Nanoparticles for biomedical applications: exploring and exploiting molecular interactions at the nano-bio interface

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A R T I C L E   I N F O

Article history:
Received 31 August 2019
Received in revised form
19 November 2019
Accepted 19 November 2019
Available online 8 January 2020

Keywords:
Nanomaterials
Protein adsorption
Protein corona
Mechanisms
Nanomedicine

A B S T R A C T

Nanomaterials have high potential as powerful tools for nanomedicine in a wide range of most promising applications as highly specific devices for diagnosis and therapy. Yet, despite enormous research activities in the design and synthesis of nanomaterials for biomedicine, only a small number of those have made their way to clinical use. The unavoidable formation of a biomolecular adsorption layer, the ‘protein corona’ or ‘bionanoscopie corona’ around nanoparticles (NPs) has been recognized as a major roadblock on the way toward the efficient design of nanomedicines. It masks the generic NP properties and creates a new ‘biological identity’ that largely controls the interactions with the biological environment. Therefore, for successful design of nanomedical devices, researchers must anticipate formation of this protein adlayer and its ensuing effects. In this review, we summarize our current knowledge in the field and focus on three topics that appear to be important for furthering progress in our ability to predict in-vivo responses to NP incorporation from in-vitro studies. First, we address fundamental physicochemical issues of protein corona formation as revealed by recent in-vitro studies, with a focus on the underlying mechanistic details. Second, we illustrate with recent examples how our present, still incomplete understanding can already be exploited to control protein corona formation in the organism, including important processes involving the immune system. Third, recent advances in the transition from in-vitro to in-vivo studies of protein adsorption will be summarized, which is obviously a key step in NP development for nanomedicine. We conclude this review with an outlook on possible future developments in the field.

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1. Introduction

Studies of interactions of artificial bulk materials with biomolecules have a long-standing history due to their importance for the development of biocompatible medical devices, e.g. catheters, joint replacements, or stents [1]. Recent years have witnessed enormous efforts to investigate such interactions with engineered nanomaterials, driven by the realization that these devices offer unprecedented capabilities as tools in biomedical research, diagnostics and therapy [2]. For example, a wide range of nanoparticles (NPs) have been developed as luminescence markers with specific targeting capabilities for optical imaging, or contrast agents for computer tomography or magnetic resonance imaging [3–7]. Another most exciting field is the design of nanocarriers that deliver drugs to specific cell types, e.g. in cancer therapy [2,3,8,9]. NPs for biological applications have been synthesized in a vast variety of ways from a broad palette of materials [2,10]; they come in many different sizes and shapes and can vary widely in their physicochemical properties (Fig. 1). Frequently, inorganic cores are coated with surface ligands, endowing the NPs with colloidal stability and functional groups for specific targeting of proteins and other biomolecules. Like bulk materials, NPs typically possess surfaces that are reactive toward biomatter, and when they are exposed to biological fluids containing biological macromolecules (biofluids), e.g. cell–culture media in in-vitro studies or blood upon intravenous injection, their surfaces become rapidly covered by dissolved components, in particular proteins. The catchy notions
protein corona' [11] and, subsequently, 'biomolecular corona' [12] (to acknowledge the presence of other biomolecules, e.g., lipids and carbohydrates) have been coined to describe this adsorption layer. It conceals the ‘physicochemical identity’ of the pristine NPs and confers a ‘biological identity’ to the NPs because cells within an organism interact with this adlayer rather than the NP surface, mainly via receptor proteins resident in the plasma membrane [12]. Accordingly, any attempt to engineer NPs for biomedical applications must take this inevitable modification into account. The processes occurring during formation of this interfacial layer are exceedingly complicated, however, and even fundamental issues are still under debate.

The physical nature of the protein corona is largely governed by the (surface) properties of the NPs and by the types and (relative) amounts of biomolecules (and their individual properties) present in the biofluid [13–16]. Moreover, it is affected by external factors such as incubation time, temperature, shear forces due to flow (e.g., in vitro in reactors or in vivo in the bloodstream), and may even reflect the history of the entire trajectory taken by a NP migrating through different compartments of an organism [12,17–20]. For any biomedical application of NPs, e.g., as drug carriers, it is crucially important to understand corona formation at the molecular level, so that it becomes possible to control and predict its effects on the biological environment [21].
Many useful reviews have appeared in recent times addressing NPs for biomedical applications and the implications of protein corona formation for NP engineering [12,22–27]. Here we have not aimed at contributing yet another exhaustive review. We instead focus on three different areas that seem particularly important for further advancement of NPs for biomedical applications. After a brief sketch introducing important aspects of the protein corona, we first address fundamental physicochemical issues of protein corona formation, which have been explored in in-vitro studies, with a special focus on the underlying mechanistic details. Second, we present recent examples of how a molecular-scale understanding can be used to control the nature of the protein corona, enabling the engineering of NPs with advantageous properties for nanomedicine applications. Third, we discuss the transition from in-vitro to in-vivo studies of protein adsorption, which is a key step in the translation from nanomaterials to nanomedical devices. Finally, we discuss ongoing challenges and give an outlook on research directions that may help enhance the effectiveness of the development of NPs for diagnostics and therapy.

