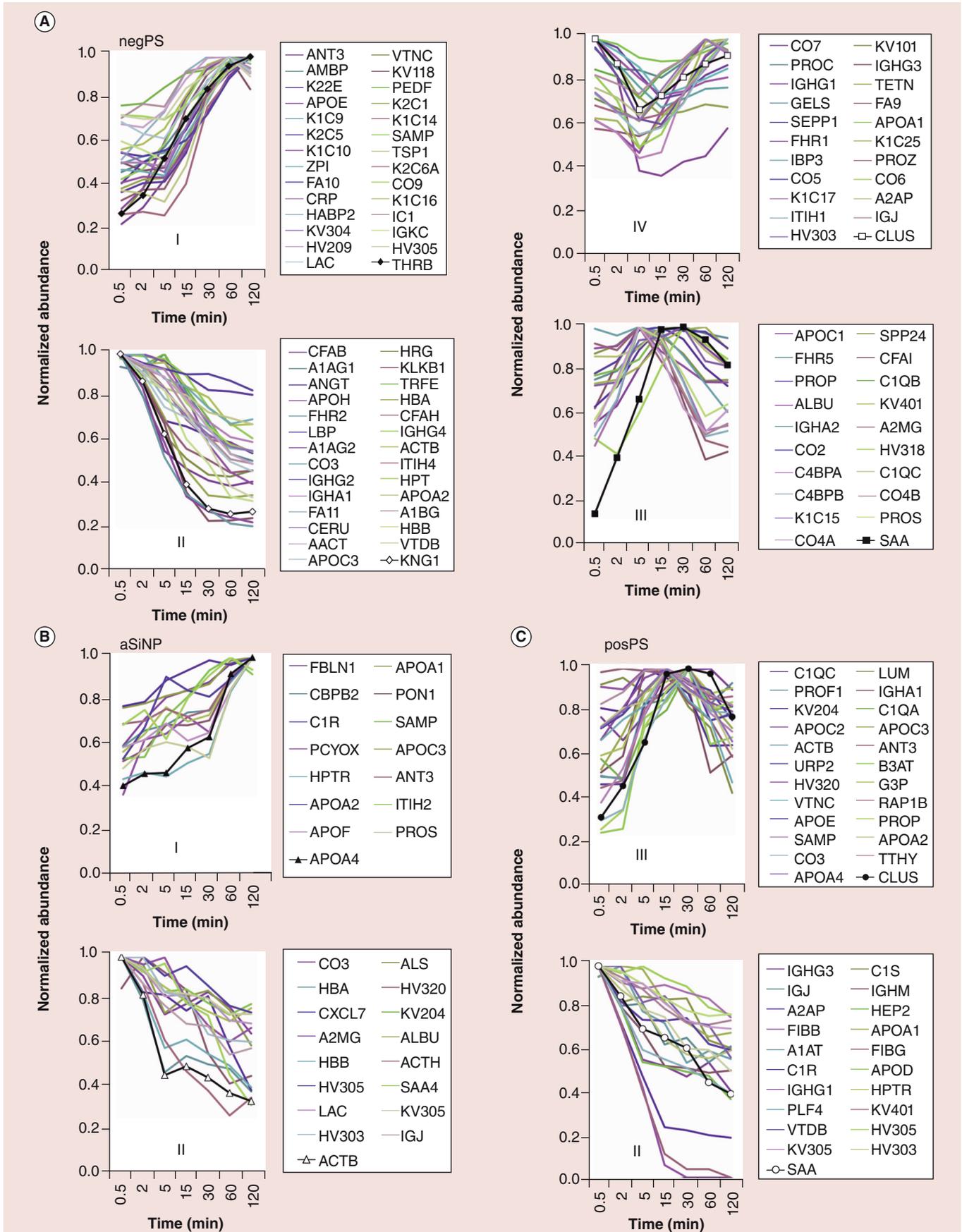


Figure 2. Correlation analysis to demonstrate distinct kinetic protein-binding modalities during the temporal evolution of the plasma protein corona (see facing page). (A–C) Time-smoothed normalized protein abundance profiles of (A) negPS, (B) aSiNP and (C) posPS. NP coronae were classified into four groups by correlation analysis and relative values were normalized to the maximum amount (set to 1) across all time points for each protein. Protein groups PG I and PG II showed increasing or decreasing binding over time, respectively. PG III, ‘Peak’ proteins, display low abundance at the beginning of plasma exposure and at later time points, but higher (peak) abundance at intermediate time points. PG IV proteins show the opposite behavior, with a high abundance at early and late time points, but low abundance at intermediate time points. A selection of representatives is displayed.
aSiNP: Amorphous silica NP; negPS: Negatively charged polystyrene NPs; NP: Nanoparticle; posPS: Positively charged polystyrene NPs.

