Check for updates

NANO · MICRO small
www.small-journal.com

Mechanistic Understanding of Protein Corona Formation around Nanoparticles: Old Puzzles and New Insights

*Karin Nienhaus and Gerd Ulrich Nienhaus**

Although a wide variety of nanoparticles (NPs) have been engineered for use as disease markers or drug delivery agents, the number of nanomedicines in clinical use has hitherto remained small. A key obstacle in nanomedicine development is the lack of a deep mechanistic understanding of NP interactions in the bio-environment. Here, the focus is on the biomolecular adsorption layer (protein corona), which quickly enshrouds a pristine NP exposed to a biofluid and modifies the way the NP interacts with the bio-environment. After a brief introduction of NPs for nanomedicine, proteins, and their mutual interactions, research aimed at addressing fundamental properties of the protein corona, specifically its mono-/multilayer structure, reversibility and irreversibility, time dependence, as well as its role in NP agglomeration, is critically reviewed. It becomes quite evident that the knowledge of the protein corona is still fragmented, and conflicting results on fundamental issues call for further mechanistic studies. The article concludes with a discussion of future research directions that should be taken to advance the understanding of the protein corona around NPs. This knowledge will provide NP developers with the predictive power to account for these interactions in the design of efficacious nanomedicines.

1. Introduction

Impressive advances in nanotechnology over the past decades have paved the way to the design and synthesis of nanomaterials in significant quantities and with the highest precision, down to the atomic scale.[1–3] Their optical, magnetic, and electrical properties can be finely tuned via changes in size, shape, and other physicochemical parameters,[4] making them highly attractive for a wide variety of biomedical applications, both in diagnosis (e.g., contrast agents, biosensors) and therapy (e.g., drug delivery, phototherapy).[5–7]

An area that has attracted special attention in academics and industry alike is the development of nanoscale drug carriers for systemic administration to patients, e.g., by intravenous injection for delivery of therapeutic agents to tumor cells to destroy them.[8,9] Nanoparticles (NPs) can have enormous advantages over the free drug, as biodistribution and pharmacokinetics can be addressed through NP design, independently of drug properties, e.g., solubility and stability.[10] With dimensions in the range of biological transport devices such as biomolecules and vesicles, NPs can invade cell and tissue compartments, and their highly programmable physical properties offer a wealth of strategies to target and manipulate specific subgroups of cells in the body. "Passive targeting" takes advantage of the aberrant vascularization of solid tumors and inflamed tissues, causing enhanced NP uptake via the "enhanced permeability and retention" (EPR) effect[11] and "vascular bursts."[12] "Active targeting" is achieved by NP surface decoration with moieties that specifically and selectively bind to cells, mainly via cell surface receptors, which are often overexpressed by tumor cells. However, in the body, drug accumulation at the target site always competes with NP clearance from the circulation through the renal or immune (MPS, mononuclear phagocytic system) systems. A fine balance is needed between target accumulation and clearance to achieve appropriate NP levels at the target site and effective removal from the organism, which minimizes systemic toxicity, e.g., immune system destruction,[13] and other off-target effects.

While NP-based drug carriers have frequently shown impressive performance in laboratory experiments, only a small number of nanoformulations have made their way from the lab bench to the clinic to date.[14–16] A key problem is their low

K. Nienhaus, G. U. Nienhaus
Institute of Applied Physics
Karlsruhe Institute of Technology
76049, Karlsruhe, Germany
E-mail: uli@uiuc.edu
G. U. Nienhaus
Institute of Nanotechnology
Karlsruhe Institute of Technology
76021, Karlsruhe, Germany
G. U. Nienhaus
Institute of Biological and Chemical Systems
Karlsruhe Institute of Technology
76021, Karlsruhe, Germany
G. U. Nienhaus
Department of Physics
University of Illinois at Urbana-Champaign
Urbana, IL 61801, USA
The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/smll.202301663.

© 2023 The Authors. Small published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

DOI: 10.1002/smll.202301663

delivery efficiency, as only 0.7% of the administered NP dose was found to reach the target site on average.[15] To solve this problem, we need a deeper understanding of the processes that NPs undergo in the bio-environment,[17–19] where they are exposed to complex aqueous media such as blood, which contains thousands of different proteins and a wide variety of lipids, carbohydrates, and small metabolites as well as blood cells. An adsorption layer quickly forms around the NP, known as the "protein corona"[20] or "biomolecular corona,"[21–23] which conceals the "physicochemical identity" of the pristine NP and confers it with a new, "biological identity."[21] This extensive transformation of the outer surface greatly modifies the interactions with the bio-environment, and thus has serious implications for the efficacy of nanomaterials in disease diagnosis, tissue regeneration and cancer therapy.[24–26] Different strategies have been pursued to cope with this extra layer of complexity: Protein adsorption onto NPs can be suppressed by coating their surfaces with, e.g., zwitterionic, PEGylated or carbohydrate moieties, rendering the NPs extremely hydrophilic.[27–29] Without proteins presented on their surfaces, these NPs can evade clearance from the organism, so they are fittingly referred to as "stealth NPs."[30] Complete rejection of proteins from the NP surface appears challenging, however.[31–35] Alternatively, the nature of the adsorption layer (structure, types, and relative amounts of proteins) can be manipulated through specific NP surface design, resulting in a tailor-made protein corona that can elicit the desired biological responses.[36–41] Furthermore, optimal targeting, pharmacokinetic, and biodistribution/clearance abilities may be achievable by combining the two approaches, balancing stealth material and targeting ligand density on the NP surface.[40,42]

A deep, quantitative understanding of the physicochemical processes at the nano–bio interface is a prerequisite for gaining full control over the behavior of NPs in the bioenvironment. After years of intense research, however, controversies are still lingering over rather fundamental questions such as, what is the basic structure of the protein corona (monolayer, bilayer, multilayer), what is the nature of the adsorbed proteins (native, partially or completely unfolded), and how does the protein corona form and evolve in time? While the diversity of NPs, proteins (and other biomolecules) as well as biofluids and sample preparation conditions suggests a certain level of variability, distinct properties of the corona are expected to arise from the general physicochemical principles governing nano–bio interactions. Focusing on these issues, we briefly summarize the basic properties of important NPs and proteins, and present mechanistic aspects of the processes occurring at the NP–protein interface. Then, we critically discuss recent investigations aimed at gaining further insights into the protein corona structure and dynamics. We conclude with a perspective on future research needed for further progress toward the aim of reaping the full benefits of nanotechnology for biomedical applications.

2. NP–Protein Interactions

2.1. NPs for Biomedical Applications

NPs can be synthesized with widely tunable characteristics, including size, shape, rigidity, surface properties such as topography (roughness, porosity etc.), hydrophobicity and charge distribution, and responsiveness to external stimuli (**Figure 1**).[43,44] Heterogeneity in size and other properties is unavoidable in most NP preparations but has to be minimized.[45,46] Precise characterization and continual quality control with a range of analytical techniques[47–49] is especially important for biomedical applications, requiring the preparation of highly defined and colloidally stable NPs in bulk quantities.[17] We briefly introduce the three major NP classes employed in the field, inorganic, polymeric, and lipid NPs, featuring different and partially complementary properties (Figure 1).[16]

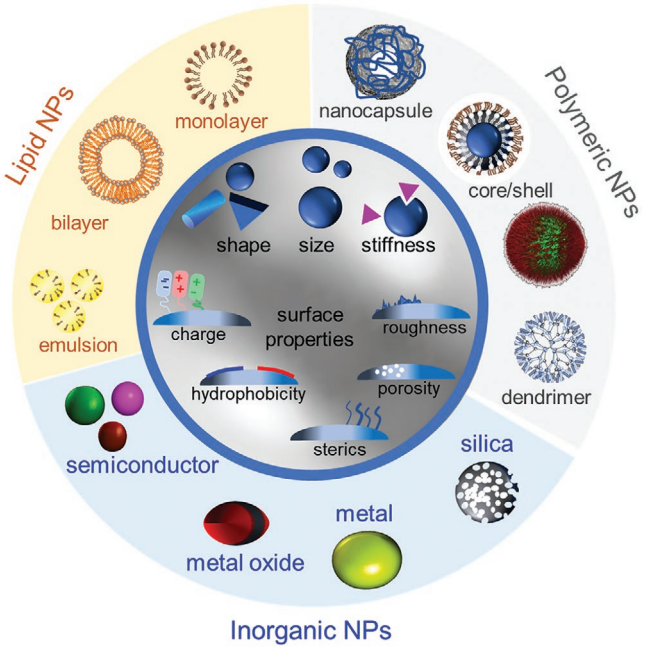


Figure 1. Engineered NPs for biomedical applications. In the center, various physicochemical properties of NPs are depicted schematically. The circumference shows the three major classes (lipid-based, polymeric and inorganic) of NPs that are important for biomedical applications.

Inorganic NPs can be synthesized from a broad range of materials, including gold (and other metals), oxides (metal oxides, silica) and several semiconductors, featuring excellent size definition (up to atomic precision) and diverse forms and shapes (nanospheres, nanorods, nanoshells, etc.). They can be endowed with interesting physical (optical absorption and luminescence, magnetic and electrical) properties that are beneficial for diagnostic and therapeutic applications. The inorganic core is frequently enclosed in a shell of organic ligands including polymers (e.g., poly(ethylene glycol), PEG), passivating the reactive core's surface and endowing the NP with colloidal stability in an aqueous solution.[50] This organic layer has to be carefully designed to ensure chemical stability during long-term exposure of the NPs to the harsh bioenvironment.[51] Notably, inorganic NPs that accumulate in the body bear the risk of long-term toxicity.[52–54]

Polymeric NPs can be prepared from materials of natural or synthetic origin, featuring solid cores (nanospheres) or internal cavities (nanocapsules).[55,56] They are viewed as good drug

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

delivery vehicles, capable of transporting considerable amounts of hydrophilic and hydrophobic cargo, by encapsulation, entrapment in the polymer matrix, or chemical conjugation to the polymer matrix or the NP surface. For in vivo applications, biocompatible and biodegradable polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLA-PGA copolymers (PLGA), and polycaprolactones (PCL) are preferable.[57] For in-vitro analytics and fluorescence imaging, NPs made from simple synthetic polymers such as poly(methyl methacrylate) (PMMA) or polystyrene (PS) are often used. NPs made from hydrophobic polymers (e.g., PS) can be electrostatically stabilized by surface functionalization with charged groups (e.g., sulfate, carboxylate, or amine). Moreover, steric stabilization can be achieved with "fuzzy" surfaces on the NPs made by polymer chains protruding from the surface.[58] Agglomeration and toxicity may cause problems in in-vivo applications and has to be carefully assessed.

Lipid NPs (LNPs) are spherical structures consisting of a lipid mono- or bilayer enshrouding an internal compartment.[59–61] They are convenient to prepare, as they self-assemble from a mixture of lipids formulated to convey the desired properties to the NP. A variety of LNP designs have been developed for the targeted delivery of hydrophobic, hydrophilic, and solid payloads. LNPs have proven effective for the treatment of various diseases and currently are the largest group of FDA-approved nanomedicines.[16,62] Especially liposomes, consisting of a membrane bilayer enclosing an aqueous compartment, have a long history as medicine delivery platforms, thanks to their good biocompatibility and bioavailability. Recently, LNPs have proven effective for the protection and delivery of mRNA in vaccines against the SARS-CoV-2 virus.[63] More complex and not solely lipid-based are cell membrane-coated NPs.[64] Their synthetic core is camouflaged by a lipid bilayer shell consisting of naturally derived cellular membranes, so that the NP displays biomolecules that are present on the cell surface and thus mimics to a certain extent the bio-interfacing properties of cell membranes.

2.2. Globular Proteins—Structure and Stability

Proteins, linear polymers varying in length from below one hundred to many thousand monomeric units, are synthesized by ribosomes, the protein factories of cells, from usually 20 different proteinogenic amino acids (**Figure 2**a). The unique amino acid sequence of each protein dictates its intricate, densely packed 3D fold that it adopts after biosynthesis. Small globular proteins spontaneously fold into their compact structure and have dimensions of a few nanometers. Larger proteins are often oligomeric and/or consist of several domains, independently folded and thermodynamically stable units arranged like beads on a string. Notably, some rather elongated (fibrous) proteins also exist, such as fibrinogen in blood plasma, with a length of ca. 50 nm. In general, the protein fold is stabilized by a large number of weak forces, mostly van der Waals interactions and hydrogen bonds; charge interactions (e.g., salt bridges) and covalent bonds (e.g., disulfide bridges) are also present in some proteins (Figure 2a). A key contributor to the thermal stability of the fold is the hydrophobic force. In

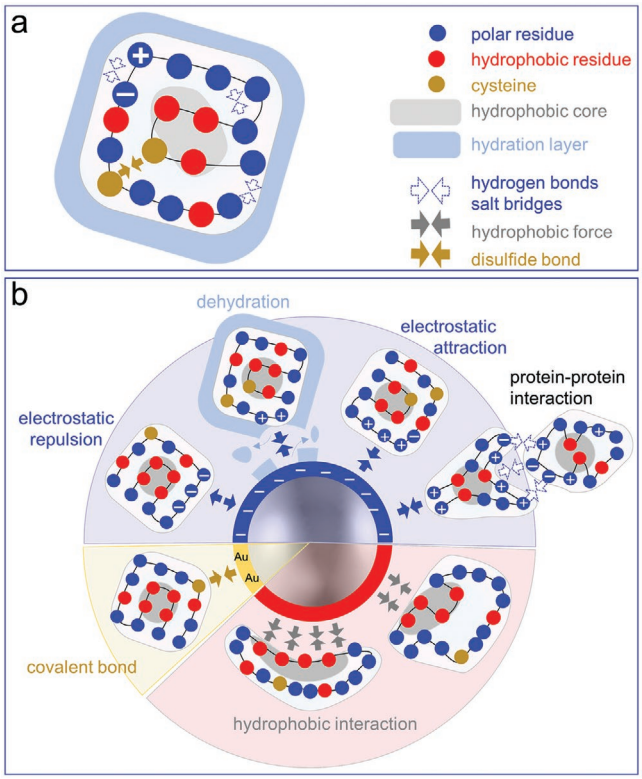


Figure 2. Interactions stabilizing proteins and NP–protein binding. a) Schematic of a folded protein, depicted by a chain of polar (blue) and hydrophobic (red) amino acids; cysteines (yellow) can form disulfide bonds. The hydrophobic core is shown in gray, the hydration layer in light blue. b) Forces between NP and proteins. Electrostatic attraction and repulsion, hydrophobic interaction, and covalent bond formation. Attractive/repulsive forces are represented by different directions of the arrows. NP surface properties are color-coded: blue, hydrophilic; red, hydrophobic; yellow, Au metal.

globular (water-soluble) proteins, hydrophobic (apolar) amino acid residues are sequestered in the protein interior, away from the polar aqueous environment, whereas hydrophilic (polar, neutral, or charged) amino acid residues preferentially reside on the surface, ensuring high colloidal stability of the protein. Frequently, distinct patterns of negative and positive charges including patches of like charges are seen on the surface. They often mediate specific, functionally important interactions with other proteins.