2. Facts and views about the nature of the protein corona

2.1. Current views of the protein corona

Upon immersion in a biological fluid, NPs randomly collide with proteins and other biomolecules. Protein association with NP surfaces will be stabilized by formation of enthalpic (covalent, electrostatic, van-der-Waals, hydrogen bond) interactions; further important stabilizing contributions come from entropic effects (liberation of small molecules from the joining surfaces, configurational entropy changes of the proteins) [28,29]. Processes occurring upon corona formation include fast reversible protein binding and unbinding events, NP-protein interactions triggering conformational adaptations especially of the proteins and possibly also of the NPs. Protein-protein interactions can modulate these structural changes and they can also lead to displacement of a protein by a more strongly adhering one. Moreover, protein adsorption can enhance NP–NP interactions, cross-linking NPs and generating agglomerates. It is evident that formation of NP clusters can have detrimental effects for the usefulness of nanomedicines.

A wealth of papers has been published reporting experimental investigations of in-vitro protein corona formation, revealing dependencies on: (1) the physicochemical nature of the NP surface; (2) the unique structural, dynamic and energetic properties of biological macromolecules, especially proteins; and (3) external parameters including temperature, shear forces, protein concentration and pH [23,25,30–32]. The initial adsorption of proteins is a concentration-dependent process and happens on fast (subsecond) time scales in typical biofluids. Subsequently occurring structural adaptations at the interface, involving the proteins and possibly also the NPs, may be much slower and can lead to very strong binding that is irreversible on any relevant time scale. These processes are already difficult to capture in experimental studies with simple model biofluids (protein solutions), but in real biofluids such as blood plasma, there are ~3700 proteins and other small molecules [33], all with different adsorption behaviors, competing for binding sites on the NPs. For example, a rigid yet weakly interacting NP surface may induce only minor structural adaptations in the proteins, reducing the binding free energy but keeping the overall protein intact. Strongly reactive surfaces, in contrast, may induce major protein conformational changes and can cause complete loss of native protein structure [34–36]. Thus, depending on the nature of the NP surface and possibly also of the proteins, the adsorption process can result in very different outcomes, and general conclusions cannot be drawn from a particular example. The notions of ‘soft’ and ‘hard’ coronae, have been introduced to capture the heterogeneity in the strengths of NP–protein interactions [12], as indicated by kinetic or equilibrium parameters. A weakly interacting, soft corona exists as long as the NPs are in equilibrium with the biofluid; it is quickly lost, however, after separation of the NPs from the immersion medium. In contrast, a hard corona refers to a persistent polypeptide layer that withstands extensive rinsing. A real protein corona may include soft and hard components. To rationalize this fact, multilayer models have been proposed, in which the hard corona is surrounded by a soft corona or even ‘protein clouds’ [25,37,38]. However, although the hard/soft dichotomy is practical from the viewpoint of experimental studies, a protein-decorated NP surface may display a wide and continuous spectrum of binding strengths, with weakly and strongly binding sites and everything in between. Notably, this does not necessarily require the presence of multilayer structures.