laboratory practice manufacturing [36,47,48,56,61,62]. From a regulatory aspect, (bio)molecule-coated nanomaterials may therefore be even considered as novel materials with different properties compared with the pristine nanomaterials during production [36,47]. Hence, the subsequent biological responses of the body as well as the particle’s biodistribution in patients may be significantly influenced by the NP-protein complexes, potentially contributing not only to favorable nanomedical reactions but also to unwanted (patho) biological side effects [36,47,63–65]. Clearly, for the rational development of nanomaterials for any kind of biological or biomedical application, it is thus the key to understand the formation and kinetic evolution of the protein corona [46,48,66–69]. Numerous studies have been conducted to generally dissect and mechanistically understand the biomolecule corona on nanoscale materials, its dependence on the NPs’ physico-chemical properties and its biomedical and/or (patho)biological relevance [36,47,54,63–65]. Typically, corona profiles differ significantly from the protein composition of the (biological) fluid investigated [47,53,54,56,70,71]. Distinct proteins will be either enriched or displayed only weak affinity for the NP surface. However, the relation between original surface functionality of the NP and the nature of the corona is far from being trivial and currently still remains impossible to predict in complex physiological environments [47,53,54,56,65,70,71]. Not only particle material, size and surface properties but also exposure time and the relative ratio of the physiological fluid to the NP dispersion have been shown to play a role in determining the composition and evolution of the corona, although the underlying physical mechanism is not yet resolved in detail (Table 1) [47,48,53,54,56,65,70–72]. Moreover, when NPs move from one biological environment to another, for example, from the blood system via different cellular uptake mechanisms into cells (e.g., monocytes or macrophages), a key issue is whether the original corona remains stable or is subjected to substantial changes [54], adding an additional level of complexity. The current model assumes that after passing through several ‘biological environments’, the final corona still contains a fingerprint of its history and keeps a

memory of its prior journey through the body [54,73]. However, as recent data demonstrate that the plasma protein corona is surprisingly stable and matures only quantitatively rather than qualitatively [47], one might however hypothesize that the corona may not be subjected to significant changes, even when passing through several ‘biological environments’, unless processing is performed by enzymatic cellular machineries. Close inspection of the literature indeed reveals that the detailed fate of the original corona, as it travels through membranes and barriers, thereby interacting with the extracellular matrix and various cellular enzymatic machineries, is still not resolved in detail, and may differ in various body organs, such as the liver or the brain.

Nevertheless, when developing nanomaterials for *in vitro* diagnostic sensors or drug delivery-/cell-targeting vehicles, one has to at least consider the formation and potential impact of the biomolecule corona on the biomedical performance of the product.

It is envisaged that dissecting the composition of the protein corona in a given biological fluid may allow predictions of the particle’s fate regarding its interactions with specific cell types and surface receptors, biodistribution, as well as predictions of its half-life in the body [43,47,65,91,92]. As the majority of NP applications in the area of biomedicine depend on their exposure to the complex protein-rich environment of the blood system, such knowledge is particularly relevant for plasma proteins [47,65,93]. As the complete plasma proteome reference set contains more than 2000 different proteins [94], the plasma protein corona has recently been shown to be indeed highly complex as well [47]. Human plasma proteins play important roles in recognizing foreign materials entering the circulation [46,86,95]. Specific proteins are involved in eliciting an immunological response to pathogens or in assisting their clearance by the reticuloendothelial system (RES) [46,86,91,95]. Also, the NP’s decoration with certain plasma proteins and/or blood components may potentially affect distribution and delivery to the intended target sites [95–97]. Hence, a deep understanding of the biological effects triggered by NP particularly in the blood system requires detailed knowledge of the particle-associated