In the context of NP–protein interactions, it is important to appreciate that properly folded globular proteins exist on the brink of stability. The 3D fold of a small protein consisting of several thousand atoms is typically stabilized by ca. 20–70 kJ mol^{-1}, corresponding to the energy of just a few hydrogen bonds.[65] At physiological temperatures, the protein structure incessantly fluctuates among a huge number of subconformations,[66] and may even completely unfold (and refold) on occasion. Upon contact with an NP surface, the internal forces supporting the structure of the protein in solution compete with NP–protein interactions, including van der Waals, hydrogen bonds and charge interactions (Figure 2b). Sulfur atoms from cysteine residues form coordination bonds to gold and other metals and thus may play a special role. The outcome of the NP-protein

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

encounter can range from minimal conformational changes to complete unfolding of the protein, depending on the nature of the NP surface and the thermodynamic stability of the protein.[67,68] Notably, for larger proteins with domain structure such as immunoglobulins (Igs), only domain(s) in contact with the NP surface may suffer structural changes, while the others retain their native architecture and functional properties.

2.3. Mechanistic Aspects of Protein Adsorption onto Solid Surfaces

Systematic investigations of protein adsorption onto solid surfaces were pioneered by Vroman in the 1960s,[69,70] and the field quickly picked up speed due to its relevance for biomedicine, e.g., in the context of implants or stents. Many of the early studies dealt with planar surfaces, allowing the use of simple yet powerful analytical methods.[71] Results from this early work are definitely pertinent to protein corona formation, although significant curvature of small NPs on the length scale of a protein may add an extra complication.[72] It was quickly recognized that protein adsorption onto solid surfaces is an enormously complex process that depends on the nanoscale properties of the surface and the adsorbing proteins, as well as external parameters such as concentration, pH, ionic strength, and temperature.[73,74] Even slight changes in the experimental protocols can lead to markedly different outcomes, jeopardizing repeatability and reproducibility. Therefore, it is not that surprising that, already in 1980, Fair and Jamieson[75] introduced their study of protein adsorption onto PS NPs by stating that "the literature pertaining to the study of protein adsorption at solid/liquid interfaces has a long and confusing history." Some but not all of the confusion has been lifted over the years, and progress in the field will enormously benefit from gaining a deeper mechanistic understanding of the processes at the nano–bio interface.

2.3.1. NP–Protein Collisional Dynamics

Upon NP immersion in the biofluid, diffusive motions will lead to incessant collisions between the NP and the dissolved biomolecules, with the bimolecular rate coefficient, k_{diff}, given by the Smoluchowski equation,

$$k_{\text{diff}} = 4\pi N_{\text{A}} R_{\text{NP}} D_{\text{protein}} \tag{1}$$

here we assume that the NP radius, R_{NP}, is much greater than the one of the biomolecule, so the diffusion coefficient, D_{protein}, is much greater than the one of the NP; N_{A} is Avogadro's constant. For a 100 nm diameter NP and human serum albumin (HSA, D_{protein} = 61 μm^2 s^{-1} in water[76]), the most abundant protein in blood (≈600 × 10^{-6} M), Equation (1) yields k_{diff} = 2.3 × 10^{10} M^{-1} s^{-1}. Consequently, immersed in blood, the 100 nm NP will be bombarded by HSA molecules at a frequency of $k_{\text{diff}} \times$ [HSA] ≈ 10^7 s^{-1}. Thus, collisional encounters are frequent even for low-abundance proteins; however, not every collision will necessarily lead to transient or persistent NP-protein association.

2.3.2. Reversible Protein Binding

As both NP and protein surfaces are structurally heterogeneous, mutual shifts and reorientations after first contact result in the formation of an NP–protein interface stabilized by multiple, typically weak (electrostatic, hydrogen bond, van der Waals and hydrophobic) interactions, which are established at the expense of NP-solvent and protein–solvent bonds (Figure 2b).[71] If the interfacial interactions are only weak, protein conformational changes will be small and reversible, and desorption will be fast on the experimental timescale. Accordingly, free and bound proteins are in thermal equilibrium, and a reversibly bound ("soft") protein corona is maintained around the NP as long as there are free proteins in solution that can replace the desorbing ones. Based on a binding model that assumes incessant binding and unbinding of protein ligands to identical binding sites on the NP surface while the ligands remain unchanged by the interaction, the Langmuir-Hill equation describes the degree of saturation, S, of the NPs equilibrated with protein ligands of concentration, [L],

$$S = \left(1 + \left(K'_{\text{D}}/[L]\right)^n\right)^{-1} \tag{2}$$

with the ligand concentration at half-saturation, K'_{D}, and the cooperativity parameter, n, which controls the steepness of the curve. The special case n = 1 describes noncooperative (Langmuir) binding of independently adsorbing ligands, n < 1 anti-cooperative and n > 1 cooperative binding due to protein–protein interactions on the NP surface.

2.3.3. Irreversible Protein Binding

Strong NP–protein binding may result in a persistent, "hard" protein corona over relevant experimental times, usually hours to days. Since the individual bonds in the NP–protein binding interface are generally non-covalent and thus weak, many of them have to be established, and all of them have to be broken simultaneously for dissociation to occur, resulting in a high binding avidity. To form a strong binding interface with the NP surface, proteins may undergo significant conformational changes, trading in their internal interactions for many, altogether stronger interactions with the surface (**Figure 3**a). Even simple model experiments with well-defined surfaces and rather simple aqueous solutions containing only one type of protein may display complex adsorption behavior due to movements, reorientation, and protein–protein interactions on the surface. At a low protein concentration, the resulting low adsorption rate gives protein molecules arriving early on the NP surface plenty of time (and space) for structural relaxations before other proteins appear in their vicinity (Figure 3b). Thus, late arrivers can only attach to the remaining gaps, where they can bind only weakly. Because of steric restrictions imposed by their neighbors, they are more likely to maintain a compact fold and to adsorb reversibly. At a high protein concentration, however, the entire NP surface becomes quickly coated with a dense protein layer, and conformational changes are restricted due to mutual interactions (Figure 3b). Thus, the nature of the protein

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

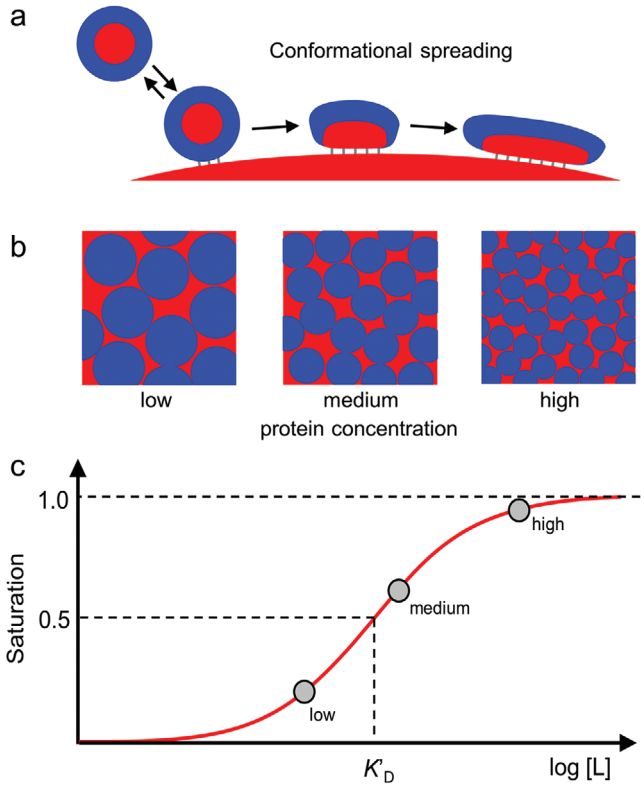


Figure 3. Protein adsorption onto hydrophobic NP surfaces. a) Schematic of conformational spreading. The protein (blue, polar residues on the surface; red, hydrophobic core) adsorbs reversibly onto the hydrophobic NP surface, and subsequently unfolds and spreads its hydrophobic core over the NP surface to increase the area (number of interactions). b) Top views onto the NP surface, depicting protein monolayers formed upon NP immersion in aqueous solutions with different protein concentrations, leading to various degrees of conformational spreading and high coverage. c) Resulting Langmuir-Hill protein adsorption curve, with saturation degrees at low, medium and high protein concentrations (see panel b) marked by gray spheres. K'_D, protein ligand concentration, [*L*], at half-saturation.

corona is affected by the protein-concentration dependent grafting density in a major way, which also gives rise to Langmuir-Hill-type saturation curves (Figure 3c).[77] The competition of proteins for the limited space on the NP surface can be visualized in single-molecule fluorescence experiments, e.g., with dye-labeled fibrinogen adsorbing onto plain and HSA-precoated glass surfaces.[78]

On hydrophobic NP surfaces, adsorbed proteins usually undergo pronounced conformational changes (conformational spreading), through which apolar amino acid residues normally buried in their hydrophobic core become exposed to the NP surface, where they are stabilized via van der Waals interactions. Thus, after the initial contact, the protein unfolds and spreads its hydrophobic core over the surface. This process is driven by the reduction of the overall hydrophobic surface area that the NP-protein complex presents to the solvent.[79] Experiments with planar self-assembled monolayer surfaces revealed substantial conformational spreading for albumin and fibrinogen on hydrophobic surfaces, with footprints expanding three- to fivefold from their initially occupied areas.[80,81] Resistance against adsorption-induced unfolding can vary greatly for different proteins, depending on their folding pattern and stability.[67,68]

On hydrophilic NP surfaces, protein binding is mediated mainly by hydrogen bonds and charge interactions. Importantly, charged and polar functional groups are unevenly distributed on the protein surface, and only charges in contact with the NP surface contribute to Coulomb interactions, as biofluids contain high levels of ions, resulting in a charge screening (Debye) length of <1 nm. On approach to the NP surface, a protein may be attracted or repelled, and it may reorient to expose an optimally arranged patch of charges to the surface for subsequent binding.[82,83] After docking, protein conformational transitions may occur and further increase the number of bonds, ensuring a high avidity of the interaction. On hydrophilic NP surfaces, structural rearrangements are spatially more confined than on hydrophobic surfaces,[81] and extensive protein spreading is avoided to preserve the hydrophobic protein core as a major contributor to the net free energy of stabilization. Thus, a hydrophilic, zwitterionic protein surface is maintained as the outer surface of the protein, providing resistance against the adsorption of additional layers.[79] Retaining a compact globular protein shape on hydrophilic surfaces may imply less adaptability of the binding interface to NP curvature.[84–86]

Charge interactions contributing to the interfacial stability on hydrophilic NP surfaces are intricate. It is important to consider that the charges on the protein (and oftentimes also on the NP) surface are due to protonatable groups, mostly amine, and carboxylic acid groups. Their charge state depends on the local electrostatic environment and not on the proton affinity in the bulk solution.[87] Furthermore, close to a charged NP, the local pH differs from the bulk pH due to the presence of a diffusive ion cloud. Thus, a protein approaching an NP surface may generally present a charge pattern different from the one in the bulk environment.

In complex biofluids containing a variety of proteins, the adsorption process is further complicated by the competition of proteins for the limited space on the NP surface (**Figure 4**a,b).[88] Small and abundant proteins have a higher collision frequency (Equation 1), so they are initially enriched on the NP surface. Less abundant and larger proteins are more likely to arrive later, adsorb onto (transiently) vacant sites, and subsequently optimize their interactions with the surface through conformational relaxation, thereby reducing their desorption rate.[89,90] Thus, the protein adlayer develops from a kinetically controlled structure toward one that is governed by equilibrium, i.e., according to the binding strengths of the proteins. In this context, there is an intriguing effect discovered by Vroman.[91–94] He noticed that larger proteins were able to displace smaller ones, even though the smaller ones alone did not show any net desorption tendency. Thus, the larger proteins apparently acted as catalysts for the desorption of the smaller ones. Put more precisely, the Vroman effect depends on differences in binding strengths. If two proteins adsorb side by side on the NP surface, their polypeptide chains compete for the many binding sites. Their weak bonds with the NP surface will continually fluctuate between open and closed states by virtue of thermal activation, and in the struggle for binding sites, the protein with the larger number of bonds has a lower probability to desorb.[95]

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

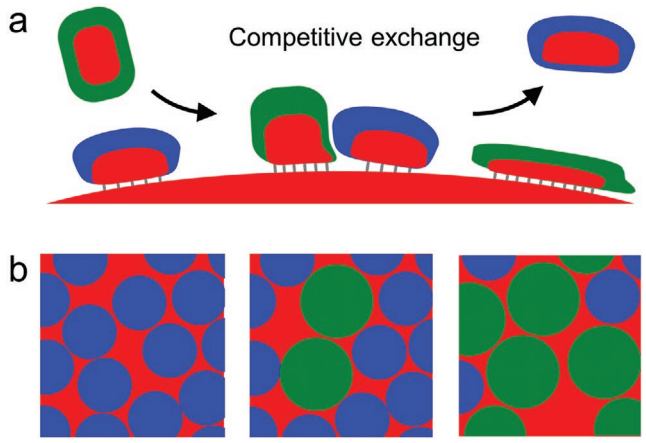


Figure 4. Competitive adsorption of two different proteins onto an NP surface. a) Schematic illustration of the Vroman effect. The initially bound protein (blue, with the hydrophobic core depicted in red) is displaced from the NP surface and replaced by the later arriving, more strongly interacting protein (green). b) Left to right: Development of the protein monolayer composition during the competitive exchange.

2.3.4. Mathematical Modeling and Computer Simulations

Various mathematical models have been developed to model the equilibrium and kinetics of surface adsorption of proteins.[71] Despite its simplicity, the Langmuir-Hill model oftentimes describes experimental binding curves very well, even in cases of irreversible adsorption.[96] More elaborate models, described briefly in ref. [71], capture some of the aspects of protein adsorption onto surfaces. All have their limitations since they reduce the complexity of the problem to a small set of (average) kinetic and equilibrium parameters. In reality, however, binding parameters are broadly distributed due to protein and NP surface heterogeneity, as was vividly shown by studies of individual adsorption events.[89] Structure-based computational modeling and simulation can also provide valuable insights into conformational changes of biomolecules interacting with surfaces.[97–101] At this stage, however, there are still severe challenges that need to be overcome to achieve realistic simulations of the adsorption process over longer times.