Strictly speaking, in the presence of widely differing protein adsorption affinities, the complete protein corona can only be studied with in-situ experiments, measurements on NPs while they are being exposed to the biofluid. Whenever the NPs are separated from the biofluid, weakly binding proteins will disassociate. Are more weakly bound proteins irrelevant for eliciting biological responses? Not necessarily – a highly abundant protein with its higher association rate can populate even a weaker binding site to a high degree. The hard component of the corona has been the focus of most experimental investigations in the field, however, because it can be studied more easily due to its quasistatic (slowly evolving) nature. After immersion in biofluids, e.g. blood serum or plasma, protein-coated NPs are separated from the dissolved protein fraction by washing, and the tightly adherent polypeptide layer can be resolubilized under harsh conditions (buffers containing sodium dodecyl sulfate (SDS), dithiothreitol (DTT), urea and 95 °C) and subjected to proteomic analysis by, e.g. gel electrophoresis or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which allows the types and relative abundances of the adsorbed peptide chains to be determined [25]. Proteomics analyses have revealed the protein compositions of the NP coronae and their dependencies on NP properties including size, charge and chemical functionalization [19,25,39,40]. In view of the widely differing binding strengths of proteins to NPs, it is not surprising that details of the separation procedures can have a strong effect on the results [41]. Typically, hundreds of different types of adsorbed polypeptide chains are identified in the adsorption layer, often more than the number of proteins that can fit onto a single NP. Importantly, this observation does not indicate that there are “multiple core-shell structures or higher order Christmas tree-like structures [25],” but rather points to a fundamental statistical problem. Bulk proteomic analysis reveals the protein composition as an average over a NP ensemble [42]. Thus, considering that thousands of proteins in serum or plasma compete for a tiny surface area, offering room for typically a few ten to a few hundred proteins, there will necessarily be large statistical variations in protein composition within the NP ensemble. Therefore, if only a subset of the NP ensemble displaying a particular protein decoration induces a biological response, knowledge of the average composition is not sufficient. Recently, Maurizi and coworkers [40] investigated the significance of a proteomics analysis of serum protein adsorption onto functionalized silica beads (with iron oxide cores). By repeating the experiments three times, they found that 60% of the identified corona proteins were statistically insignificant. Still, although it appears that some technical issues deserve further attention, proteomics analyses are exceedingly powerful for protein corona exploration.
2.2. Temporal evolution of the protein corona

Hard coronae are believed to develop within seconds. In fact, formation of a persistent protein layer has been observed after 30 s immersion of NPs in human blood plasma [19]. However, the structure and composition of the corona may still continue to change for hours or days. There are only a few experimental studies aimed at elucidating structural changes during the long-time evolution of the protein corona [35,37,43–45]. For example, Piella et al. [43] have tracked the red shift of the localized plasmon resonance band of citrate-stabilized Au NPs by, which reports an increase of the refractive index and/or covalent bond formation between the Au NP and the polypeptide chain (especially via cysteine, histidine, or lysine sidechains). Usually, the slow evolution of the protein composition in the corona is explained as a transition from an early non-equilibrium state toward thermodynamic equilibrium, referred to as the ‘Vroman effect’ [46]. The initial composition of the corona is governed by (bimolecular) association rates, which depend on the concentrations of different proteins in a biofluid and, to a lesser extent, on their diffusional properties. Subsequently, the adlayer relaxes to an equilibrium protein composition by exchange of fast-binding proteins-rich coronae with strongly binding ones. Eventually, this simple model does not account for other important effects due to protein-protein and protein-surface interactions [47]. Thus, one should not be too surprised to find experimental results that are at odds with the simple Vroman mechanism [47,48]. Moreover, it appears feasible that approach to equilibrium, which is — according to the physicist R. Feynman — when “all the fast things have happened but the slow things have not” [49], may be completely suppressed under given conditions. For example, a densely grafted layer of proteins may be able to prevent access of more strongly binding proteins to the surface on all relevant experimental time scales, so equilibration will not be observed. This example also shows that it is crucially important to obtain a better understanding of the molecular level structure of the protein corona. The corona around NPs migrating through different compartments of an organism may never reach equilibrium because of the concomitant change of biofluid composition between those compartments. In fact, it has been suggested that the protein corona may keep signatures of its entire history of formation [32].

2.3. Characterization of the protein corona

There are a wide variety of analytical techniques available for protein corona characterization. In general, they offer only limited views, and complementary techniques should be combined to analyze different aspects. Importantly, methods for nanoscale characterization are often sophisticated and require considerable technical skills to produce meaningful results. Extensive descriptions of available techniques, including their advantages and disadvantages, have been compiled in recent reviews [50–57]. In the following, we only briefly mention a selected set of methods relevant to the topics of this review.