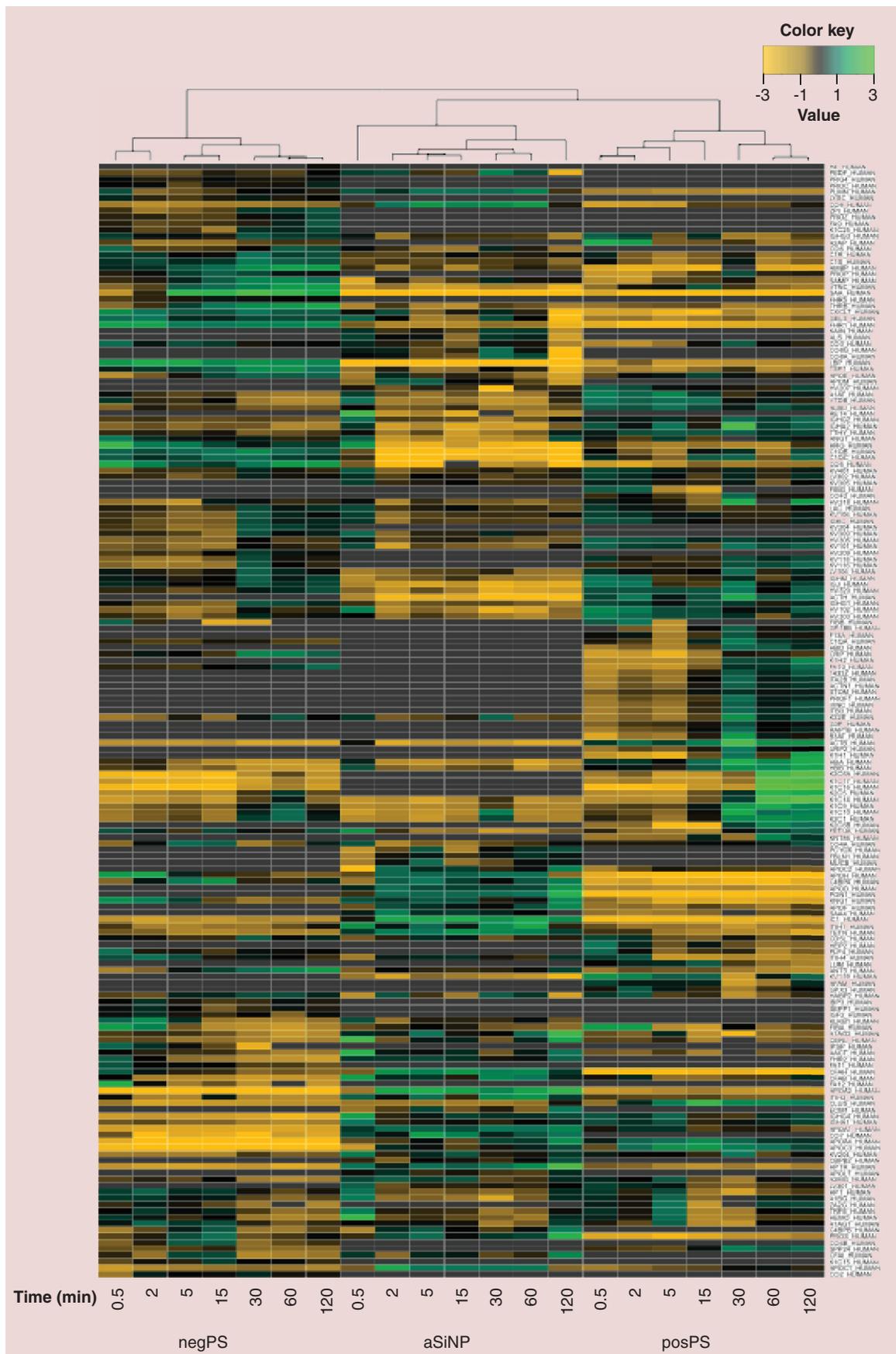


Figure 3. Corona protein binding profiles are affected by the nanoparticle's physico-chemical characteristics as well as plasma incubation time (see facing page). Unsupervised hierarchical cluster analysis of the relative abundance of plasma proteins bound to negatively charged aSiNPs, and negPS or posPS. Dendrograms illustrate sample similarities. Color scheme is based on \log_2 of the ratio (protein amount at the respective time point/ average protein amount across all time points). Green indicates proteins with higher than average abundance, yellow indicates proteins with lower than average abundance. Exposure time periods to human plasma are indicated. For further details, see [47].

aSiNP: Amorphous silica NP; negPS: Negatively charged polystyrene NPs; NP: Nanoparticle; posPS: Positively charged polystyrene NPs.

proteins, a fundamental prerequisite for nanomedicine, nanobiology and nanotoxicology [46,47,64,98,99].

Requirements for a comprehensive analysis of protein corona profiles

To date, numerous studies have been conducted to identify plasma proteins (specifically) associating with (specific) NPs and attempted to correlate their binding with the NPs' physico-chemical properties [47,53,54,56,58,65,66,70,71,100]. Protein adsorption on nanoscale materials has been investigated by quite varying experimental methods and in addition has mostly not been analyzed quantitatively [47,54]. Collectively, the heterogeneity of these studies resulted not only in a still incoherent picture of how the composition and evolution of the protein corona is affected by various factors (Table 1) but sometimes even led to conflicting conclusions. Also, albeit most physiological systems and the blood system in particular are highly dynamic, snap-shot time-resolved NP-specific fingerprints were hardly resolved [47,54,66]. Based on classic analytical procedures, it was suggested that the blood plasma protein corona consists of only a few tens of proteins and changes significantly over time [54]. Notably, albeit protein coronas can in principle be obtained by standard centrifugation methods, those involve mostly relatively long centrifugation steps (for an overview, see [54]). Therefore, while the 'intended exposure times' of NPs to biological fluids were reported to be relatively short, the centrifugation times required to pellet the NP-protein complexes (10–20 min) represent an 'unintended exposure time', during which the NPs are still in contact with the biological fluid, potentially resulting in the additional binding and/or dissociation of corona proteins [54,56]. Thus, most previous studies did not precisely control (short) exposure times of NPs to the biological fluid of interest. These limitations precluded so far a rapid and high-resolution kinetic analysis of corona profiles, necessitating the development of standardized protocols to quantitatively and qualitatively analyze the (blood plasma) protein corona of NPs [47]. To minimize the contact time of NPs with biological fluids of interest, a sucrose cushion-based centrifugation method was recently introduced to resolve the plasma corona

[47]. Also, many previous studies describing the protein corona of various NPs used protein separations through 1D and/or 2D gel electrophoresis followed by in gel tryptic digestion and mass spectrometry (MS) [47,54]. Such approaches are though not only tedious, but are likely to reveal only a partial view of the protein corona profile [47,54,56], and do not allow a global quantification of corona constituents. Over the last years, precursor-intensity based label-free quantification is rapidly gaining popularity [101]. Compared to other techniques including those that use labeling, label-free LC-MS provides a wide range of benefits. Time-consuming and expensive protocol steps, as for example the introduction of a label into proteins and peptides, can be omitted. Indeed, the use of label-free quantification by LC-MS was shown to achieve a reliable and highly reproducible comprehensive quantification of plasma protein corona components [47,54]. In Figure 1, the proteomic workflow for the quantitative and qualitative characterization is schematically summarized. The basic steps described are suitable for various biotic and (abiotic) protein-containing (patho)physiological fluids, such as ichor, cerebrospinal fluid, cell/organ lysates as well as for exposure with different NP formulations.