3. Key Issues of Current Debate

The structure and organization of the biomolecules in the protein corona as well as the dynamics are only poorly understood; we are still "scratching the surface of the protein corona."[102] Here we discuss important issues that are currently under debate as well as recent results aimed at further clarification.

3.1. Monolayer or Multilayer?

Many studies have asked the question if more layers of polypeptide chains form on top of the first shell surrounding the NP.[103–108] In fact, the hard protein corona was originally introduced as a near-monolayer of strongly binding biomolecules overcoated with a soft corona, an additional layer of loosely associated and rapidly exchanging proteins.[21] Although the multilayer concept is prevalent in the community, the majority (75%) of publications report thin protein adlayers compatible with monolayers, as Latreille et al.[102] recently emphasized in their extensive survey of the literature.

This issue can be addressed by determining the increase of the hydrodynamic radius of NPs due to adsorbing proteins directly in the biofluid via Brownian motion,[109] including dynamic light scattering (DLS),[110,111] fluorescence correlation spectroscopy (FCS),[107,112] and NP tracking analysis (NTA).[113–115] A key advantage of such in situ experiments is that no further sample processing is needed that could potentially affect the results. Data are collected typically within a few minutes after mixing NP solutions and biofluids, and the average (hydrodynamic) radius of NPs can be determined from the measured diffusivity via the Stokes-Einstein relation with (sub)nanometer precision. The low NP concentrations used in these experiments alleviate concerns about colloidal stability. Despite their conceptual simplicity, these techniques can pose challenges and pitfalls.[116] In fact, inter-laboratory comparisons of NP sizing measurements have shown considerable variations in the results from different laboratories, and standardized procedures were recommended to achieve reproducible results.[115,117]

In our own work, we have extensively used FCS and DLS to study protein adsorption onto small inorganic NPs (radius <10 nm) as well as large PS NPs (radius up to 50 nm), and ubiquitously observed saturation of the size increase at the highest concentrations (Figure 3c) for various important serum proteins, including albumin, transferrin (Tf), various apolipoproteins, complement protein 3 (C3). For small hydrophilic NPs coated with polar/charged functional groups (amine, carboxyl, hydroxyl),[82,83,112,118–121] the maximal thickness of the protein adlayer was always in the range expected from the molecular structures of the proteins. Distinct thickness variations for differently charged NP surfaces could even be traced back to different protein orientations on the surface induced by specific electrostatic interactions.[82,83,112,118,120] We also investigated the adsorption of blood serum onto small hydrophilic quantum dots (QDs) and measured a corona thickness of 6 –7 nm, suggesting a thin, compact protein adlayer.[122] Similar layer thicknesses, in line with monolayer formation, were found for large (diameter 30–110 nm) PS NPs.[123,124] We have argued that dense grafting of blood proteins on NP surfaces should generate a hydrophilic, zwitterionic outer surface that minimizes further adsorption of proteins, as blood proteins are colloidally very stable.[107,125]

On a critical note, the presence of a thin protein shell does not per se prove the presence of a monolayer. For example, if proteins undergo strong conformational spreading upon adsorption, the resulting thin layer may go unnoticed if a second protein layer forms on top. However, protein coronas of several ten nanometers based on DLS data were reported in the literature, way too thick for a protein monolayer, and thus attributed to multilayers.[104,126–130] We are skeptical about these findings because such large increases of the average hydrodynamic radius can also result from the presence of NP agglomerates induced by protein adsorption (see Subsection 3.4).[125]

Structural information beyond the overall thickness is needed for gaining a deeper understanding of the protein

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

corona. Imaging methods offer opportunities to directly visualize the adsorption layer. Unlike conventional light microscopy, superresolution optical fluorescence microscopy features the spatial resolution necessary to resolve nanoscale structures,[131,132] but such studies are challenging and still scarce.[133–137] Transmission electron microscopy (TEM) can provide images with sufficient resolution but involves extensive sample preparation, fixation and possibly staining procedures that may significantly modify the adlayer structure. In a pioneering model study with differently functionalized PS NPs and blood plasma, Kokkinopoulou et al.[138] observed a loose, patchy network of proteins extending up to 15 nm from the NP surface instead of the expected dense protein adlayer. Recently, Mahmoudi and co-workers[139] scrutinized these data by using cryo-TEM with flash freezing for cryofixation of the protein-adsorbed NP samples, which is believed to perfectly preserve the room temperature structure. They confirmed the presence of a patchy structure, but also stated that a dense yet not so apparent layer of small proteins was present that was overlooked in the earlier study.[138] Importantly, they associated the prominent patches with protein clusters formed during sample preparation, based on the observation that these were absent in plasma-only samples and less prevalent in NP-protein preparations with fivefold reduced NP numbers. It was concluded that the conventional procedure of preparing hard protein coronas, i.e., incubation (and agitation) of NPs in plasma at 37 °C for ca. 1 h, may cause proteins to flocculate and form these clusters. This appears quite conceivable, as the protein molecules in, e.g., 1 mL of plasma or serum exposed to PS NPs (radius ≈50 nm) at the usual (sub)nanomolar concentrations will be exposed to a few hundred square centimeters of an extremely hydrophobic and thus highly denaturing surface. In this context, it would be most interesting to see if similar effects occur in vivo, e.g., upon intravenous injection. The large structures observed by TEM are definitely incommensurate with the thin protein adlayers found in the in situ experiments, and future studies should address these discrepancies.

At the end of this subsection, we briefly bring up two well-known biological mechanisms through which proteins can persistently overcoat an already formed protein layer. 1) A major change in protein conformation, which may occur upon adsorption onto NP surfaces, can trigger formation of long fibrils of agglomerating peptide chains.[140–142] Fibrillation has been seen for many proteins and has significant biological relevance, for instance in various neuropathologies. 2) Although blood proteins are colloidally stable in the circulation, a number of strong protein–protein interactions can become activated as part of the coagulation cascade and immune responses. Certain proteins, including antibodies, complement proteins and a few other circulating proteins, can tightly bind to structures recognized as "non-self" to mediate opsonization, i.e., to tag them for elimination by the immune system.[143] Thus, they will associate with the NP-bound protein layer if its structure is recognized as "non-self," e.g., due to adsorption-induced denaturation.[143,144] To explore such specific protein–protein interactions, Chan and co-workers[145] investigated the protein corona around 60 nm diameter gold NPs (AuNPs) formed after immersion in human serum, using immunoassays with antibodies against 24 different proteins. They proposed a three-layer corona stabilized by specific protein–protein interactions, based on two pieces of evidence: 1) A large fraction (72.6%) of the adsorbed protein molecules was inaccessible to antibody binding, which could be due to an additional screening layer concealing the antibody binding epitopes. However, it is expected that the number of antibody-binding epitopes will also be reduced in a dense monolayer. 2) Sandwich immunoassays showed a persistent association for 42.4% of the $24^2 = 576$ possible protein-protein pairs. Notably, only a tiny fraction of ca. 4000 serum proteins[146] was tested, and false positives are likely to occur. Although the three-layer hypothesis is premature in our opinion, this work demonstrates that immunoassaying is a powerful technique for gaining detailed information on the arrangement of binding motifs within the protein corona and their mutual interactions.

3.2. Soft or Hard Corona?

Residence times of proteins on an NP surface may vary over many orders of magnitude, depending on the local properties of the NP–protein binding interface.[89,147,148] Weakly interacting NP materials will lead to fast protein desorption (within seconds or less), whereas highly reactive ones will cause essentially irreversible binding. In the literature, the distinction between soft and hard coronas is often based on the specifics of the experimental procedures and thus somewhat arbitrary and prone to semantic confusion. In our work, we define a soft corona as a weakly bound adsorption layer that is only maintained in the presence of free proteins in the biofluid around the NPs, so that a dynamic equilibrium is established. Therefore, direct analyses require in situ experiments, in which the NP samples are immersed in biofluid. Alternatively, one can attempt to "freeze in" the corona, e.g., by flash freezing[139] or chemical cross-linking.[149] In contrast, hard coronas can be studied ex situ, i.e., after separating the protein-coated NPs from the biofluid to remove all weak binders. In general, both soft and hard components may coexist, and to which extent they contribute to the biological identity of the protein-coated NP is still under discussion.[21,149–152]

3.2.1. Soft Corona

Soft protein coronas have received less research attention than hard coronas, presumably because in-situ experiments are more challenging yet essential for their direct investigation. While Brownian motion-based NP sizing methods using visible light such as FCS[153] and NTA[113] have been successfully employed for quite some time, a number of new in situ techniques have appeared in recent years. For example, ^{19}F diffusion-ordered nuclear magnetic resonance (NMR) spectroscopy is a diffusion-based technique applicable to turbid media.[154] Two other recently proposed, spectroscopy-based in-situ methods are Förster resonance energy transfer (FRET)[155,156] and synchrotron-radiation far-UV circular dichroism (CD) spectroscopy.[157] Fluorescence imaging within microfluidic devices is an elegant technique that allows in-situ tracking of protein adsorption in space/time along a flow channel after mixing NPs and fluorescently labeled proteins.[158] Recently, an interesting

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

fluorescence image correlation-based approach dubbed differential dynamic microscopy (DDM) has proven to be effective for in situ protein corona studies.[124] Furthermore, small angle x-ray scattering (SAXS)[159,160] and neutron scattering (SANS)[161] enable NP sizing experiments via the structure factor.

We have extensively used FCS for quantitative in-situ studies of the soft corona forming on fluorescent NPs with different core materials and decorated with differently charged (negative, positive, zwitterionic, uncharged) moieties.[82,107] These NPs were incubated in high ionic strength buffer solutions containing one type of serum protein (e.g., albumin, Tf, C3 and various apolipoproteins). In these experiments, the protein concentration dependence of the average NP radius always followed the Langmuir-Hill isotherm. As this behavior has also been observed for irreversible adsorption,[77] an additional experiment is necessary that can explicitly probe reversibility. To this end, a sudden dilution step can be applied in situ. If binding is reversible, the NP radius will decrease as expected from the binding curve, whereas it will stay constant for irreversible binding. We observed the expected size decrease that proves reversibility already at the shortest times accessible with FCS (a few minutes), and it did not change over the ensuing hours.[118,123] We note in passing that a high excess of proteins over NPs should be maintained after the concentration jump to ensure first-order binding conditions, so NP binding has no effect on the concentration of free proteins in the solution. Alternatively, competition experiments have been employed to study reversibility, in which a second type of protein is added to the solution that competes with the first one for the binding sites on the NP surface.[162–164] To visualize the exchange with NP sizing experiments, the two proteins must have markedly different sizes; alternatively, fluorescence labeling can be used.[156,165] Notably, as the Vroman effect can accelerate the desorption of a protein that binds persistently on its own (see Subsubsection 2.2.3, Figure 4),[88] a competition experiment bears the risk of inducing rather than proving reversible binding of the protein under study.

Although soft coronas often show half-saturation concentrations in the lower micromolar range, their presence can nevertheless have a strong effect on the yield of NP uptake by cells.[166–168] We note that equilibrium binding implies that protein conformational changes must either be absent upon adsorption or reversed upon desorption, so that the leaving ligand species is identical to the incoming one. Protein adsorption onto NP surfaces is likely to induce protein conformational changes, but their reversibility is difficult to assess. For example, Sanchez-Guzman et al.[157] combined in-situ synchrotron-radiation CD with cryoTEM to study weak adsorption of hemoglobin onto silica NPs. They revealed significant changes in the structure and stability of the protein and analyzed these at the molecular level using molecular dynamics simulations. Based on the thermodynamic analysis, they proposed that enthalpy-driven NP–protein interactions induce a shift of the protein equilibrium conformations toward partially unfolded ones. The issue of reversibility of these changes upon desorption was not addressed, however.

In general, we can envision that reversible and irreversible protein binding may coexist, as has been frequently seen for planar surfaces.[79,169,172] Dawson and co-workers[21] originally proposed that a soft corona adsorbs on top of a hard corona. Alternatively, weakly and strongly binding proteins may exist side-by-side in the same protein adlayer. For example, proteins undergoing conformational spreading after adsorption onto denaturing surfaces may leave small patches of bare surface, allowing for weak binding of further proteins.

Quantitative kinetic studies of soft NP coronas in the presence of a persistently bound protein fraction are scarce.[20] Instead, the distinction between soft and hard corona components is frequently heuristically based on the observation of desorption under certain conditions. For example, Baimanov et al.[173] investigated the protein corona on ultrasmall chiral Cu_2S NPs (immobilized on IgG-coated biosensors) in situ using bio-layer interferometry. In their proteomic analysis, they assigned proteins that could be eluted with an aqueous, highly dilute (0.005%) trifluoroacetic acid (TFA) solution to the soft corona, whereas hard corona proteins were released from the NPs with a more concentrated (0.1%) TFA solution. In a similar vein, Kari et al.[174] studied immobilized liposomes adsorbing proteins from human plasma under flow conditions. For proteomic analysis, the solution was first replaced by the plain buffer to elute soft corona proteins; afterward, surfactant was added to detach the tightly binding hard corona proteins.

An interesting new approach was pursued by Mohammad-Beigi et al.[149] in their recent study on protein adsorption on silica and PS NPs. They employed in situ click chemistry to fix weakly interacting soft corona proteins via the highly specific, strain-promoted alkyne azide cycloaddition reaction to the hard corona adsorbed onto the NPs, with subsequent ex-situ proteomic analysis using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Notably, the same types of proteins were identified in the hard and soft coronas. They reported distinctly different cell adhesion behavior for the normal (dynamic) and chemically fixed soft coronas, suggesting that the protein exchange dynamics is important for cell recognition and further emphasizing the importance of weakly binding proteins for the biological identity of NPs.

Other ex situ studies have used the notion of a soft corona to describe the fraction of proteins that remain on the NPs upon separation from the solvent, but come off more easily in the ensuing (repetitive) washing steps to remove the proteins (see, e.g., ref. [175]). A minireview by Weber et al.[176] gives an overview of different sample treatments (centrifugation, magnetism, chromatography) for the analysis of the protein corona and evaluates their influence on the outcome, especially with regard to binding persistence.

The studies presented in this section exemplify that the notion of a soft corona is used very loosely in the literature, ranging from truly reversibly binding proteins on weakly interacting NP surfaces to protein fractions that remain attached upon NP isolation from the solvent but are more easily washed off from the NP surface than others. The heuristic distinction between soft and hard coronas merely reflects the capabilities and conditions of the experiment.