Mass spectrometry (MS) is a highly sensitive method that allows the identification of protein types in a mixture [58]. Thus, it has been widely employed to identify different kinds of polypeptide chains adsorbed onto NPs ex situ, i.e. after separation of the NPs from the immersion fluid. Among the MS variants, liquid chromatography-MS (LC-MS) has become the method of choice for qualitative and quantitative determination of the types of proteins in the hard corona [19,59]. This is crucial information but does not reveal how the polypeptide chains are physically arranged on the NP surface, which is indispensable for a detailed understanding of the adsorption layer. Transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) can provide (usually) ex-situ images of NPs coated with adsorbed proteins. For in-situ measurement of NPs immersed in the biofluid, techniques that quantify translational diffusion of NPs have been widely employed, especially dynamic light scattering (DLS) [60], but also fluorescence correlation spectroscopy (FCS) [61], nanoparticle tracking analysis (NTA) [62] and diffusion-ordered nuclear magnetic resonance spectroscopy (DOSY-NMR) [63]. These techniques have in common that they reveal diffusion coefficients in the biofluid, from which hydrodynamic radii can be calculated via the Stokes–Einstein relation. Thus, the protein corona thickness can be determined from the radius increase due to protein adsorption as a single structural parameter, averaged over the whole ensemble. This is a small yet important piece of structural information. Furthermore, there are spectroscopic techniques sensitive to the conformation of the adsorbed proteins. Secondary structure can be analyzed via the circular dichroism (CD) spectrometry of the peptide bond resonance in the ultraviolet (UV) region [35,64], and by Fourier transform infrared (FTIR) spectrometry in the amide vibrational bands of the peptide group [65,66]. Fluorescence spectroscopy of the near-UV–infrared bands reveals their sensitivity to the conformational changes [67].

In recent years, sophisticated new experimental methods have been introduced for the study of structural aspects of protein adsorption onto NPs. Importantly, fluorescence-based optical microscopy techniques [68,69] offer nanometric resolution and/or single molecule sensitivity, and enable the direct visualization of biomolecular adsorption at the level of individual NPs and proteins, so that ensemble averaging is avoided. However, they require fluorescence labeling and thus are preferably applied in simplified model studies. Landes, Link and coworkers [34] have observed the adsorption, unfolding and spreading of albumin on Au nanorods and studied the resulting effects on NP aggregation. In other studies, single molecule localization microscopy of proteins adsorbing onto NPs has been used to analyze the formation and time evolution of the protein corona [70–73]. Förster resonance energy transfer (FRET) spectroscopy is a powerful method for structural studies of the protein corona with nanometric resolution [74]. Raouf et al. [75] used FRET to monitor the conformation of fibronectin on corona-coated Au NPs. Single-molecule sensitivity can also be achieved by label-free approaches that rely on single molecule detection using plasmonic metal nanostructures as a sensing platform [76,77]. For example, surface-enhanced Raman scattering (SERS) uses the huge amplification of Raman scattering that occurs only within a few nanometers from a plasmonic NP and, thus, can be used for in-situ studies [78,79].

The steady growth of computational power is likely to further spawn computer simulations aimed at modelling molecular dynamics at the nano-bio interface [80]. Computer simulations of protein adsorption are still at an early stage but have already provided valuable atomic-scale insight into early steps of protein structural changes upon adsorption to artificial surfaces [80–87]. The ensuing, slower unfolding processes and rearrangements in the adsorption layer, however, have not yet been simulated due to the large systems and long times involved.
3. Factors and processes controlling protein corona formation

3.1. NP properties

A wide variety of NPs have been synthesized from many different materials, giving rise to widely different interfacial properties that govern the reactions with biomolecules (Fig. 1). Much insight into these interactions has already been gained from studies of proteins with bulk surfaces dating back to the 1960s and 1970s, revealing how parameters such as surface free energy, morphology, charge and hydrophobicity affect the protein adsorption process [48,88,89]. By virtue of their size, NPs feature new and distinct properties that have to be taken into account. As NP dimensions approach the atomic scale, NP surface properties become inevitably heterogeneous. Inorganic NPs are mostly small crystals, and their surface properties depend on the exposed crystalline facets, edges and vortices, the presence of surface defects, and the structures can be unstable and prone to reconstruction. For biological applications, the crystalline NP cores are typically coated with ligands bearing, e.g. carboxy, amine or other groups, which endow them with chemical and colloidal stability. A coating with polymeric ligands such as polyethylene glycol (PEG) can provide hydrophilic and protein-repellant properties [90,91]. Polymeric NPs comprise a class of highly versatile NPs that may feature outstanding biodegradability and biocompatibility [92]. In many cases, they consist of a hydrophobic core-forming polymer and a shell-forming polymer (typically charged or hydrophilic), which may be functionalized with targeting and/or protein-repellent moieties. To enhance the overall stability of the NPs, the core and/or shell polymers can be crosslinked. The linkers between core and shell or shell and functional groups can be utilized to introduce responsiveness (i.e. assembly/disassembly) towards internal stimuli such as pH, enzymatic or reductive/oxidative conditions of the bioenvironment or external parameters such as temperature changes, light irradiation, or ultrasound [93,94]. Widely used synthetic polymers are poly(lactic acid) (PLA), poly(-glycolic acid) (PGA), PLA-PGA copolymers, polycaprolactones (PCL), and polyacrylates such as poly(cyanoacrylate) (PCA). Alginate, albumin, and chitosan are among the most widely explored natural polymers [95]. Lipid-based particles, called liposomes, were described as early as 1965 [96] and soon afterwards proposed as drug delivery systems [97,98]. In the meantime, small liposomes with diameters of ~100 nm have evolved into a well-studied class of drug carriers consisting of a lipid bilayer that is primarily composed of amphiphatic phospholipids enclosing an interior aqueous space loaded with a chemotherapeutic agent [99,100]. A PEGylated liposome-based formulation of the antiancancer drug doxorubicin, Doxil, was the first NP-based drug approved for clinical application [101]. Liposomes can be engineered to have a high drug-to-liquid ratio, excellent retention of the encapsulated drug, and a long (>6 h) circulation lifetime, which facilitates their accumulation at target sites such as tumors, where the endothelial layer is ‘leaky’ and allows extravasation of small particles [102].