Dynamics of corona formation, evolution & complexity under physiological conditions

Several studies addressing the composition of the protein corona revealed that not only physico-chemical properties of NPs, but also the exposure time of NPs to biological environments significantly affects the protein and most likely the biomolecule corona in general. Such corona modulations may lead to altered biodistribution, as well as to effects impacting therapeutic and (patho)physiological responses (Table 1) [46,47,54,102,103]. A current model suggests that particularly at short exposure times the 'soft' protein corona is initially formed around the NPs, which is highly dynamic and subsequently rather slowly matures to the 'hard' corona by significantly changing its composition over time [47,54,76]. Most studies however focused on corona formation upon prolonged nanomaterial exposure to complex biological environments [47,54,56,76]. These studies often failed to recognize that physiological systems are highly dynamic

and might need to react instantly to external stimuli. In particular, in the blood system, flow velocities in the ascending aorta can reach up to 60 cm s^{-1} , in contrast to 0.1 mm s^{-1} within tumors, and processes controlling hemostasis and thrombosis can be triggered within minutes or even seconds [16,86,91,95].

In contrast to nanoscale objects, the formation and evolution of protein layers on flat surfaces was first analyzed by Vroman already in 1962 [104]. The so-called 'Vroman effect' describes a time-dependent composition of the bio-coating, in which the early state is dominated by highly abundant proteins, adsorbing only weakly. Subsequently, these adsorbed proteins are replaced by less abundant proteins, which however bind with higher affinity, resulting in a complex series of adsorption and displacement steps [104]. In several models, the Vroman effect was thus directly used to explain the evolution of the protein corona around NPs, resulting in the concept of a 'dynamic protein corona evolution' [54,71,73,76]. However, to fully understand the interaction of NPs with biological systems, a high-resolution time-resolved knowledge of NP-specific protein adsorption is required, as various protein groups are expected to display increased or reduced binding over time. In this context, it was recently shown by Tenzer and coworkers for silica and polystyrene NPs of various sizes ($\text{Ø} \approx 35, 120, 140 \text{ nm}$), and surface functionalization (amine, carboxylate, unmodified) that already the formation of a complex 'hard' protein corona is an unexpectedly very rapid process [47]. Whether on top of such a complex protein adsorption layer an additional 'soft corona' may indeed play a physiologically relevant role remains to be fully proven [105]. As predicted from the Vroman effect, corona protein groups that displayed increased or reduced binding over time were observed [47]. Interestingly, novel binding kinetics for biologically relevant protein groups were discovered by the study, which cannot be solely explained by the Vroman effect [47]. Classification of protein-binding modalities identified proteins characterized by low abundance at the beginning of plasma exposure and at later time points, but displaying 'peak' abundance at intermediate time points and vice versa (Figure 2). Most kinetic studies did not employ quantitative LC-MS-based proteomics, which may explain why these complex binding kinetics have been unnoticed so far. However, whether similar protein binding kinetics exist for NPs in general needs to be investigated experimentally. Due to the coronas' complexity of more than hundred different proteins, which adsorbed to all studied NPs already 30 s following exposure to human plasma [47], the study also demonstrated

that the binding patterns observed in human blood plasma cannot be explained by current mathematical protein corona evolution models derived from highly simplified experimental systems [54,71,73,76]. Interestingly, the number of different corona proteins exceeds in most cases the number of proteins that theoretically could be accommodated on a single NP, giving first indications that the corona exists most likely not as a simple monolayer but may be composed of multiple core-shell structures or higher order 'Christmas tree-like' structures. Also, in contrast to previous models [54,71,73], the corona composition changed almost exclusively quantitatively but not qualitatively [47]. Nevertheless, the compositions of the protein corona signatures were clearly affected by NP properties, such as size, material and surface functionalization [47], and also the plasma exposure time was identified as a factor significantly affecting NP-bound protein abundance (Figure 3). As none of the above-mentioned factors, such as physicochemical properties of the NPs or exposure time, alone is able to determine formation, composition and evolution of the protein corona, a multi-parameter classifier will most probably be required to generally model and predict NP-protein interaction profiles in biomedical relevant environments [47]. Indeed, a recent study indicated that protein signatures alone without the kinetic information seem not to allow prediction of the hemato-compatibility of NPs [65].

Impact of corona formation on blood system physiology

The above discussed parameters affecting corona formation are not only of pure academic interest, but also have significant impact on (patho)biological effects. As illustrated in Figure 4, proteins involved in complement activation and coagulation have been identified in the coronas of negatively charged amorphous silica (SNP35) NPs [47,106]. Identified proteins span about three to four orders of magnitude dynamic range, most likely covering most of biologically relevant corona proteins [47]. Here, the respective abundance of all of these proteins was affected by plasma exposure time and NP characteristics, such as size and surface functionalization. Clearly, obtaining comprehensive quantitative and qualitative protein corona signatures (Figure 1) [47,56], and ideally the implementation of an international standardized corona profile database resource, will finally allow the bioinformatic analysis and exploitation of signatures to guide a subsequent rational *in vitro* in *vivo* investigation of the potential impact of corona proteins in physiological systems.

Employing primary human cell models of the blood system, it was recently indeed demonstrated

that already the early corona formation affected processes at the nano–bio interface [47]. From the study it was proposed that although pristine NPs in general exist only for a short period in the blood system, these are still able to affect vitality of endothelial cells, trigger thrombocyte activation and may induce hemolysis. Formation of the biomolecule corona rapidly modulates the NPs' decoration with bioactive proteins, thereby protecting cellular components of the blood system against NP-induced (patho)biological processes, and in addition also influencing cellu-

lar uptake (Figure 5). However, whether this general statement is indeed valid for all existing and future NP formulations as well as for every biomedical relevant environment remains to be experimentally confirmed.