3.2.2. Hard Corona

Characterization of hard coronas at the level of the molecular processes is truly challenging.[177–180] Computer simulations are

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

becoming ever more powerful, yet are presently still limited to protein docking and early structural relaxations accompanying protein adsorption onto planar surfaces,[97–101,181] and considerable technical difficulties need to be overcome to model the ensuing major conformational changes on longer timescales in the future. Therefore, researchers have to resort to less detailed experimental characterizations, focusing on parameters such as corona thickness, morphology, and protein composition.

In situ FCS experiments with small NPs immersed in human blood serum revealed a thin (6–7 nm) layer of irreversibly bound proteins.[122] Notably, the serum experiments were carried out with the same NP types that earlier showed a soft, reversible corona when adsorbing only one type of protein, e.g., HSA. Interestingly, HSA was also found in the hard serum corona. Thus, there are proteins in serum that interact irreversibly with the NP surfaces, and protein–protein interactions presumably mediate the association of HSA with the hard corona. Despite the complete lack of a binding equilibrium, the concentration dependence of the hydrodynamic radius was in line with the Langmuir-Hill equation. For each concentration, the thickness of the serum corona was time-invariant (within the precision of the experiment) from a few minutes (the earliest time point) up to several hours. Notably, this observation does not exclude a slow evolution due to protein exchange and other dynamics that do not affect the thickness. It appears that a soft corona forms at early times and then quickly evolves into a hard corona, which likely involves the conformational spreading of the adsorbed proteins, passivating the NP surface against further protein adsorption.[123,77]

In recent years, there has been a strong focus on the characterization of the protein composition of the hard corona forming around NPs incubated with blood plasma (or serum) due to its relevance for biomedical diagnostics.[182–185] Another reason is that sophisticated, mass spectrometry-based technology has become available that allows for facile identification and quantification of the adsorbed protein species after isolation of the NPs from the biofluid. This approach has been employed to study the dependence of the protein composition in the corona on NP properties such as size, charge and surface functionalization as well as time.[186,187] The typical workflow is such that NPs are first exposed to the biofluid (mostly blood plasma or serum) for a certain duration (often 1 h) at 37 °C under gentle agitation, in the hope to reach a biologically relevant stationary state. Afterward, the NPs are separated from the biofluid using either (gradient) centrifugation, field flow fractionation, magnetic separation, or size exclusion chromatography. Most often, loosely bound proteins are removed by one or more centrifugation and washing steps, and the polypeptides in the pellet are redissolved by harsh treatment (solvents containing surfactants, denaturants, high temperature) and processed by enzymatic digestion. For the identification of the peptide fragments, LC-MS/MS has become the workhorse technique, yielding a list of identified proteins as well as their abundances. Importantly, these data are averages over a macroscopic NP ensemble. As the loading capacity of an NP may range from a few to up to a few hundred protein molecules, depending on its size, protein adsorption from a complex biofluid containing many different proteins will in general lead to statistical assemblies of protein types and numbers on each individual NP.[188,189] Accordingly, the biological identity of the NPs will be very heterogeneous.

In recent years, awareness has risen that the results of a proteomic analysis can sensitively depend on the sample preparation and further treatment.[190–193] Typically, NPs are incubated in 1–2 mL of blood plasma (or dilute plasma). Considering the huge total surface area even at low NP concentrations, there is the risk that rare but tightly binding protein species become depleted during NP incubation, so they will be underrepresented in the obtained protein composition. Accordingly, the results crucially depend on the protein-to-NP stoichiometric ratios of the individual proteins in the biofluid. A vivid example of this effect was presented by Fedeli et al.,[194] who studied the persistent plasma protein corona forming around silica NPs (28 nm diameter). At the smallest NP concentrations, a protein of low abundance, histidine-rich glycoprotein (HRG), was predominantly enriched in the hard corona due to its high affinity to the NPs mediated by its histidine-rich region; kininogen-1 and fibrinogen were also present in significant proportions. Increasing the NP concentration shifted the protein composition toward the more abundant fibrinogen, as there were not enough HRG molecules to coat the NP surfaces.

Another problem that may arise during long-term plasma incubation of NPs with protein-destabilizing surfaces such as PS is NP-induced formation of protein clusters not associated with the hard corona,[139] which may lead to false positives in the proteomic analysis (see below). Sheibani et al.[139] recommended using only small amounts of NPs in the sample preparation to alleviate the effect. The NP separation procedures from the biofluid after incubation can also introduce significant errors. In their review of different isolation methods (centrifugation, magnetism, chromatography), Böhmert et al.[190] concluded that the apparent composition of the protein corona greatly depends on the chosen method. Centrifugation involves the risk of false negatives, as proteins may desorb due to centrifugal forces, and false positives, as unrelated protein species may sediment together with the protein-coated NPs. Under conditions of high NP concentration and/or low colloidal stability, proteins may become entrapped in interstitial regions of NP agglomerates.[139,182] Accordingly, the measured protein composition depends on the centrifugation speed, time, temperature, and the number of washing steps.

To avoid the post-incubation artifacts mentioned above, Wang et al.[195] used paraformaldehyde (PFA) fixation for arresting the protein corona around 15 nm AuNPs internalized by HepG2 cells. Unlike the work by Mohammad-Beigiet al.,[149] they reported that PFA fixation did not immobilize a soft corona. We recommend further scrutinization of (unspecific) chemical cross-linking methods for protein corona analysis, as these methods have an inherent risk of producing false positives, especially when applied in the crowded environment of cells.

Apart from the problems with sample processing, there have been critical discussions about low repeatability and reproducibility of mass spectrometry-based approaches for corona analysis.[189,196] To exclude sample preparation-induced variations, Mahmoudi and co-workers[197] sent identical, fully washed, protein corona-coated PS NP (diameter 80 nm) samples to 17 core facilities in the United States for analysis. Comparison of the

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

results revealed a large variability among the data from the different labs, judged to be greater than the reported effect sizes of most published NP–protein corona studies in the literature.[197] Remarkably, only 73 of the overall 4022 identified proteins were shared among those 12 centers providing semi-quantitative analyses. The variability was not attributed to the LC-MS/MS method itself, but rather to different sample handling procedures, instrument settings, raw data processing, and other factors. The authors recommended precise documentation and standardization of protocols as means to ensure high-quality proteomic analysis of hard coronas.[197]

3.3. Dynamics of the Protein Corona

Protein corona properties will change significantly over time, e.g., during the journey of NPs through an organism, and so will their biological identity. Therefore, early protein corona research has emphasized the importance of quantitative assessment of (time-dependent) rate coefficients of protein association with and dissociation from NPs, in addition to affinities and stoichiometries, for gaining a profound understanding of the nature of the protein corona interacting with the functional machinery of cells.[20,119] To date, the number of kinetic studies has remained small and limited to a few model systems, however. Within fractions of a second after NP exposure to a biofluid, the initial protein layer forms, and subsequently evolves further due to protein movements (translation and reorientation), protein exchange with the biofluid, as well as conformational changes of the adsorbed proteins (and possibly the NP surface as well). The Vroman effect is expected to play an important role for the development of the adsorption layer, as it accelerates protein exchange, so that rare yet strongly binding proteins can win the competition for the limited number of attachment sites on the NP surfaces.[194] We are not aware of experimental studies of the early (subsecond) dynamics, but coarse-grained molecular dynamics simulations indicated that fibrinogen (5 μg mL^{-1}) can replace HSA in a preformed corona around silica NPs within milliseconds.[165]

Slower processes (seconds to hours) are experimentally more easily accessible. Recent work has focused less on structural aspects of the hard protein corona, but mainly on temporal changes of the protein composition via mass spectrometry-based proteomics.[183,186,187,198–201] In these in vitro studies, NPs were incubated in human plasma for varying lengths of time. After subsequent isolation of the protein-coated NPs, proteomic analysis revealed significant changes of the fractional abundance of specific proteins, attesting to the dynamics of the hard corona.

In vivo, additional effects come into play that are not captured by simpler laboratory experiments. NPs injected into the blood stream experience hydrodynamic forces and come into contact with circulating blood cells and endothelial cells lining the vasculature. Furthermore, the biofluid environment changes considerably as NPs migrate from the blood stream into tissues and finally into cells and subcellular compartments. Studies aimed at more closely mimicking in-vivo conditions have intensified in recent years, including investigations of protein corona formation in flowing media,[180,202,203] and complex 3D tissue culture models of, e.g., the blood-brain barrier[204] or the epithelial airway barrier.[205]

Lundqvist et al.[206] performed a simple yet illustrative in-vitro experiment that addresses the effect of a changing environment upon cellular uptake of NPs injected into the bloodstream. They monitored the evolution of the protein corona by comparing the protein compositions of hard coronas around silica (9 nm), "plain" (presumably detergent-stabilized) PS (50 nm), and carboxylated PS (PS-COOH, 50 nm) NPs after immersion in human plasma, cytosolic fluid, and first in plasma and then in the cytosolic fluid. Proteomic analysis revealed significant changes in the protein composition after transfer from plasma to cytosolic fluid. Notably, the final corona was markedly different from the one found upon NP immersion in cytosolic fluid only, and thus retained a "fingerprint" of its two-step history.

Cai et al.[207] recently emulated a more complex scenario that explicitly includes cellular uptake via the endosomal-lysosomal pathway. They incubated AuNPs first with human blood plasma, then with freshly prepared lysosomal extracts and finally with cytosolic extracts from human cervical carcinoma (HeLa) cells. LC-MS/MS-based proteomic analysis showed that the total protein amount on the surface of the AuNPs decreased upon transfer from plasma to lysosomal extract due to the degradation of blood plasma-derived proteins, and increased again slightly upon subsequent immersion in the cytosolic extract. In this step, there was a specific enrichment in chaperone and glycolysis proteins, including heat shock cognate protein 70, heat shock protein 90, and pyruvate kinase M2 [PKM2], a key regulator of cell metabolism that is upregulated in tumor cells.[208] This replacement of blood proteins by cytosolic proteins was also found upon internalization of the AuNPs by HeLa cells, where it induced elevated chaperone-mediated autophagy activity and disruption of the cell metabolism, leading to cell aging and death. Thus, the cellular responses to AuNP uptake were shown to depend on the intracellular protein corona rather than the one in blood plasma, emphasizing the need to anticipate the effects of the changing bioenvironment that nanomedicines experience as they migrate to their target cells.

Studies of in vivo-formed coronas have recently appeared as well.[201,209–212] In this setting, other types of biomolecules including nucleic acids,[213] lipids,[214,215] sugars,[23] and small metabolites[216] are present and adsorb to the NPs, and take part in exchange processes that continuously modify the composition of the biomolecular corona. In their comprehensive LC-MS/MS proteomic analysis, Hadjidemetriou et al.[209] observed significant differences in protein composition in the coronas formed on PEGylated liposomes (with and without attached antibodies for targeting) in vitro (10 min incubation in CD-1 mouse plasma) and in vivo (NP recovery from CD-1 mice 10 min after intravenous injection). Notably, a greater variety of protein species was found in the in-vivo corona. In follow-up work focusing on the time evolution over 3 h,[201] the total amount of adsorbed proteins stayed constant, whereas the relative fractions of proteins changed considerably, attesting to the dynamic nature of the protein corona.

In contrast to the many studies addressing the changing protein composition within the corona, Han et al.[217] focused on the long-term fate of the protein corona inside a cell. By

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

combining confocal laser scanning microscopy (CLSM) with electron microscopy in a correlative fashion, they tracked the subcellular location of green-emitting PS-COOH NPs (diameter 116 nm) with pre-adsorbed plasma proteins (red-emitting due to Cy5 labeling) in murine RAW264.7 macrophages as a function of time. After internalization through the endosomal-lysosomal pathway, they observed a spatial separation of the NPs from the protein corona. Corona proteins were sorted to multivesicular bodies for further processing, whereas the NPs were routed to recycling endosome for exocytosis.

3.4. NP Agglomeration

Especially for biomedical applications, colloidal stability of the NPs under all conditions of preparation and application is of utmost importance. Protein adsorption, however, may bear the risk that the NPs become colloidally unstable, e.g., due to charge compensation[218,219] or protein-mediated bridging (**Figure 5**a), as shown by Cedervall and co-workers[220] in their insightful study of IgG and fibrinogen adsorption onto PS-COOH and sulfated PS (PS-OSO_3H) NPs in phosphate-buffered saline (PBS). Using DLS for size determination, they observed extensive agglomeration depending on the IgG concentration or, more to the point, the protein:NP stoichiometric ratio. Already at very low IgG concentrations (stoichiometric ratio ≈1), they found a mixture of pristine NPs, NPs with only a single IgG molecule bound, and small agglomerates where IgG forms a bridge between two NPs. At intermediate stoichiometric ratios, IgG-induced NP–NP cross-linking occurred as well; only for large IgG excess, stable NP suspensions were obtained due to rapid and essentially complete NP coating with proteins. CD spectroscopy showed that the overall secondary structure of the IgG molecules was only slightly affected, and no shift in the tryptophan emission was observed. These results indicate only limited conformational changes, perhaps involving just a few of the altogether twelve IgG domains. For fibrinogen, the same agglomeration effect was observed, but different from IgG, fibrinogen underwent extensive structural changes in the concentration regime where protein-induced NP aggregation occurred.

Tf adsorption onto PS-OSO_3H NPs in PBS showed qualitatively similar NP agglomeration effects as those discussed above, with extremely sharp transitions between concentration/stoichiometry ranges of stability and agglomeration (Figure 5b).[123] For lower ionic strength of the solvent (reduced charge screening), the onset of agglomeration shifted toward higher Tf concentrations, as expected for enhanced NP-NP repulsion of the electrostatically stabilized PS-OSO_3H NPs. In highly (20-fold) diluted PBS, repulsion was strong enough to ensure colloidal stability at all Tf concentrations. Tf is negatively charged at physiological pH, yet its bridging interaction can overcome the electrostatic repulsion of the negatively charged NPs under the charge screening conditions of typical biofluids.