For biomedical applications, reliable bulk production of highly defined, i.e. monodisperse and colloidal stable NPs with minimal heterogeneity of surface properties (surface chemistry, charge, morphology), is a key requirement [67]. Although atomically precise NPs have been synthesized [103,104], most NPs in use are heterogeneous in size, density of surface functionalizing groups and other properties [105,106]. In addition, precise characterization of the physicochemical properties of NPs at the nanoscale is far from being a routine procedure. To this end, many techniques have been made available [56], but their application can be challenging and require considerable experience [55]. New analytical methods are under continuous development [34,61,107], and progress in this area will be essential to further advance the field.

3.2. Protein properties

Proteins are linear polymer chains made from 20 different amino acids that fold (often spontaneously) into specific, bioactive 3D structures in the proper environment. They are flexible macromolecules with a large inherent structural complexity [108], which is often essential for their biological function. Proteins are thermodynamically only marginally stable; the stabilization free energy of a small protein consisting of several thousand atoms typically amounts to the equivalent of only a few hydrogen bonds [109]. Moreover, thermodynamic stability can differ considerably among proteins, affecting their structural responses onto artificial surfaces [110,111]. In globular proteins, hydrophobic and hydrophilic amino acid residues are preferentially located in the interior and on the surface, respectively. In fact, sequestration of hydrophobic residues into the core is a key driving force for folding. The presence of polar (neutral and positively or negatively charged) residues on the protein surface ensures their high colloidal stability. The charge state of a particular protonatable residue depends on the pH and its local electrostatic environment. The overall protein charge and its charge distribution can be changed by pH variation within a considerable range, outside of which the protein unfolds. Charge modification can strongly affect NP-protein and protein-protein interactions [112,113], and can be exploited for the design of protein adsorption layers. Larger proteins typically consist of several domains, individually folding subunits that are arranged like beads on a string. Thus, in a multidomain protein such as an immunoglobulin (Ig), a particular domain may unfold due to surface interactions, while others still retain their native 3D architecture and functional properties.

3.3. NP-protein interactions

In general, NP-protein interactions can cause modifications of the physicochemical properties of both NP and protein. Structural changes (reconstruction) of small crystalline NPs or removal of solubilizing ligands (e.g. citrate on Au NPs, surfactants on polymer NPs) can occur upon protein adsorption, and charge compensation due to protein adsorption can compromise the colloidal stability of electrostatically stabilized NPs. Proteins inevitably change their structure upon adsorption if the internal forces that stabilize the 3D structure upon adsorption if the internal forces that stabilize the 3D architecture are weaker than the interactions with the NP surface. Then, the polypeptide chain abandons its native fold in order to establish optimal interactions with the NP surface. These structural adaptations are exceedingly complex and can completely change the nature of the polypeptides. Notably, partial or complete unfolding on a NP surface can expose the hydrophilic core of a protein globule and render the unfolded polypeptide chain much less hydrophilic than the original folded species, compromising the colloidal stability in aqueous solutions.

Due to their complexity, the structural adaptations induced by NP–protein interactions cannot be predicted. They depend on the physical nature of the NPs and proteins and, to a great extent, on the external conditions (temperature, pH, concentration, flow conditions) [23,25,30–32]. In fact, the notion of a protein corona appears unifying when referring to an assemblage of completely unfolded polypeptide chains on a NP surface. This lack of detailed information about the molecular-level structure of the adsorption layer is one of the key impediments to an in-depth understanding of the protein corona; colorful schematic depictions of the adsorption layer (Fig. 1)[12,25,27,38,114–116] are no substitutes for
profound knowledge based on experimental facts. Spectroscopic experiments such as circular dichroism (CD) spectrometry and Fourier transform infrared (FTIR) spectroscopy reveal secondary structure alterations upon protein adsorption, but this is a very limited piece of information. NP sizing experiments, based on the precise quantification of NP translational diffusion, are conceptually simple and yield the corona thickness as a simple yet important structural parameter. Model experiments with simple biofluids such as solutions of purified proteins and well characterized NPs can provide general insight into the adsorption process.