As the nature of the protein corona is currently a still unpredictable complex factor potentially triggering not only desired (nano)medical reactions but also undesired biological responses [43,47,65,107], there are currently numerous attempts to chemically completely prevent protein adsorption [28,78,108–110]. NPs func-

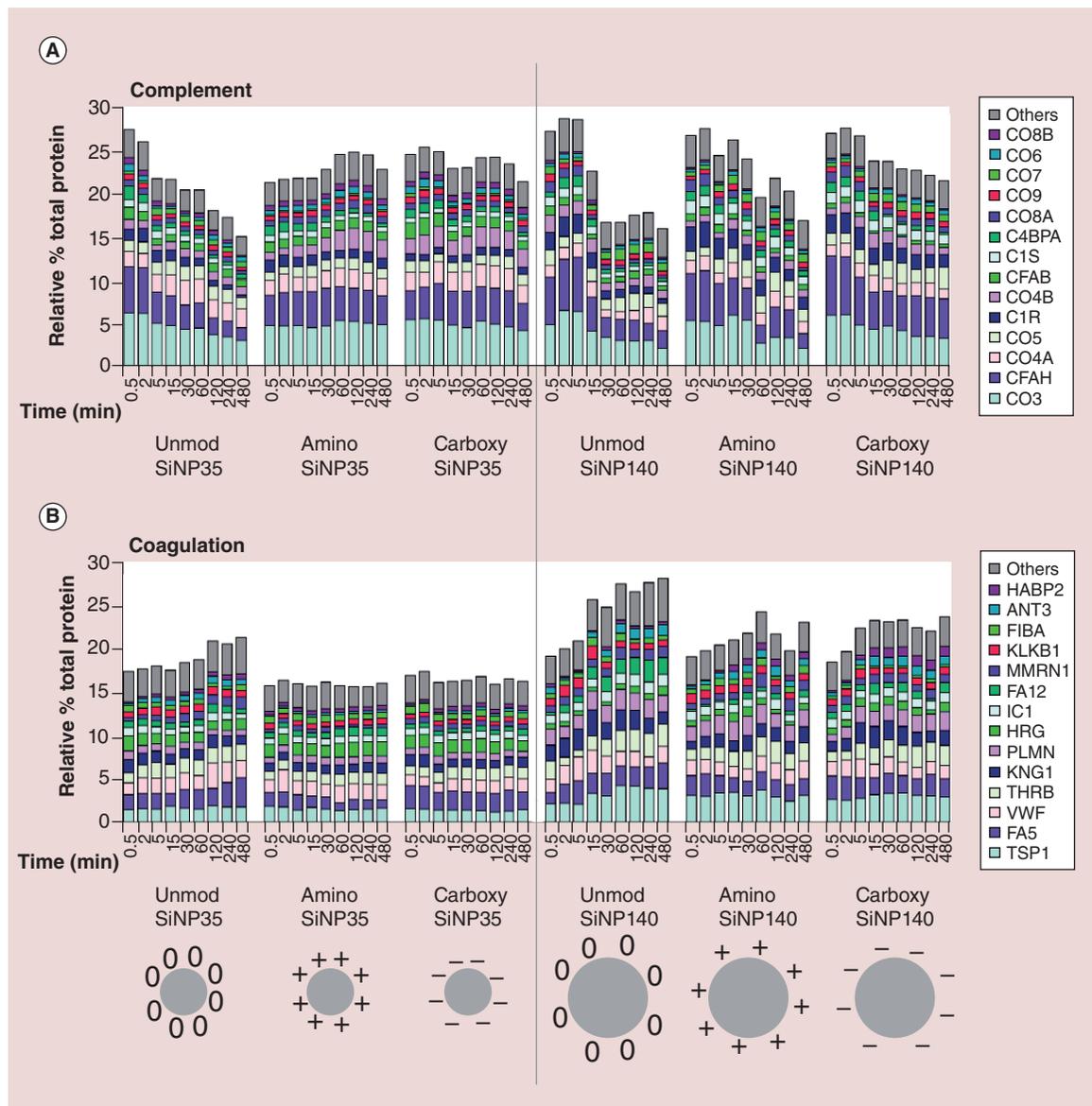


Figure 4. Nanoparticle- and time-dependent corona protein profiles involved in complement activation and coagulation. (A) Complement activation. (B) Coagulation. Exposure time-dependent functional classification of corona proteins on negatively charged amorphous SiNP. SiNP of various sizes ($\varnothing \approx 35$ and 140 nm) and various surface-functionalization (unmodified (0, unmodified), amine (+, amino) and carboxylate (-, carboxy)) were analyzed.

SiNP: Amorphous silica nanoparticle.