Similar agglomeration behavior was reported by Link and co-workers[177] for bovine serum albumin (BSA) adsorption onto gold nanorods suspended in 1×10^{-3} M phosphate buffer, pH 7.2. At high BSA concentration (20×10^{-6} M), the nanorods were completely stable, whereas extensive NP–NP cross-linking

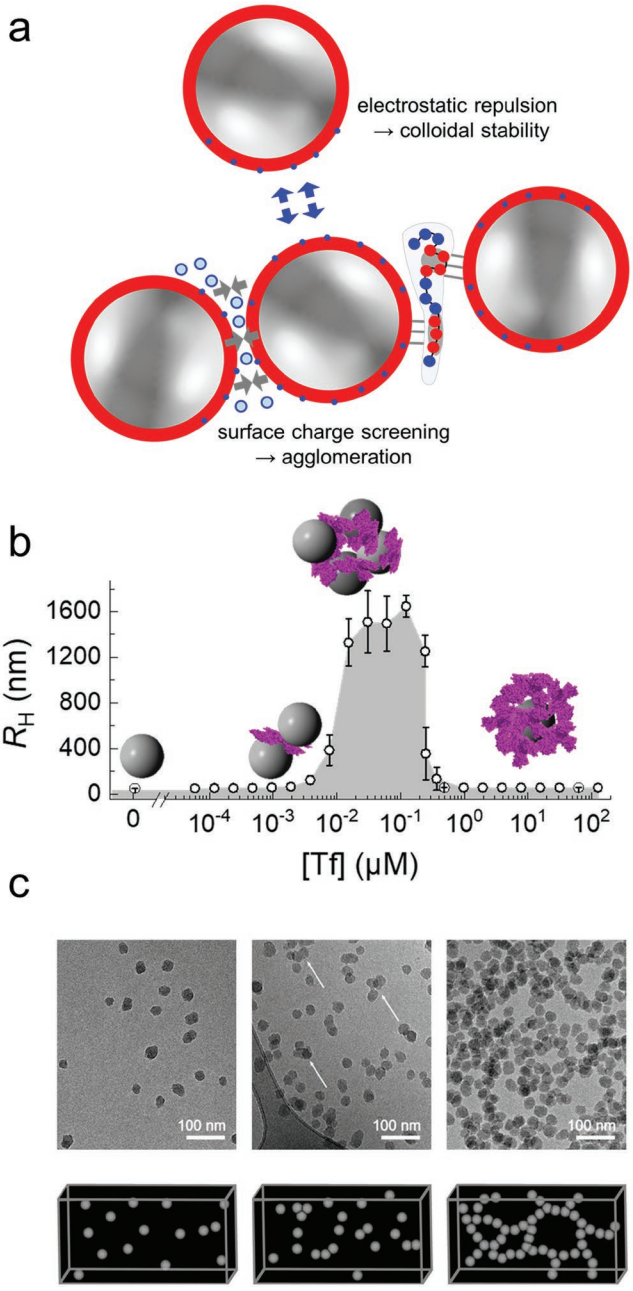


Figure 5. Colloidal instability of NPs due to protein bridging. a) Hydrophobic NPs (red shell) are shown as an example. They are colloidally stabilized in aqueous solution by electrostatic repulsion due to sparse decoration with surface charges (blue spheres). NP aggregation can be induced by counterion charge screening (light blue) and protein crosslinking. b) NP aggregation as a function of protein concentration. Radius of diffusing entities (PS-OSO_3H NPs or clusters thereof) as a function of Tf concentration, determined by DLS. Adapted with permission.[123] Copyright 2019, Wiley-VCH. c) Cryo-TEM images (top) and schematic structures (bottom) for 25 nm silica NPs and (left to right) exposed to BSA, fetal bovine serum, and lysozyme, resulting in colloidally stable NPs, small clusters, and mass fractals. Reproduced with permission.[221] Copyright 2022, American Chemical Society.

was measured in a dilute BSA solution (2×10^{-9} M) by DLS. Single-molecule imaging revealed an enormous spreading of individual polypeptide chains on the gold surface.

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Recently, Ferreira et al.[221] studied corona formation and agglomeration of 25 nm silica NPs in low and high ionic strength buffers containing a variety of proteins as well as bovine serum and human plasma. By combining NP sizing (SAXS) with imaging (cryoTEM), they established clear correlations between size changes and aggregation phenomena and obtained information on the structural, specifically, fractal nature of the NP agglomerates (Figure 5c).

Wei et al.[222] used in vivo flow cytometry to monitor the concentration and aggregation of PEG-modified PLA NPs (diameter 100 nm, fluorescently labeled with coumarin-6) in the bloodstream of live male BALB/c mice. NPs were injected into the tail vein of the anesthetized mouse. The animal was placed on the microscope sample stage, the excitation laser was focused onto an artery of ≈50 µm diameter in the ear and the fluorescence signal emitted from the circulating NPs was recorded as a function of time. The resulting intensity–time traces provided information both on the overall NP clearance kinetics and on NP concentration and aggregation at certain time points.

These studies illustrate that NP agglomeration induced by protein adsorption is a prevalent phenomenon. It affects protein corona formation as well as its characterization and may lead to erroneous results, e.g., in DLS or proteomics studies.[194,223–225] Notably, incomplete NP coverage with proteins bears the risk that protein bridging interactions may overcome NP–NP repulsion. Thus, careful experimentation is needed to identify conditions under which NP agglomeration can be avoided, thereby excluding its potentially detrimental effects in most biomedical applications.

4. Conclusions and Perspectives

Without doubt, NP-based devices have enormous potential to revolutionize medicine, in diagnosis as well as in therapy.[5,7] They are in the appropriate size range to take advantage of the transport machinery of the body on all spatial scales, from the systemic scale (blood circulation) all the way down to subcellular compartments. The huge diversity of NP design strategies available today offers almost unlimited opportunities for controlling their travel within the human body and to exert specific effects on selected cells. Many proof-of-concept studies have vividly illustrated the impressive potential of NP-based devices for medicine; their translation into safe and efficacious nanomedicine products, however, has remained remarkably inefficient.[14,15] A key obstacle in nanomedicine development is the lack of a deep mechanistic understanding of NP interactions with the bio-environment. This knowledge would provide NP developers with predictive power, enabling them to anticipate and account for these interactions in the design process, so that NPs can exert their intended biological responses.

Migrating through the body, NPs pass through different bio-environments, which have been likened to "chemical reactors"[226] modifying these nanoscale objects. NPs interact with cells via receptor-mediated endocytosis, a key process involved in both transcytotic migration and internalization by target cells. It is triggered by (multiple) plasma membrane receptors binding to their cognate ligands on the NP surface. Importantly, entire nanoscale surface patches are recognized by the cell, and the distinct arrangement of binding motifs within these patches dictates the cellular response. Structural changes of the adsorbing proteins due to NP–surface interactions can greatly modify the presented motifs, and protein denaturation may lead to the activation of scavenger and pattern recognition receptors causing inflammatory responses. When looking at the many artful depictions of well-structured NPs enwrapped in well-structured adsorbed layers of well-folded proteins in the literature, we feel that there is still only insufficient awareness for the delicate, weakly stabilized 3D architectures of proteins that require careful NP surface design to preserve them. Future progress will necessitate detailed structural characterizations of the corona as an essential step toward specific control of cellular responses, including mapping of recognition motifs on NP surfaces and matching them with receptor patterns on the cells of interest.[227–229]

In this review, we have focused on fundamental yet still disputed questions regarding the physicochemical nature of the protein corona around NPs, including NP–protein binding strengths, the vertical structure of the protein adlayer as well as its temporal evolution. Early research reports have emphasized the importance of determining association and dissociation rate coefficients, affinities, and stoichiometries of NP–protein interactions for understanding the behavior of NPs in the changing bioenvironment.[20] Yet, recent years have seen little progress in this direction. In the future, efforts should be intensified that aim at quantitative mechanistic explorations of protein corona formation as well as its (changing) structure in the bioenvironment. An impressive range of experimental methods has become available for this purpose, and novel techniques are continuously appearing.[47–49] In our view, microscopic and spectroscopic techniques have not yet been exploited to the fullest extent. Ex situ visualization of protein corona structure by TEM appears promising; however, further systematic investigation of sample preparation procedures appears necessary to ensure that TEM images indeed reproduce the biologically relevant state of the corona. In the future, we expect to see more in-situ super-resolution optical fluorescence microscopy studies of protein corona structure and dynamics, providing data with high resolution in both space and time. A wide range of spectroscopic techniques including X-ray, CD, visible absorption and fluorescence, infrared and NMR spectroscopies have been pivotal for the exploration of protein structure and dynamics over several decades and proven effective for studying conformational changes of proteins upon adsorption onto NPs as well. In this context, experiments that are sensitive to single NPs and single (bio)molecules are very important, and we expect to see more of them in the future, as they open avenues to address central issues in NP research, such as the physicochemical heterogeneity of NPs as well as the unavoidable variations of the protein corona.

In general, protein corona exploration is a challenging endeavor that requires profound expertise across a broad range of disciplines including physics, chemistry, nanotechnology, biology, and medicine. Among the experimental techniques, some are straightforward to use and thus frequently employed. However, non-expert users are often not aware of the inherent limitations, problems and pitfalls, and may arrive at

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

wrong conclusions. A case in point is NP size determination by (fixed-angle) DLS, which can lead to misinterpretations in various ways, especially when samples tend to agglomerate.[125] Likewise, LC-MS/MS provides an apparently robust pipeline for quantitative proteomic analysis of the protein corona. However, recent literature indicates that the results sensitively depend on many factors, starting from details of the corona preparation over NP isolation to parameter settings of the instrument.[223] Using standardized protocols may alleviate some of these problems, but in our view cannot replace specific expertise in each of the scientific areas involved. The high susceptibility of the reported outcomes to variations in the experimental parameters appears to be ubiquitous in this research field. Criticism has been voiced that crucial details are frequently not included in scientific publications. To solve the problem of varying reporting standards, guidelines for minimum information reporting have been proposed to make sure that relevant procedural details are presented,[216,230] so that experiments can be reproduced and compared with related work. A recent discussion among researchers in the field has vividly shown the pros and cons of submitting checklists together with publications.[231] We tend to agree with the view that "focusing on strategies to better train interdisciplinary scientists in biological and analytical techniques, including validation approaches to methodology optimization, is a more important solution."[231]

Precise and highly reproducible data are prerequisites for the compilation of databases with the essential physicochemical parameters of the pristine NPs, the adsorbed coronas and their subsequent changes in response to time or environmental changes. Based on these data, computational modeling may reveal qualitative and quantitative structure-activity relationships.[232,233] Elaborate compilations may also allow the training of machine learning algorithms to predict biological responses even without understanding the underlying processes.[234]

So far, there has been a strong research focus on protein adsorption onto NPs, which is motivated by the abundance of proteins and their ability to engage in specific interactions. However, there are other relevant classes of biological molecules (lipids, nucleic acids, metabolites), and a comprehensive view of the bio–nano interface requires us to also examine their role in the biomolecular corona and the ensuing biological responses, e.g., cell signaling and metabolism.[216,235] Lipids are insoluble in aqueous solvents and thus transported in blood as roughly spherical lipoprotein particles with sizes in the range <10–1000 nm, featuring a lipid core that is solubilized by surface functionalization with apolipoproteins. Little is known about the mechanisms through which these natural particles interact with synthetic NPs: Will lipoproteins attach to NPs as whole particles, or transfer their material, lipids and apolipoproteins, partially or completely to the NPs? Another important group of molecules is metabolites, small (<1 kDa) organic molecules that may adsorb onto NPs and thereby alter their physicochemical properties and the biological effects.[216] Even smaller entities such as (metal) ions, e.g., Zn^{2+}, can affect corona formation in a major way due to their ability to form stable complexes with various chemical groups on the surfaces of the NPs and proteins. We expect that efforts to characterize and quantify the full biomolecular corona will intensify in the near future.

There is general agreement that in vitro studies of protein corona formation and the ensuing biological responses in simple cell cultures have only limited significance for predicting outcomes in the in-vivo environment. In the future, using more complex in-vitro environments such as cell co-cultures, organoids, and flow reactors that simulate, e.g., the blood circulation will be valuable intermediate steps. In the end, however, in vivo studies will be essential to explore the molecular-level architecture of the protein corona and its evolution as the NPs migrate through chemically very different environments (circulation, tissue, subcellular compartments), while ensuring that their continuously varying biological identity is still capable of triggering the intended responses at the target site. Formidable challenges still lie ahead of us on this road, but the perspective toward a versatile platform for developing highly specific and efficacious, possibly even patient-specific nanomedicines is definitely worth the effort.

Acknowledgements

This work was supported by the Helmholtz Association, program Materials Systems Engineering (MSE).

Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

conformational changes, nano–bio interface, nanoparticle agglomeration, nanoparticles, protein adsorption, protein corona

Received: February 24, 2023
Revised: March 14, 2023
Published online:

[1] A. E. Nel, L. Mädler, D. Velegol, T. Xia, E. M. Hoek, P. Somasundaran, F. Klaessig, V. Castranova, M. Thompson, *Nat. Mater.* **2009**, *8*, 543.
[2] R. Jin, C. Zeng, M. Zhou, Y. Chen, *Chem. Rev.* **2016**, *116*, 10346.
[3] P. D. Jadzinsky, G. Calero, C. J. Ackerson, D. A. Bushnell, R. D. Kornberg, *Science* **2007**, *318*, 430.
[4] E. Roduner, *Chem. Soc. Rev.* **2006**, *35*, 583.
[5] C. Domingues, A. Santos, C. Alvarez-Lorenzo, A. Concheiro, I. Jarak, F. Veiga, I. Barbosa, M. Dourado, A. Figueiras, *ACS Nano* **2022**, *16*, 9994.
[6] K. Nienhaus, Y. M. Xue, L. Shang, G. U. Nienhaus, *Nanotechnology* **2022**, *33*, 262001.
[7] B. Pelaz, C. Alexiou, R. A. Alvarez -Puebla, F. Alves, A. M. Andrews, S. Ashraf, L. P. Balogh, L. Ballerini, A. Bestetti, C. Brendel, S. Bosi, M. Carril, W. C. W. Chan, C. Chen, X. Chen, X. Chen, Z. Cheng, D. Cui, J. Du, C. Dullin, A. Escudero, N. Feliu, M. Gao, M. George, Y. Gogotsi, A. Grünweller, Z. Gu, N. J. Halas, N. Hampp, R. K. Hartmann, et al., *ACS Nano* **2017**, *11*, 2313.
[8] M. Sohail, W. Guo, Z. Li, H. Xu, F. Zhao, D. Chen, F. Fu, *Curr. Med. Chem.* **2021**, *28*, 3753.