3.3.1. In-situ model studies of corona formation on hydrophilic NPs

To explore the structure of the protein corona, we have used particle sizing experiments based on FCS to measure the NP size increase (with subnanometer resolution) due to protein adsorption while small (5–10 nm) and fluorescent NPs were immersed in phosphate-buffered saline (PBS) solutions containing a single type of protein. As a representative example, Fig. 2 shows data of human serum albumin (HSA) adsorption onto CdSe/ZnS quantum dots (QDs) with systematically varied surface ligands, negatively charged dihydrolipoic acid (DHLA), zwitterionic L-penicillamine (DPA), and PEG end-functionalized with cationic amino groups [117]. Each data point represents a single experiment, in which the NP size was measured in the HSA (in PBS) immersion fluid of the indicated concentration (10 min incubation). For PEG-coated NPs, no change of the NP size is visible over the entire HSA concentration range, attesting to the protein-repellant effect of PEG. For DHLA- and DPA-coated NPs, the size increases smoothly up to a saturation level, in accord with an equation based on the Langmuir adsorption isotherm or, more generally, a Hill isotherm, which includes an additional steepness parameter to capture binding cooperativity (protein-protein interactions) [118,119]. This functional form is theoretically based on a simple adsorption/desorption equilibrium. Reversible protein adsorption was tested with concentration jump experiments. Like other relaxation methods such as temperature or pressure jump, this approach can reveal whether a dynamic equilibrium exists and allows, in principle, the kinetic coefficients to be determined [120]. The protein concentration was changed in situ (up or down), resulting in a concomitant RH change as predicted by the isotherm, proving reversibility of protein adsorption. Needless to say that those so-called ‘soft’ coronae can only be studied in situ; every attempt to isolate the protein-enshrouded NP from the solution will quickly lead to protein desorption. We have found Langmuir/Hill isotherms without a single exception for a wide selection of serum proteins adsorbing onto a range of small model NPs with diverse surface ligands [112,121–123].

Quantitative analysis of the protein concentration dependence of the hydrodynamic radius, RH, yields the corona thickness, ΔRH, a midpoint protein concentration, K_0, which is a measure of the binding affinity, a Hill parameter n, and an estimate of the maximum number of protein molecules, N_{max}, that fit into the volume of the protein adlayer [118]. With increasing HSA

Fig. 2. Protein adsorption onto (a, b, c) DHLA-QDs, (d, e, f) DPA-QDs, and (g, h, i) PEG-QDs immersed in human serum and HSA dissolved in PBS (10 min incubation). (a, d, g) Schematic depictions of QDs (green spheres) with their coating layers (gray) and the serum protein coronae. (b, e, h) Hydrodynamic radii of QDs, determined by using FCS, are plotted as functions of serum (black) and HSA (gray) concentration, respectively; solid lines represent fits based on the Hill equation. Error bars indicate standard deviations from three independent measurements. Green and purple symbols mark the concentrations selected for the dilution experiments. (c, f, i) Dilution experiments after preparing a full (purple) or half (green) corona by immersion in serum solution of the marked concentrations. The QD sizes were measured before (at time 0) and after quantitative dilution. Dilution factors: (c) DHLA-QDs, 32-fold (purple), 4-fold (green); (f) DPA-QDs, 23-fold (purple), 4-fold (green); (i) PEG-QDs, 64-fold (purple). Error bars denote standard deviations from three independent measurements. Adapted from Ref. [117].
to the NPs, possibly due to unfolding. There could also be cooperatively different properties, some of which may adhere very tightly explored. Obviously, serum contains a vast number of proteins with reasons for the strong binding of proteins from serum are yet to be possible to separate the protein-coated NPs from the serum to although the adsorption curve followed an equilibrium binding nanometer thickness, compatible with monolayers. Importantly, however, not governed by the net charges of the NPs and the proteins but rather by electrostatic interactions between localized charged patches on the protein and NP surfaces [121, 122]. Whereas other studies reported multilayer coronae on relatively 'flat' nanomaterials, e.g. nanorods or larger NPs [43, 115, 124 – 127]. Comparison of the binding affinities, quantified by K_0, of different proteins to model NPs revealed the important role of charge interactions on the protein surfaces mediating the interaction. The K_0 values were widely different (0.02 – 37 μM [211]), depending on the particular NP-protein combination. They were, however, not governed by the net charges of the NPs and the proteins but rather by electrostatic interactions between localized charged patches on the protein and NP surfaces [121, 122]. At typical ionic strengths of biological media (~150 mM), Debye screening is efficient, limiting charge interactions to distances <1 nm, which explains why NP-protein association is mediated by local charge patterns on the NPs and proteins rather than overall charges. Experiments with hydrophilic NPs and a complex biofluid, i.e. blood serum, revealed similarities but also important differences to the model studies described above (Fig. 2). Again, the concentration dependence followed binding isotherms, saturating at a size increase of a few nanometers, suggesting formation of a protein monolayer. Notably, there are apparently proteins in blood serum that, unlike HSA, are able to adsorb onto PEGylated NPs (Fig. 2g and h). For the examples shown, we observed thin coronae of only a few nanometer thickness, compatible with monolayers. Importantly, although the adsorption curve followed an equilibrium binding isotherm, the serum proteins adsorbed irreversibly, as confirmed by concentration jump experiments (Fig. 2c, f, i). Thus, it was possible to separate the protein-coated NPs from the serum to identify the adsorbed proteins by mass spectrometry. The exact reasons for the strong binding of proteins from serum are yet to be explored. Obviously, serum contains a vast number of proteins with widely different properties, some of which may adhere very tightly to the NPs, possibly due to unfolding. There could also be cooperative interactions between different types of proteins (including opsonization reactions) that can cause these effects.