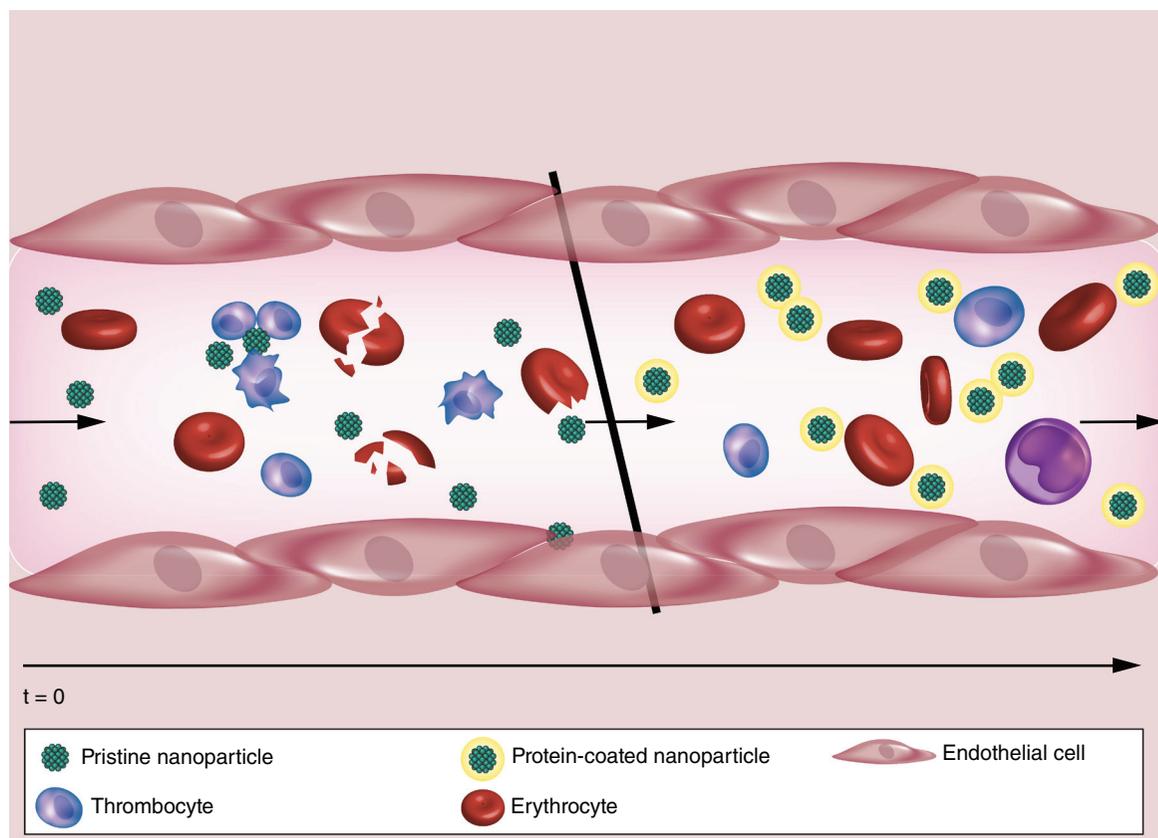


Figure 5. Cartoon illustrating how rapid corona formation may kinetically impact early nanopathology in the human blood system. Upon entry or parenteral application, pristine nanoparticles only exist for a short period of time, but are still capable to immediately affect vitality of endothelial cells, trigger thrombocyte activation and aggregation, and may induce hemolysis. Formation of the biomolecule corona rapidly modulates the nanoparticles' decoration with bioactive proteins protecting cells of the blood system against nanoparticle-induced (patho)biological processes, and also can promote cellular uptake. Elements not drawn to scale.

tionalized with certain polymer chains, such as the addition of various polyethylene glycol-based chains ('PEGylation') onto the NP surface, are often referred to be highly 'biocompatible', as unspecific interactions with biological components are minimized [78,108,109]. Nanomaterials functionalized with PEG offer high colloidal stability under physiological salinity conditions caused by interparticular repulsion [78,108,109]. As protein adsorption is suppressed, numerous cellular responses are affected, including opsonization by cells of the RES. Thus, the circulation time in the blood system as well as the biodistribution of NPs may be modulated via PEGylation, although the detailed mechanisms are not yet resolved [86,91,111]. The extension of the circulation time by excessive PEGylation can lead to a strong inhibition/reduction of cellular uptake and thereby reducing the potential of the NP drug delivery system [110]. For an effective drug delivery, a compromise has to be achieved between a prolonged circulation time, minimum opsonization by the cells of the RES and an efficient uptake by the target cells.

Remarkably, even the arming of NPs with various antibodies tailored to achieve specific targeting of (cancer) cells did not prevent rapid corona formation (Figure 6), and in many cases reduced cell-targeting efficacy *in vitro* as well as *in vivo* [12,97,103,112]. Notably, also for the diagnostic detection and treatment of circulating tumor cells, an 'undesired' protein corona may be of disadvantage [97,113].

Collectively, although sophisticated surface modifications, such as PEGylation, reduce the binding of biomolecules, some association with biomolecules does still occur. Indeed, we are currently unaware of any nanomaterial functionalization, which will completely prevent the formation of a biomolecule corona in biomedical relevant environments. The design of such nanodevices represents certainly one of the key challenges promoting successful nanomedicine.

Conclusion & future perspective

Despite the fact that the relationship between nanomaterial design and the physiological response has been studied intensely for more than two decades,

only some general principles have emerged. Changes in colloidal stability are expected to occur most likely in the ambient ion concentrations in biological fluids. The reliable determination of physical and chemical parameters of NPs in biological fluids therefore remains still a challenging task. Currently, reliable characterization data can only be obtained by employing multiple, complementing standardized methods. Nevertheless, the nanomedicine field has reached an important stage by now accepting that the protein corona does exist for the majority of nanomaterials used in biomedicine, seems to be established rapidly and is highly complex in most if not all physiological relevant environments. Results compiled over many studies and particularly recent comprehensive proteomic investigations unambiguously demonstrated that a 'typical' plasma protein corona consists of more than hundred different proteins adsorbed at abundance potentially capable of modulating biological responses. Recent bioinformatic comparisons start to uncover that not only highly abundant proteins, such as serum albumin, are most likely present in the coronas of all nanomaterials, albeit many more data need to be compiled in order to define even the 'plasma adsorbome'.