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

[9] P. M. Cevaal, A. Ali, E. Czuba-Wojnilowicz, J. Symons, S. R. Lewin, C. Cortez-Jugo, F. Caruso, *ACS Nano* **2021**, *15*, 3736.
[10] V. Balasubramanian, Z. Liu, J. Hirvonen, H. A. Santos, *Adv. Healthcare Mater.* **2018**, *7*, 1700432.
[11] U. Prabhakar, H. Maeda, R. K. Jain, E. M. Sevick-Muraca, W. Zamboni, O. C. Farokhzad, S. T. Barry, A. Gabizon, P. Grodzinski, D. C. Blakey, *Cancer Res.* **2013**, *73*, 2412.
[12] Y. Matsumoto, J. W. Nichols, K. Toh, T. Nomoto, H. Cabral, Y. Miura, R. J. Christie, N. Yamada, T. Ogura, M. R. Kano, Y. Matsumura, N. Nishiyama, T. Yamasoba, Y. H. Bae, K. Kataoka, *Nat. Nanotechnol.* **2016**, *11*, 533.
[13] J. Crawford, D. C. Dale, G. H. Lyman, *Cancer* **2004**, *100*, 228.
[14] K. Park, *J. Controlled Release* **2019**, *305*, 221.
[15] S. Wilhelm, A. J. Tavares, Q. Dai, S. Ohta, J. Audet, H. F. Dvorak, W. C. W. Chan, *Nat. Rev. Mater.* **2016**, *1*, 16014.
[16] M. J. Mitchell, M. M. Billingsley, R. M. Haley, M. E. Wechsler, N. A. Peppas, R. Langer, *Nat. Rev. Drug Discovery* **2021**, *20*, 101.
[17] J. B. Coty, C. Vauthier, *J. Controlled Release* **2018**, *275*, 254.
[18] M. Mahmoudi, *Trends Biotechnol.* **2018**, *36*, 755.
[19] M. A. Younis, H. M. Tawfeek, A. A. H. Abdellatif, J. A. Abdel-Aleem, H. Harashima, *Adv. Drug Delivery Rev.* **2022**, *181*, 114083.
[20] T. Cedervall, I. Lynch, S. Lindman, T. Berggard, E. Thulin, H. Nilsson, K. A. Dawson, S. Linse, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 2050.
[21] M. P. Monopoli, C. Åberg, A. Salvati, K. A. Dawson, *Nat. Nanotechnol.* **2012**, *7*, 779.
[22] A. J. Chetwynd, I. Lynch, *Environ. Sci.: Nano* **2020**, *7*, 1041.
[23] L. Abarca-Cabrera, P. Fraga-García, S. Berensmeier, *Biomater. Res.* **2021**, *25*, 12.
[24] M. Yang, E. Wu, W. Tang, J. Qian, C. Zhan, *J. Mater. Chem. B* **2021**, *9*, 6713.
[25] J. Ren, N. Andrikopoulos, K. Velonia, H. Tang, R. Cai, F. Ding, P. C. Ke, C. Chen, *J. Am. Chem. Soc.* **2022**, *144*, 9184.
[26] M. Martinez-Negro, G. Gonzalez-Rubio, E. Aicart, K. Landfester, A. Guerrero-Martinez, E. Junquera, *Adv. Colloid Interface Sci.* **2021**, *289*, 102366.
[27] S. Ashraf, J. Park, M. A. Bichelberger, K. Kantner, R. Hartmann, P. Maffre, A. H. Said, N. Feliu, J. Lee, D. Lee, G. U. Nienhaus, S. Kim, W. J. Parak, *Nanoscale* **2016**, *8*, 17794.
[28] N. Bertrand, P. Grenier, M. Mahmoudi, E. M. Lima, E. A. Appel, F. Dormont, J. M. Lim, R. Karnik, R. Langer, O. C. Farokhzad, *Nat. Commun.* **2017**, *8*, 777.
[29] B. Kang, T. Opatz, K. Landfester, F. R. Wurm, *Chem. Soc. Rev.* **2015**, *44*, 8301.
[30] R. Gref, M. Lück, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk, R. H. Müller, *Colloids Surf., B* **2000**, *18*, 301.
[31] Y. Ju, J. Cui, M. Müllner, T. Suma, M. Hu, F. Caruso, *Biomacromolecules* **2015**, *16*, 807.
[32] J. Cui, M. Björnmalm, Y. Ju, F. Caruso, *Langmuir* **2018**, *34*, 10817.
[33] B. Pelaz, P. del Pino, P. Maffre, R. Hartmann, M. Gallego, S. Rivera-Fernández, J. M. de la Fuente, G. U. Nienhaus, W. J. Parak, *ACS Nano* **2015**, *9*, 6996.
[34] I. Alberg, S. Kramer, M. Schinnerer, Q. Hu, C. Seidl, C. Leps, N. Drude, D. Mockel, C. Rijcken, T. Lammers, M. Diken, M. Maskos, S. Morsbach, K. Landfester, S. Tenzer, M. Barz, R. Zentel, *Small* **2020**, *16*, e1907574.
[35] M. Li, S. Jiang, J. Simon, D. Passlick, M. L. Frey, M. Wagner, V. Mailänder, D. Crespy, K. Landfester, *Nano Lett.* **2021**, *21*, 1591.
[36] K. Nienhaus, H. Wang, G. U. Nienhaus, *Mater. Today Adv.* **2020**, *5*, 100036.
[37] J. Ren, R. Cai, J. Wang, M. Daniyal, D. Baimanov, Y. Liu, D. T. Yin, Y. Liu, Q. Miao, Y. Zhao, C. Chen, *Nano Lett.* **2019**, *19*, 4692.
[38] J. Simon, L. K. Müller, M. Kokkinopoulou, I. Lieberwirth, S. Morsbach, K. Landfester, V. Mailänder, *Nanoscale* **2018**, *10*, 10731.
[39] M. Tonigold, J. Simon, D. Estupiñán, M. Kokkinopoulou, J. Reinholz, U. Kintzel, A. Kaltbeitzel, P. Renz, M. P. Domogalla, K. Steinbrink, I. Lieberwirth, D. Crespy, K. Landfester, V. Mailänder, *Nat. Nanotechnol.* **2018**, *13*, 862.
[40] Q. Dai, N. Bertleff-Zieschang, J. A. Braunger, M. Björnmalm, C. Cortez-Jugo, F. Caruso, *Adv. Healthcare Mater.* **2018**, *7*, 1700575.
[41] N. Singh, C. Marets, J. Boudon, N. Millot, L. Saviot, L. Maurizi, *Nanoscale Adv.* **2021**, *3*, 1209.
[42] Q. Xiao, M. Zoulikha, M. Qiu, C. Teng, C. Lin, X. Li, M. A. Sallam, Q. Xu, W. He, *Adv. Drug Delivery Rev.* **2022**, *186*, 114356.
[43] T. A. Saleh, *Environ. Technol. Innovation* **2020**, *20*, 101067.
[44] N. Joudeh, D. Linke, *J. Nanobiotechnol.* **2022**, *20*, 262.
[45] A. Bekdemir, S. Liao, F. Stellacci, *Colloids Surf., B* **2019**, *174*, 367.
[46] R. A. J. Post, D. van der Zwaag, G. Bet, S. P. W. Wijnands, L. Albertazzi, E. W. Meijer, R. W. van der Hofstad, *Nat. Commun.* **2019**, *10*, 1663.
[47] C. Carrillo-Carrion, M. Carril, W. J. Parak, *Curr. Opin. Biotechnol.* **2017**, *46*, 106.
[48] S. Mourdikoudis, R. M. Pallares, N. T. K. Thanh, *Nanoscale* **2018**, *10*, 12871.
[49] M. M. Modena, B. Ruhle, T. P. Burg, S. Wuttke, *Adv. Mater.* **2019**, *31*, 1901556.
[50] A. Heuer-Jungemann, N. Feliu, I. Bakaimi, M. Hamaly, A. Alkilany, I. Chakraborty, A. Masood, M. F. Casula, A. Kostopoulou, E. Oh, K. Susumu, M. H. Stewart, I. L. Medintz, E. Stratakis, W. J. Parak, A. G. Kanaras, *Chem. Rev.* **2019**, *119*, 4819.
[51] M. A. Boles, D. Ling, T. Hyeon, D. V. Talapin, *Nat. Mater.* **2016**, *15*, 141.
[52] B. B. Manshian, J. Jimenez, U. Himmelreich, S. J. Soenen, *Biomaterials* **2017**, *127*, 1.
[53] L. S. Arias, J. P. Pessan, A. P. M. Vieira, T. M. T. de Lima, A. C. B. Delbem, D. R. Monteiro, *Antibiotics* **2018**, *7*, 46.
[54] L. Q. Chen, L. Fang, J. Ling, C. Z. Ding, B. Kang, C. Z. Huang, *Chem. Res. Toxicol.* **2015**, *28*, 501.
[55] E. Rideau, R. Dimova, P. Schwille, F. R. Wurm, K. Landfester, *Chem. Soc. Rev.* **2018**, *47*, 8572.
[56] A. Zielińska, F. Carreiró, A. M. Oliveira, A. Neves, B. Pires, D. N. Venkatesh, A. Durazzo, M. Lucarini, P. Eder, A. M. Silva, A. Santini, E. B. Souto, *Molecules* **2020**, *25*, 3731.
[57] A. George, P. A. Shah, P. S. Shrivastav, *Int. J. Pharm.* **2019**, *561*, 244.
[58] T. L. Moore, L. Rodriguez-Lorenzo, V. Hirsch, S. Balog, D. Urban, C. Jud, B. Rothen-Rutishauser, M. Lattuada, A. Petri-Fink, *Chem. Soc. Rev.* **2015**, *44*, 6287.
[59] Y. Duan, A. Dhar, C. Patel, M. Khimani, S. Neogi, P. Sharma, N. Siva Kumar, R. L. Vekariya, *RSC Adv.* **2020**, *10*, 26777.
[60] R. Tenchov, R. Bird, A. E. Curtze, Q. Zhou, *ACS Nano* **2021**, *15*, 16982.
[61] L. Xu, X. Wang, Y. Liu, G. Yang, R. J. Falconer, C.-X. Zhao, *Adv. Biomed. Res.* **2022**, *2*, 2100109.
[62] A. C. Anselmo, S. Mitragotri, *Bioeng. Transl. Med.* **2019**, *4*, e10143.
[63] X. Hou, T. Zaks, R. Langer, Y. Dong, *Nat. Rev. Mater.* **2021**, *6*, 1078.
[64] R. H. Fang, W. Gao, L. Zhang, *Nat. Rev. Clin. Oncol.* **2023**, *20*, 33.
[65] T. E. Creighton, *Biochem. J.* **1990**, *270*, 1.
[66] G. U. Nienhaus, J. D. Müller, B. H. McMahon, H. Frauenfelder, *Phys. D* **1997**, *107*, 297.
[67] T. Arai, W. Norde, *Colloids Surf.* **1990**, *51*, 1.
[68] D. Coglitore, J.-M. Janot, S. Balme, *Adv. Colloid Interface Sci.* **2019**, *270*, 278.
[69] L. Vroman, *Nature* **1962**, *196*, 476.
[70] L. Vroman, A. Lukosevicius, *Nature* **1964**, *204*, 701.
[71] M. Rabe, D. Verdes, S. Seeger, *Adv. Colloid Interface Sci.* **2011**, *162*, 87.
[72] E. Gonzalez Solveyra, I. Szleifer, *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2016**, *8*, 334.
[73] Y. Yu, Y. Luan, W. Dai, *Int. J. Biol. Macromol.* **2022**, *205*, 731.