3.3.2. In-situ studies of corona formation on large, hydrophobic NPs
Recently, we extended our protein adsorption studies to large PS-OSO_3H NPs (R_H ~50 nm) [72]. Polystyrene (PS) NPs, sparsely functionalized with sulfate (PS-OSO_3H NPs) for electrostatic colloidal stabilization in aqueous solution, have extremely hydrophobic surfaces covered with benzene moieties and are popular model NPs for nano-bio studies [14]. Their non-polar surfaces adsorb proteins more strongly than polar surfaces and tend to severely destabilize and unfold proteins, which leads to effective NP-protein and protein-protein interactions. In addition, the strongly acidic sulfate groups may also play a role in the adsorption process [128]. For in-situ NP sizing experiments, we used DLS rather than FCS because the strong scattering contribution from large NPs is well separable from the one of the much smaller proteins. For small NPs, this is not the case and FCS offers clear advantages if the analysis is based only on the fluorescence of the NPs, as the (non-fluorescent) proteins are invisible. We selected TF and HSA for our studies onto these NPs because they had already been widely employed in other protein adsorption studies.

Whereas the concentration dependence of TF adsorption onto carboxyl-functionalized, hydrophilic PS NPs (PS-COOH NPs, R_H ~35 nm) showed the usual adsorption isotherm discussed in the previous subsection, a completely different behavior was found with PS-OSO_3H NPs (in regular phosphate buffered saline (PBS) solvent, Fig. 3a and b) [72]. The observed size changes were all due to irreversible adsorption of TF to the hydrophobic surfaces, as confirmed by concentration jump experiments (Fig. 3a and b). In very dilute (subnanomolar) TF solutions, the overall size of the NPs (0.7 nM) increased moderately with increasing TF concentration. At intermediate concentrations, up to 1.6 μM large particles were present. Total internal reflection fluorescence (TIRF) microscopy (Fig. 3a, insets) revealed that these particles are NP agglomerates. This tendency was already noticeable at very low TF concentrations, corresponding to a NP:protein ratio of roughly one to one. Consequently, already a few Tf molecules (or even single ones) can act as glue in connecting the PS NPs. This protein-induced stickiness can – in our view – only result from denaturation of the proteins and subsequent adsorption of their hydrophobic moieties to two NPs. These findings are in nice agreement with results by Link and coworkers on protein-mediated crosslinking of Au nanorods [34]. However, T4 lysozyme was reported to trigger Au NP aggregation while maintaining its native structure and activity [129]. Thus, protein-induced aggregation may occur without denaturation, depending on the thermodynamic stability and structural properties of the specific protein.