Currently, we cannot yet predict how the synthetic identity of a nanomaterial influences the structure, composition and evolution of the protein corona. We do understand that when a cell or an organ is presented with an NP, it does not primarily see the bare particle, but also the NP with its ligand plus its entire surrounding biomolecule corona profile. Still, the mechanisms governing the interaction of pristine and/or corona-covered nanomaterials with cells and the question that which of the corona proteins involved in cell recognition and uptake are critically involved have not been resolved in detail. At present, it is still unclear whether every corona protein or only a certain subset is accessible to and/or biologically active in the physiological environment, and ultimately influencing responses at the nano-bio interface. Without such a detailed understanding as a guide, it is still challenging to rationally design nanomaterials to interact with defined corona proteins and cells in a controlled and predictable way. As a result, nanomaterials are currently often modified with anti-fouling polymers to overall suppress protein adsorption in order to reduce off-target cell uptake and improve targeting efficiency. However, detailed relationships between the synthetic identity, biological identity and physiological response to nanomaterial are not clear.

We here have discussed latest developments leading to novel insights, which however intriguingly demonstrate that current models of competitive adsorption

fall short of explaining the factual physiological situation where NPs are subjected to thousands of different proteins competing for binding to their surface. Fundamentally, the challenge of deciphering these detailed relationships is the complexity inherent in the system [114]. Large gaps still exist in the understanding of the fundamental physico-chemical aspects of corona formation and we are even having larger deficiencies in applying this knowledge to a realistic (patho) physiological situation.

Moving forward clearly requires standard operating procedures, detailed enough to ensure data qual-

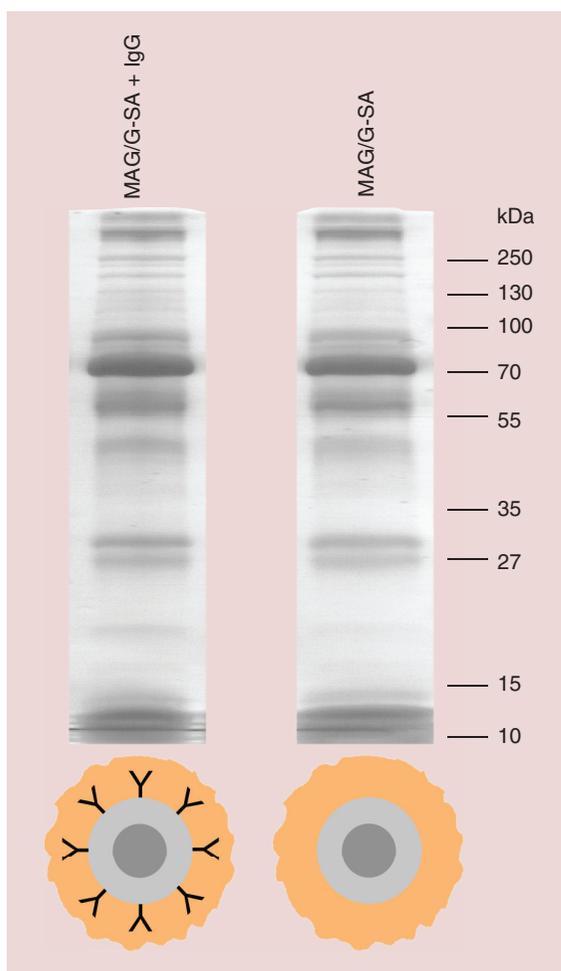


Figure 6. Antibody functionalization of nanoparticles does not prevent rapid plasma protein corona formation. 1D SDS-PAGE to visualize NP-bound human plasma proteins on IgG-conjugated superparamagnetic magnetite NPs (MAG/G-SA+IgG; left lane) or pristine superparamagnetic magnetite NPs (MAG/G-SA; right lane). MW is indicated. Magnetite core is depicted in dark gray, starch matrix in light gray and protein corona in orange. Sizes not drawn to scale. MAG/G-SA: Superparamagnetic magnetite NPs; MAG/G-SA + IgG: Superparamagnetic magnetite NPs conjugated with IgG; MW: Molecular weight; NP: Nanoparticle.

Executive summary

Background

- In the field of nanomedicine, nanomaterials and nanoparticles (NPs) are increasingly considered as new promising tools.
- NPs can be engineered to be small enough to directly interact with the cellular machinery.
- Development will lead to an increasing exposure of humans and the environment to nanomaterials.

Fate of NPs in biological environments – friend or foe for medical applications?

- In physiological environments NPs are exposed to relatively high ion concentrations, drastic pH changes and a huge variety of complex biomolecules.
- Changes in ionic strength, physical and chemical interactions with proteins and/or other biomolecules will in most cases significantly affect the NPs' behavior and fate in a physiological environment.
- By enshrouding the particle, the protein adsorption layer (protein corona) defines the NP surface and mediates further interactions between the NPs and the biological environment.
- The biomolecular corona primarily interacts with biological systems and thereby constitutes a major element of the NPs' biological identity affecting multiple molecular-scale interactions.
- For the rational development of nanomaterials for biological or biomedical application, it is the key to understand the formation and kinetic evolution of the protein corona.
- Particle material, size, surface properties and exposure time play a role in determining the composition and evolution of the corona.
- Dissection of the composition of the protein corona in a given biological fluid may allow predictions of the particle's fate regarding its interactions with specific cell types and surface receptors, biodistribution, as well as predictions of its half-life in the body.
- Deep understanding of the biological effects triggered by NPs in a physiological system requires detailed knowledge of the particle-associated proteins.