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

[74] S. J. Park, *Int. J. Nanomed.* **2020**, *15*, 5783.
[75] B. D. Fair, A. M. Jamieson, *J. Colloid Interface Sci.* **1980**, *77*, 525.
[76] L. Medda, M. Monduzzi, A. Salis, *Chem. Commun.* **2015**, *51*, 6663.
[77] R. A. Latour, *Colloids Surf., B* **2020**, *191*, 110992.
[78] L. A. Warning, Q. Zhang, R. Baiyasi, C. F. Landes, S. Link, *J. Phys. Chem. Lett.* **2020**, *11*, 1170.
[79] R. A. Latour, in *Encyclopedia of Biomaterials and Biomedical Engineering* (Eds: G. Wnek, G. Bowlin), Vol. *1*, Taylor & Francis, London **2005**, p. 270.
[80] M. M. Santore, C. F. Wertz, *Langmuir* **2005**, *21*, 10172.
[81] C. F. Wertz, M. M. Santore, *Langmuir* **2001**, *17*, 3006.
[82] P. Maffre, K. Nienhaus, F. Amin, W. J. Parak, G. U. Nienhaus, *Beilstein J. Nanotechnol.* **2011**, *2*, 374.
[83] P. Maffre, S. Brandholt, K. Nienhaus, L. Shang, W. J. Parak, G. U. Nienhaus, *Beilstein J. Nanotechnol.* **2014**, *5*, 2036.
[84] A. A. Vertegel, R. W. Siegel, J. S. Dordick, *Langmuir* **2004**, *20*, 6800.
[85] P. Roach, D. Farrar, C. C. perry, *J. Am. Chem. Soc.* **2006**, *128*, 3939.
[86] M. Lundqvist, I. Sethson, B. H. Jonsson, *Langmuir* **2004**, *20*, 10639.
[87] R. A. Hartvig, M. van de Weert, J. Ostergaard, L. Jorgensen, H. Jensen, *Langmuir* **2011**, *27*, 2634.
[88] S. Angioletti-Uberti, M. Ballauff, J. Dzubiella, *Mol. Phys.* **2018**, *116*, 3154.
[89] K. Lyu, H. Chen, J. Gao, J. Jin, H. Shi, D. K. Schwartz, D. Wang, *Biomacromolecules* **2022**, *23*, 4709.
[90] K. S. Lavery, C. Rhodes, A. Mcgraw, M. J. Eppihimer, *Adv. Drug Delivery Rev.* **2017**, *112*, 2.
[91] L. Vroman, A. L. Adams, *Surf. Sci.* **1969**, *16*, 438.
[92] L. Vroman, A. L. Adams, *J. Biomed. Mater. Res.* **1969**, *3*, 43.
[93] L. Vroman, A. L. Adams, M. Klings, *Fed. Proc.* **1971**, *30*, 1494.
[94] C. A. LeDuc, L. Vroman, E. F. Leonard, *Ind. Eng. Chem. Res.* **1995**, *34*, 3488.
[95] S. L. Hirsh, D. R. McKenzie, N. J. Nosworthy, J. A. Denman, O. U. Sezerman, M. M. Bilek, *Colloids Surf., B* **2013**, *103*, 395.
[96] R. A. Latour, *J. Biomed. Mater. Res., Part A* **2015**, *103*, 949.
[97] M. Ozboyaci, D. B. Kokh, S. Corni, R. C. Wade, *Q. Rev. Biophys.* **2016**, *49*, e4.
[98] J. Kim, *Colloids Surf., B* **2018**, *162*, 370.
[99] X. Quan, J. Liu, J. Zhou, *Curr. Opin. Colloid Interface Sci.* **2019**, *41*, 74.
[100] V. P. Zhdanov, *Curr. Opin. Colloid Interface Sci.* **2019**, *41*, 95.
[101] Q. Shao, C. K. Hall, *J. Phys.: Condens. Matter* **2016**, *28*, 414019.
[102] P. L. Latreille, M. L. Goas, S. Salimi, J. Robert, G. De Crescenzo, D. C. Boffito, V. A. Martinez, P. Hildgen, X. Banquy, *ACS Nano* **2022**, *16*, 1689.
[103] D. V. Sotnikov, A. N. Berlina, V. S. Ivanov, A. V. Zherdev, B. B. Dzantiev, *Colloids Surf., B* **2019**, *173*, 557.
[104] M. Waghmare, B. Khade, P. Chaudhari, P. Dongre, *J. Nanopart. Res.* **2018**, *20*, 185.
[105] D. Docter, D. Westmeier, M. Markiewicz, S. Stolte, S. K. Knauer, R. H. Stauber, *Chem. Soc. Rev.* **2015**, *44*, 6094.
[106] S. Milani, F. B. Bombelli, A. S. Pitek, K. A. Dawson, J. Rädler, *ACS Nano* **2012**, *6*, 2532.
[107] L. Shang, G. U. Nienhaus, *Acc. Chem. Res.* **2017**, *50*, 387.
[108] D. Docter, U. Distler, W. Storck, J. Kuharev, D. Wünsch, A. Hahlbrock, S. K. Knauer, S. Tenzer, R. H. Stauber, *Nat. Protoc.* **2014**, *9*, 2030.
[109] K. Nienhaus, G. U. Nienhaus, *Biointerphases* **2020**, *15*, 061201.
[110] P. M. Carvalho, M. R. Felicio, N. C. Santos, S. Goncalves, M. M. Domingues, *Front. Chem.* **2018**, *6*, 237.
[111] H. Kato, in *Nanomaterials: Processing and Characterization with Lasers* (Eds: S. C. Singh, H. Zeng, C. Guo, W. Cai), Wiley-VCH, Berlin **2012**, p. 535.
[112] G. U. Nienhaus, P. Maffre, K. Nienhaus, *Methods Enzymol.* **2013**, *519*, 115.
[113] X. Tan, K. Welsher, *Angew. Chem., Int. Ed.* **2021**, *60*, 22359.
[114] V. Filipe, A. Hawe, W. Jiskoot, *Pharm. Res.* **2010**, *27*, 796.
[115] P. Hole, K. Sillence, C. Hannell, C. M. Maguire, M. Roesslein, G. Suarez, S. Capracotta, Z. Magdolenova, L. Horev-Azaria, A. Dybowska, L. Cooke, A. Haase, S. Contal, S. Manø, A. Vennemann, J. J. Sauvain, K. C. Staunton, S. Anguissola, A. Luch, M. Dusinska, R. Korenstein, A. C. Gutleb, M. Wiemann, A. Prina-Mello, M. Riediker, P. Wick, *J. Nanopart. Res.* **2013**, *15*, 2101.
[116] K. Fischer, M. Schmidt, *Biomaterials* **2016**, *98*, 79.
[117] D. Langevin, O. Lozano, A. Salvati, V. Kestens, M. Monopoli, E. Raspaud, S. Mariot, A. Salonen, S. Thomas, M. Driessen, A. Haase, I. Nelissen, N. Smisdom, P. P. Pompa, G. Maiorano, V. Puntes, D. Puchowicz, M. Stępnik, G. Suárez, M. Riediker, F. Benetti, I. Mičetić, M. Venturini, W. G. Kreyling, M. van der Zande, H. Bouwmeester, S. Milani, J. O. Rädler, S. Mülhopt, I. Lynch, *NanoImpact* **2018**, *10*, 97.
[118] L. Treuel, S. Brandholt, P. Maffre, S. Wiegele, L. Shang, G. U. Nienhaus, *ACS Nano* **2014**, *8*, 503.
[119] C. Röcker, M. Pötzl, F. Zhang, W. J. Parak, G. U. Nienhaus, *Nat. Nanotechnol.* **2009**, *4*, 577.
[120] X. Jiang, S. Weise, M. Hafner, C. Röcker, F. Zhang, W. J. Parak, G. U. Nienhaus, *J. R. Soc., Interface* **2010**, *7*, S5.
[121] Y. Klapper, P. Maffre, L. Shang, K. N. Ekdahl, B. Nilsson, S. Hettler, M. Dries, D. Gerthsen, G. U. Nienhaus, *Nanoscale* **2015**, *7*, 9980.
[122] H. Wang, L. Shang, P. Maffre, S. Hohmann, F. Kirschhofer, G. Brenner-Weiss, G. U. Nienhaus, *Small* **2016**, *12*, 5836.
[123] H. Wang, R. Ma, K. Nienhaus, G. U. Nienhaus, *Small* **2019**, *15*, 1900974.
[124] P. L. Latreille, J. M. Rabanel, M. L. Goas, S. Salimi, J. Arlt, S. A. Patten, C. Ramassamy, P. Hildgen, V. A. Martinez, X. Banquy, *Adv. Mater.* **2022**, *34*, 2203354.
[125] H. Wang, Y. Lin, K. Nienhaus, G. U. Nienhaus, *WIREs Nanomed. Nanobiotechnol.* **2018**, *10*, e1500.
[126] J. Piella, N. G. Bastus, V. Puntes, *Bioconjugate Chem.* **2017**, *28*, 88.
[127] S. H. Lacerda, J. J. Park, C. Meuse, D. Pristinski, M. L. Becker, A. Karim, J. F. Douglas, *ACS Nano* **2010**, *4*, 365.
[128] S. Goy-Lopez, J. Juarez, M. Alatorre-Meda, E. Casals, V. F. Puntes, P. Taboada, V. Mosquera, *Langmuir* **2012**, *28*, 9113.
[129] E. Casals, T. Pfaller, A. Duschl, G. J. Oostingh, V. F. Puntes, *Small* **2011**, *7*, 3479.
[130] M. A. Dobrovolskaia, A. K. Patri, J. Zheng, J. D. Clogston, N. Ayub, P. Aggarwal, B. W. Neun, J. B. Hall, S. E. McNeil, *Nanomed.: Nanotechnol. Biol. Med.* **2009**, *5*, 106.
[131] K. Nienhaus, G. U. Nienhaus, *J. Mol. Biol.* **2016**, *428*, 308.
[132] S. Pujals, L. Albertazzi, *ACS Nano* **2019**, *13*, 9707.
[133] M. Battaglini, N. Feiner, C. Tapeinos, D. De Pasquale, C. Pucci, A. Marino, M. Bartolucci, A. Petretto, L. Albertazzi, G. Ciofani, *Nanoscale* **2022**, *14*, 13292.
[134] A. M. Clemments, P. Botella, C. C. Landry, *J. Am. Chem. Soc.* **2017**, *139*, 3978.
[135] N. Feiner-Gracia, M. Beck, S. Pujals, S. Tosi, T. Mandal, C. Buske, M. Linden, L. Albertazzi, *Small* **2017**, *13*, 1701631.
[136] Y. Wang, P. E. D. Soto Rodriguez, L. Woythe, S. Sanchez, J. Samitier, P. Zijlstra, L. Albertazzi, *ACS Appl. Mater. Interfaces* **2022**, *14*, 37345.
[137] T. Andrian, P. Delcanale, S. Pujals, L. Albertazzi, *Nano Lett.* **2021**, *21*, 5360.
[138] M. Kokkinopoulou, J. Simon, K. Landfester, V. Mailänder, I. Lieberwirth, *Nanoscale* **2017**, *9*, 8858.
[139] S. Sheibani, K. Basu, A. Farnudi, A. Ashkarran, M. Ichikawa, J. F. Presley, K. H. Bui, M. R. Ejtehadi, H. Vali, M. Mahmoudi, *Nat. Commun.* **2021**, *12*, 573.
[140] S. Linse, C. Cabaleiro-Lago, W. F. Xue, I. Lynch, S. Lindman, E. Thulin, S. E. Radford, K. A. Dawson, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 8691.

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

[141] S. Mirsadeghi, R. Dinarvand, M. H. Ghahremani, M. R. Hormozi-Nezhad, Z. Mahmoudi, M. J. Hajipour, F. Atyabi, M. Ghavami, M. Mahmoudi, *Nanoscale* **2015**, *7*, 5004.
[142] Y. Chen, Q. Liu, F. Yang, H. Yu, Y. Xie, W. Yao, *Colloids Surf., B* **2022**, *218*, 112736.
[143] E. Papini, R. Tavano, F. Mancin, *Front. Immunol.* **2020**, *11*, 567365.
[144] H. Mohammad-Beigi, C. Scavenius, P. B. Jensen, K. Kjaer-Sorensen, C. Oxvig, T. Boesen, J. J. Enghild, D. S. Sutherland, Y. Hayashi, *ACS Nano* **2020**, *14*, 10666.
[145] Y. Zhang, J. L. Y. Wu, J. Lazarovits, W. C. W. Chan, *J. Am. Chem. Soc.* **2020**, *142*, 8827.
[146] R. Pieper, C. L. Gatlin, A. J. Makusky, P. S. Russo, C. R. Schatz, S. S. Miller, Q. Su, A. M. McGrath, M. A. Estock, P. P. Parmar, M. Zhao, S. T. Huang, J. Zhou, F. Wang, R. Esquer-Blasco, N. L. Anderson, J. Taylor, S. Steiner, *Proteomics* **2003**, *3*, 1345.
[147] J. S. Weltz, D. K. Schwartz, J. L. Kaar, *ACS Nano* **2016**, *10*, 730.
[148] Y. Cai, D. K. Schwartz, *ACS Appl. Mater. Interfaces* **2016**, *8*, 511.
[149] H. Mohammad-Beigi, Y. Hayashi, C. M. Zeuthen, H. Eskandari, C. Scavenius, K. Juul-Madsen, T. Vorup-Jensen, J. J. Enghild, D. S. Sutherland, *Nat. Commun.* **2020**, *11*, 4535.
[150] C. D. Walkey, W. C. W. Chan, *Chem. Soc. Rev.* **2012**, *41*, 2780.
[151] C. Corbo, R. Molinaro, A. Parodi, N. E. Toledano Furman, F. Salvatore, E. Tasciotti, *Nanomedicine* **2016**, *11*, 81.
[152] D. Walczyk, F. B. Bombelli, M. P. Monopoli, I. Lynch, K. A. Dawson, *J. Am. Chem. Soc.* **2010**, *132*, 5761.
[153] L. Zemanova, A. Schenk, M. J. Valler, G. U. Nienhaus, R. Heilker, *Drug Discovery Today* **2003**, *8*, 1085.
[154] M. Carril, D. Padro, P. del Pino, C. Carrillo-Carrion, M. Gallego, W. J. Parak, *Nat. Commun.* **2017**, *8*, 1542.
[155] S. Qu, F. Sun, Z. Qiao, J. Li, L. Shang, *Small* **2020**, *16*, 1907633.
[156] S. Qu, Z. Qiao, W. Zhong, K. Liang, X. Jiang, L. Shang, *ACS Appl. Mater. Interfaces* **2022**, *14*, 44147.
[157] D. Sanchez-Guzman, G. Giraudon-Colas, L. Marichal, Y. Boulard, F. Wien, J. Degrouard, A. Baeza-Squiban, S. Pin, J. P. Renault, S. Devineau, *ACS Nano* **2020**, *14*, 9073.
[158] A. C. G. Weiss, K. Krüger, Q. A. Besford, M. Schlenk, K. Kempe, S. Förster, F. Caruso, *ACS Appl. Mater. Interfaces* **2019**, *11*, 2459.
[159] F. E. Galdino, A. S. Picco, L. B. Capeletti, J. Bettini, M. B. Cardoso, *Nano Lett.* **2021**, *21*, 8250.
[160] F. Otto, X. Sun, F. Schulz, C. Sanchez-Cano, N. Feliu, F. Westermeier, W. J. Parak, *Small* **2022**, *18*, 2201324.
[161] L. Marichal, G. Giraudon–Colas, F. Cousin, A. Thill, J. Labarre, Y. Boulard, J.-C. Aude, S. Pin, J. P. Renault, *Langmuir* **2019**, *35*, 10831.
[162] S. Khan, A. Gupta, N. C. Verma, C. K. Nandi, *J. Chem. Phys.* **2015**, *143*, 164709.
[163] N. Welsch, J. Dzubiella, A. Graebert, M. Ballauff, *Soft Matter* **2012**, *8*, 12043.
[164] X. Zhang, H. Shi, R. Zhang, J. Zhang, F. Xu, L. Qiao, S. Yu, *Part. Part. Syst. Charact.* **2019**, *36*, 1800257.
[165] O. Vilanova, J. J. Mittag, P. M. Kelly, S. Milani, K. A. Dawson, J. O. Rädler, G. Franzese, *ACS Nano* **2016**, *10*, 10842.
[166] L. Shang, L. Yang, J. Seiter, M. Heinle, G. Brenner-Weiss, D. Gerthsen, G. U. Nienhaus, *Adv. Mater. Interfaces* **2014**, *1*, 1300079.
[167] L. Shang, K. Nienhaus, G. U. Nienhaus, *J Nanobiotechnology* **2014**, *12*, 5.
[168] L. Treuel, K. A. Eslahian, D. Docter, T. Lang, R. Zellner, K. Nienhaus, G. U. Nienhaus, R. H. Stauber, M. Maskos, *Phys. Chem. Chem. Phys.* **2014**, *16*, 15053.
[169] M. F. Engel, A. J. Visser, C. P. van Mierlo, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11316.
[170] R. Kurrat, J. J. Ramsden, J. E. Prenosil, *J Chem Soc* **1994**, *90*, 587.
[171] M. Wahlgren, T. Arnebrant, I. Lundstrom, *J. Colloid Interface Sci.* **1995**, *175*, 506.
[172] L. Wannerberger, M. Wahlgren, A. C. Eliasson, *Cereal Chem.* **1996**, *73*, 499.
[173] D. Baimanov, J. Wang, J. Zhang, K. Liu, Y. L. Cong, X. M. Shi, X. H. Zhang, Y. F. Li, X. M. Li, R. R. Qiao, Y. L. Zhao, Y. L. Zhou, L. M. Wang, C. Y. Chen, *Nat. Commun.* **2022**, *13*, 5389.
[174] O. K. Kari, J. Ndika, P. Parkkila, A. Louna, T. Lajunen, A. Puustinen, T. Viitala, H. Alenius, A. Urtti, *Nanoscale* **2020**, *12*, 1728.
[175] K. N. L. Hoang, K. E. Wheeler, C. J. Murphy, *Anal. Chem.* **2022**, *94*, 4737.
[176] C. Weber, S. Morsbach, K. Landfester, *Angew. Chem., Int. Ed.* **2019**, *58*, 12787.
[177] S. Dominguez-Medina, L. Kisley, L. J. Tauzin, A. Hoggard, B. Shuang, A. Indrasekara, S. Chen, L. Y. Wang, P. J. Derry, A. Liopo, E. R. Zubarev, C. F. Landes, S. Link, *ACS Nano* **2016**, *10*, 2103.
[178] C. C. Fleischer, C. K. Payne, *Acc. Chem. Res.* **2014**, *47*, 2651.
[179] P. Satzer, F. Svec, G. Sekot, A. Jungbauer, *Eng. Life Sci.* **2016**, *16*, 238.
[180] D. T. Jayaram, S. M. Pustulka, R. G. Mannino, W. A. Lam, C. K. Payne, *Biophys. J.* **2018**, *115*, 209.
[181] M. Ozboyaci, D. B. Kokh, R. C. Wade, *Phys. Chem. Chem. Phys.* **2016**, *18*, 10191.
[182] M. Lundqvist, C. Augustsson, M. Lilja, K. Lundkvist, B. Dahlbäck, S. Linse, T. Cedervall, *PLoS One* **2017**, *12*, e0175871.
[183] A. L. Barran-Berdon, D. Pozzi, G. Caracciolo, A. L. Capriotti, G. Caruso, C. Cavaliere, A. Riccioli, S. Palchetti, A. Lagana, *Langmuir* **2013**, *29*, 6485.
[184] K. Partikel, R. Korte, D. Mulac, H. U. Humpf, K. Langer, *Beilstein J. Nanotechnol.* **2019**, *10*, 1002.
[185] R. Frost, C. Langhammer, T. Cedervall, *Nanoscale* **2017**, *9*, 3620.
[186] S. Tenzer, D. Docter, J. Kuharev, A. Musyanovych, V. Fetz, R. Hecht, F. Schlenk, D. Fischer, K. Kiouptsi, C. Reinhardt, K. Landfester, H. Schild, M. Maskos, S. K. Knauer, R. H. Stauber, *Nat. Nanotechnol.* **2013**, *8*, 772.
[187] R. L. Pinals, D. Yang, D. J. Rosenberg, T. Chaudhary, A. R. Crothers, A. T. Iavarone, M. Hammel, M. P. Landry, *Angew. Chem., Int. Ed.* **2020**, *59*, 23668.
[188] V. Forest, J. Pourchez, *Nano Today* **2016**, *11*, 700.
[189] S. Galmarini, U. Hanusch, M. Giraud, N. Cayla, D. Chiappe, N. von Moos, H. Hofmann, L. Maurizi, *Bioconjugate Chem.* **2018**, *29*, 3385.
[190] L. Böhmert, L. Voß, V. Stock, A. Braeuning, A. Lampen, H. Sieg, *Nanoscale Adv.* **2020**, *2*, 563.
[191] M. Brückner, J. Simon, S. Jiang, K. Landfester, V. Mailänder, *Acta Biomater.* **2020**, *114*, 333.
[192] M. Ilett, O. Matar, F. Bamiduro, S. Sanchez-Segado, R. Brydson, A. Brown, N. Hondow, *Sci. Rep.* **2020**, *10*, 5278.
[193] S. Winzen, S. Schoettler, G. Baier, C. Rosenauer, V. Mailaender, K. Landfester, K. Mohr, *Nanoscale* **2015**, *7*, 2992.
[194] C. Fedeli, D. Segat, R. Tavano, L. Bubacco, G. De Franceschi, P. P. de Laureto, E. Lubian, F. Selvestrel, F. Mancin, E. Papini, *Nanoscale* **2015**, *7*, 17710.
[195] C. Wang, B. B. Chen, M. He, B. Hu, *ACS Nano* **2021**, *15*, 3108.
[196] D. L. Tabb, L. Vega-Montoto, P. A. Rudnick, A. M. Variyath, A. J. Ham, D. M. Bunk, L. E. Kilpatrick, D. D. Billheimer, R. K. Blackman, H. L. Cardasis, S. A. Carr, K. R. Clauser, J. D. Jaffe, K. A. Kowalski, T. A. Neubert, F. E. Regnier, B. Schilling, T. J. Tegeler, M. Wang, P. Wang, J. R. Whiteaker, L. J. Zimmerman, S. J. Fisher, B. W. Gibson, C. R. Kinsinger, M. Mesri, H. Rodriguez, S. E. Stein, P. Tempst, A. G. Paulovich, et al., *J. Proteome Res.* **2010**, *9*, 761.
[197] A. A. Ashkarran, H. Gharibi, E. Voke, M. P. Landry, A. A. Saei, M. Mahmoudi, *Nat. Commun.* **2022**, *13*, 6610.
[198] M. P. Monopoli, D. Walczyk, A. Campbell, G. Elia, I. Lynch, F. B. Bombelli, K. A. Dawson, *J. Am. Chem. Soc.* **2011**, *133*, 2525.