Requirements for a comprehensive analysis of protein corona profiles

- Previous studies did not precisely control (short) exposure times of NPs to the biological fluid of interest and limited a rapid and high-resolution kinetic analysis of corona profiles.
- Necessity of the development of standardized protocols to quantitatively and qualitatively analyze the protein corona of NPs.

Dynamics of corona formation, evolution & complexity under physiological conditions

- Previous studies often failed to recognize that physiological systems are highly dynamic and need to react instantly to external stimuli.
- Previous studies used the Vroman effect to directly explain the evolution of the protein corona around NPs, resulting in the concept of a 'dynamic protein corona evolution'.
- Recent studies revealed novel binding kinetics for biologically relevant protein groups which cannot be solely explained by the Vroman effect.
- Observed binding patterns cannot be explained by current mathematical protein corona evolution models derived from highly simplified experimental systems.
- Corona composition changed almost exclusively quantitatively but not qualitatively.
- None of the factors (size, material, surface functionalization and the exposure time) alone is able to determine formation, composition and evolution of the protein corona.
- Multiparameter classifier will be required to generally model and predict NP–protein interaction profiles in biomedical relevant environments.

Impact of corona formation on blood system physiology

- Pristine NPs exist only for a short period in the blood system but are still able to affect vitality of endothelial cells, trigger thrombocyte activation and may induce hemolysis.
- Formation of the biomolecule corona rapidly modulates the NPs' decoration with bioactive proteins, thereby protecting cellular components of the blood system against NP-induced (patho)biological processes, and also influencing cellular uptake.
- NPs functionalized with polymer chains ('PEGylation') onto the NP surface are referred to be highly 'biocompatible', as unspecific interactions with biological components are minimized and the (blood) circulation time is prolonged.
- Although sophisticated surface modifications, such as PEGylation, reduce the binding of biomolecules some association with biomolecules does still occur.

Conclusion & outlook

- Acceptance in the field of nanomedicine that the protein corona does exist for nanomaterials used in biomedicine, establishes rapidly, and is highly complex in physiologically relevant environments.

Executive summary (cont.)**Conclusion & outlook (cont.)**

- Cell or organ does not primarily see the bare particle, but the NPs with its ligand plus its entire surrounding biomolecule corona profile.
- Current models of competitive adsorption fall short of explaining the factual physiological situation where NPs are subjected to thousands of different proteins competing for binding to their surface.
- Large gaps still exist in the understanding of the fundamental physico-chemical aspects of corona formation.
- Establishing nanostructure activity relationships linking NP/corona properties to (patho)physiological responses remains a still distant goal but such knowledge is needed to develop NPs for improved future nanomedical applications.
- Protein coronas have unique properties that may be exploited to confer novel and advantageous NP properties.
- Relationships between synthetic versus biological identity and the resulting physiological response will ultimately aid the innovation of more effective therapies and biological applications of engineered nanomaterials.

ity and allow inter-laboratory comparison. Quantitative, high-resolution LC-MS/MS has been shown to dramatically reduce the protein corona characterization time. General application of such 'gold standards' across laboratories worldwide will enable the implementation of a general corona profile database resource. *In silico* exploitation of such a database combined with experimental high-throughput verification will significantly contribute not only to understanding the physico-chemical mechanisms but also enable the prediction of the (patho)biological relevance and biomedical impact of the nanomaterial-protein corona in general. Establishing nanostructure activity-relationships linking NP/corona properties to (patho)physiological responses remains a still distant goal despite the achievements of the past years. Such knowledge is ultimately needed not only to understand and minimize nanotoxicity but also to develop NPs for improved future nanomedical applications.

So far, the corona has mainly been shown to hinder the biomedical properties for which NPs were designed, constituting a substantial reason for many of the current nanomedical and nanotherapeutic failures [103,115]. However, we need to appreciate that protein coronas have unique properties that may be exploited to confer novel and advantageous NP properties (e.g., influencing their targeting and/or reducing its toxicity) [116], which could be strategically harnessed in design and 'NP-reprogramming' strategies. As we learn more about the corona and its biological ramifications, we may exploit our knowledge and perhaps reach unprecedented capabilities in nanomedicine. The ability to (fine-)tune the corona composition itself could potentially enable new possibilities in overcoming biological barriers, such as the blood-brain-barrier, help to mitigate toxicity, improve uptake, and direct bioavailability and clearance. In

addition, as certain proteins in the corona may not be fully functional, this denaturation could be exploited by tuning NP surface chemistry to force the way in which a protein on the NP is denatured and inhibited, potentially altering corona composition and biological fate.

Nanomedicine has so far mainly relied on the knowledge of sophisticated nanomaterial synthesis and engineering. It is mandatory to now join forces and to additionally exploit the NPs' biological identity, which is substantially determined by the biomolecule corona. Notably, besides proteins there are numerous additional molecules present in biomedical relevant environments, such as lipids and sugars, offering even more ways to (fine-)tune effects at the nano-bio interface. Once fully mapped, the relationships between synthetic versus biological identity and the resulting physiological response will ultimately aid the innovation of novel more effective therapies and biological applications of engineered nanomaterials. However, successfully complete such an endeavor requires the interdisciplinary cooperation of experts working in the areas of physics, chemistry, biology, pharmacy and medicine.

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