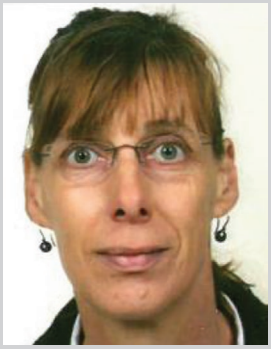
© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

[199] N. P. Mortensen, G. B. Hurst, W. Wang, C. M. Foster, P. D. Nallathamby, S. T. Retterer, *Nanoscale* **2013**, *5*, 6372.
[200] C. Pisani, J.-C. Gaillard, M. Odorico, J. Nyalosaso, C. Charnay, Y. Guari, J. Chopineau, J.-M. Devoisselle, J. Armengaud, O. Prat, *Nanoscale* **2016**, *9*, 1840.
[201] M. Hadjidemetriou, Z. Al-Ahmady, K. Kostarelos, *Nanoscale* **2016**, *8*, 6948.
[202] D. Pozzi, G. Caracciolo, L. Digiacomo, V. Colapicchioni, S. Palchetti, A. L. Capriotti, C. Cavaliere, R. Zenezini Chiozzi, A. Puglisi, A. Laganà, *Nanoscale* **2015**, *7*, 13958.
[203] S. Palchetti, D. Pozzi, A. L. Capriottic, G. La Barbera, R. Z. Chiozzi, L. Digiacomo, G. Peruzzi, G. Caracciolo, A. Lagana, *Colloids Surf., B* **2017**, *153*, 263.
[204] A. Cox, P. Andreozzi, R. Dal Magro, F. Fiordaliso, A. Corbelli, L. Talamini, C. Chinello, F. Raimondo, F. Magni, M. Tringali, S. Krol, P. J. Silva, F. Stellacci, M. Masserini, F. Re, *ACS Nano* **2018**, *12*, 7292.
[205] A. D. Lehmann, W. J. Parak, F. Zhang, Z. Ali, C. Röcker, G. U. Nienhaus, P. Gehr, B. Rothen-Rutishauser, *Small* **2010**, *6*, 753.
[206] M. Lundqvist, J. Stigler, T. Cedervall, T. Berggard, M. B. Flanagan, I. Lynch, G. Elia, K. Dawson, *ACS Nano* **2011**, *5*, 7503.
[207] R. Cai, J. Ren, M. Guo, T. Wei, Y. Liu, C. Xie, P. Zhang, Z. Guo, A. J. Chetwynd, P. C. Ke, I. Lynch, C. Chen, *Proc. Natl. Acad. Sci. USA* **2022**, *119*, e2200363119.
[208] R. A. Cairns, I. S. Harris, T. W. Mak, *Nat. Rev. Cancer* **2011**, *11*, 85.
[209] M. Hadjidemetriou, Z. Al-Ahmady, M. Mazza, R. F. Collins, K. Dawson, K. Kostarelos, *ACS Nano* **2015**, *9*, 8142.
[210] J. Lazarovits, S. Sindhwani, A. J. Tavares, Y. Zhang, F. Song, J. Audet, J. R. Krieger, A. M. Syed, B. Stordy, W. C. W. Chan, *ACS Nano* **2019**, *13*, 8023.
[211] U. Sakulkhu, L. Maurizi, M. Mahmoudi, M. Motazacker, M. Vries, A. Gramoun, M.-G. Ollivier Beuzelin, J.-P. Vallée, F. Rezaee, H. Hofmann, *Nanoscale* **2014**, *6*, 11439.
[212] J. Simon, G. Kuhn, M. Fichter, S. Gehring, K. Landfester, V. Mailänder, *Cells* **2021**, *10*, 132.
[213] C. Vidaurre-Agut, E. M. Rivero-Buceta, C. C. Landry, P. Botella, *Nanomaterials* **2021**, *11*, 1196.
[214] J. Müller, D. Prozeller, A. Ghazaryan, M. Kokkinopoulou, V. Mailänder, S. Morsbach, K. Landfester, *Acta Biomater.* **2018**, *71*, 420.
[215] M. Lundqvist, T. Cedervall, *Small* **2020**, *16*, e2000892.
[216] A. J. Chetwynd, W. Zhang, J. A. Thorn, I. Lynch, R. Ramautar, *Small* **2020**, *16*, e2000295.
[217] S. Han, R. da Costa Marques, J. Simon, A. Kaltbeitzel, K. Koynov, K. Landfester, V. Mailänder, I. Lieberwirth, *Nat. Commun.* **2023**, *14*, 295.
[218] K. Abstiens, S. Maslanka Figueroa, M. Gregoritza, A. M. Goepferich, *Soft Matter* **2019**, *15*, 709.
[219] B. D. Johnston, W. G. Kreyling, C. Pfeiffer, M. Schäffler, H. Sarioglu, S. Ristig, S. Hirn, N. Haberl, S. Thalhammer, S. M. Hauck, M. Semmler-Behnke, M. Epple, J. Hühn, P. Del Pino, W. J. Parak, *Adv. Funct. Mater.* **2017**, *27*, 1701956.
[220] R. Cukalevski, S. A. Ferreira, C. J. Dunning, T. Berggård, T. Cedervall, *Nano Res.* **2015**, *8*, 2733.
[221] L. F. Ferreira, A. S. Picco, F. E. Galdino, L. J. C. Albuquerque, J. F. Berret, M. B. Cardoso, *ACS Appl. Mater. Interfaces* **2022**, *14*, 28559.
[222] D. Wei, K. Pang, Q. Song, Y. Suo, H. He, X. Weng, X. Gao, X. Wei, *J. Controlled Release* **2018**, *278*, 66.
[223] M. Mahmoudi, *Nat. Commun.* **2022**, *13*, 49.
[224] S. B. Gunnarsson, K. Bernfur, A. Mikkelsen, T. Cedervall, *Nanoscale* **2018**, *10*, 4246.
[225] S. B. Gunnarsson, K. Bernfur, U. Englund-Johansson, F. Johansson, T. Cedervall, *PLoS One* **2019**, *14*, e0218211.
[226] W. C. W. Chan, *Acc. Chem. Res.* **2017**, *50*, 627.
[227] M. C. Lo Giudice, L. M. Herda, E. Polo, K. A. Dawson, *Nat. Commun.* **2016**, *7*, 13475.
[228] P. M. Kelly, C. Åberg, E. Polo, A. O'Connell, J. Cookman, J. Fallon, Ž. Krpetić, K. A. Dawson, *Nat. Nanotechnol.* **2015**, *10*, 472.
[229] L. M. Herda, D. R. Hristov, M. C. Lo Giudice, E. Polo, K. A. Dawson, *J. Am. Chem. Soc.* **2017**, *139*, 111.
[230] M. Faria, M. Björnmalm, K. J. Thurecht, S. J. Kent, R. G. Parton, M. Kavallaris, A. P. R. Johnston, J. J. Gooding, S. R. Corrie, B. J. Boyd, P. Thordarson, A. K. Whittaker, M. M. Stevens, C. A. Prestidge, C. J. H. Porter, W. J. Parak, T. P. Davis, E. J. Crampin, F. Caruso, *Nat. Nanotechnol.* **2018**, *13*, 777.
[231] H. S. Leong, K. S. Butler, C. J. Brinker, M. Azzawi, S. Conlan, C. Dufés, A. Owen, S. Rannard, C. Scott, C. Chen, M. A. Dobrovolskaia, S. V. Kozlov, A. Prina-Mello, R. Schmid, P. Wick, F. Caputo, P. Boisseau, R. M. Crist, S. E. McNeil, B. Fadeel, L. Tran, S. F. Hansen, N. B. Hartmann, L. P. W. Clausen, L. M. Skjolding, A. Baun, M. Ågerstrand, Z. Gu, D. A. Lamprou, C. Hoskins, et al., *Nat. Nanotechnol.* **2019**, *14*, 629.
[232] S. Y. Shaw, E. C. Westly, M. J. Pittet, A. Subramanian, S. L. Schreiber, R. Weissleder, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 7387.
[233] T. Puzyn, B. Rasulev, A. Gajewicz, X. K. Hu, T. P. Dasari, A. Michalkova, H. M. Hwang, A. Toropov, D. Leszczynska, J. Leszczynski, *Nat. Nanotechnol.* **2011**, *6*, 175.
[234] M. R. Findlay, D. N. Freitas, M. Mobed-Miremadi, K. E. Wheeler, *Environ. Sci.: Nano* **2018**, *5*, 64.
[235] L. Li, X. Jiang, J. Gao, *Adv. Mater. Interfaces* **2022**, *9*, 2201442.

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License



Karin Nienhaus is a research associate at the Institute of Applied Physics, Karlsruhe Institute of Technology (KIT), Germany. She studied chemistry at the University of Münster, Germany, and received her Ph.D. in chemistry at the University of Ulm, Germany, in 2003. From 2000 to 2009, she worked as a research assistant at the Institute of Biophysics in Ulm, where she mainly pursued spectroscopic investigations of fluorescent proteins and heme proteins. Since 2009, she has been employed at KIT. Currently, her research focuses on biomolecular interactions and photophysical processes of nanoscale luminescence markers.

Gerd Ulrich Nienhaus is a professor and director of the Institute of Applied Physics at the Karlsruhe Institute of Technology (KIT), Germany, and an adjunct professor at the Department of Physics, University of Illinois Urbana-Champaign, USA. He studied physics and physical chemistry at the University of Münster, Germany, and received his Ph.D. degree in 1988. Earlier, he held professorships at the University of Illinois and the University of Ulm, Germany, where he was director of the Institute of Biophysics. Presently, his research focuses on advanced optical microscopy, luminescence marker development, and fundamental molecular and cellular biophysics.

© 2023 The Authors. Small published by Wiley-VCH GmbH

16136829, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/smll.202301663 by Karlsruher Inst F. Technologie, Wiley Online Library on [03/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License