At 0.5 μM TF, there is a remarkably sharp transition from agglomeration to complete passivation against agglomeration (Fig. 3a). Apparently, grafting the adlayer with a sufficiently high protein concentration generates non-sticky surfaces, suggesting that protein-protein interactions acting during adlayer formation are responsible for the effect. As we argue in the next subsection, conformational changes may be restricted under these conditions so that the adsorbed proteins expose more hydrophilic surfaces that prevent agglomeration. Above the passivation transition and up to the highest TF concentration (125 μM), there were individual NPs, enshrouded by a protein corona of ~10 nm thickness, which is compatible with a TF monolayer [72]. Remarkably, despite the constant thickness, the number of adsorbed TF proteins increased between 0.5 and 62.5 μM from 175 to 985 and thus more than fivefold, as quantified by using dual-color single molecule localization microscopy. Therefore, the protein corona is by no means very dense when prepared from a protein solution with a concentration of ~1 μM. The constant corona thickness (Fig. 3a) in the presence of largely varying protein numbers suggests that solvent can form a major part of the protein layer. In fact, swelling of proteins due to incorporation of solvent molecules is a typical phenomenon observed in protein denaturation studies [130–132]. Apparently, in contrast to studies on reversibly adsorbed proteins, the corona thickness does not scale with the number of adsorbed proteins for these strongly denaturing NP surfaces.

The electrostatic stabilization of the PS-OSO_3H NPs allows variation of NP-NP repulsion by changing the ionic strength of the solvent. Indeed, NP-NP repulsion is sufficiently high under reduced charge screening, i.e. in 20-fold diluted PBS (0.05 × PBS, pH 7.4), to completely suppress agglomeration (Fig. 3c and d). The TF concentration dependence of R_H can again precisely be modeled by a binding isotherm. However, decreasing the TF concentration in situ...
showed that the proteins cannot desorb from the NP surface (Fig. 3c). Increasing the Tf concentration yielded an increase in corona thickness, indicating that still more proteins can be loaded onto the NP surface. Finally, there was no time evolution of the corona thickness after the concentration jumps on time scales from a few minutes to a few days (Fig. 3e and f).

All measurements discussed here have in common that the observed coronae were only a few nanometers thick, which is compatible with formation of protein monolayers. In view of the fact that globular proteins, especially blood proteins, are colloidaly very stable, we are convinced that formation of thin, essentially monolayer coronae on NPs is the rule [72,133]. By contrast, protein coronae of several 10 nm have been reported by DLS experiments and interpreted as multiple layers [43,126,134]. We note that large increases of the average hydrodynamic radius can also result from a small fraction of NP agglomerates forming upon protein adsorption [124]. For biomedical applications, colloidal stability of NPs in biofluids is essential, and systematic studies of the effect are still scarce [135,136]. For certain proteins, however, major structural changes as those occurring during adsorption onto NPs may trigger protein-protein association (e.g. via hydrophobic epitopes that become exposed due to denaturation) and fibrillation [137,138]. Furthermore, components of the immune system (antibodies, complement components) contained in blood serum or plasma can attach themselves covalently or non-covalently to the exposed protein surface of the corona and, thereby, form an additional layer.

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Fig. 3. Probing protein corona formation on PS-O5SO3H NPs (Rg = 50 nm, 0.7 nM) by DLS. All measurements were performed after 10 min incubation. (a, b) Corona formation in PBS. Black symbols: Tf concentration dependence of RH. The area under the curve is shown in gray. Colored symbols: RH measured before (closed symbols) and after 8-fold (open symbols) (a) decrease and (b) increase of the Tf concentration (see arrows). Initial concentrations: 8 nM (blue), 61 nM (red), 0.49 μM (orange). Insets in (a): TIRF images of bare NPs and after immersion in 61 nM and 0.49 μM Tf. (c–f) Corona formation in 0.05 × PBS. (c, d) Blue symbols: Tf concentration dependence of RH. The area under the curve is shown in blue. Colored symbols: RH measured before (closed symbols) and after (open symbols) 64-fold (c) decrease and (d) increase of the Tf concentration, as indicated by the solid arrows. Initial concentrations: 15.3 nM (magenta), 980 nM (green), 62.5 μM (purple). Dashed arrows in (d) indicate a 64-fold decrease after the initial 64-fold increase. (e, f) RH in 0.05 × PBS, measured as a function of time before (t = 0) and after (e) decreasing and (f) increasing the Tf concentration 64-fold. Error bars indicate standard deviations from at least three independent measurements. Lines mark the average RH. Adapted from Ref. [72].
3.3.3. Mechanistic aspects of protein corona formation and implications for NP engineering

There is an important question raised by the data in Figs. 2 and 3. Why do we measure binding isotherms (Fig. 3c and d), which are based on adsorption/desorption equilibria, even if protein adsorption is irreversible? A simple mechanistic model that involves two sequential steps may help to give an explanation. NPs immersed in concentrated protein solutions such as serum are incessantly bombarded with protein molecules. There are multiple protein binding sites on the NPs with rather heterogeneous binding strengths [36,139]. In a first step, a properly folded protein engages a weakly and reversibly bound complex with the NP, characterized by relatively fast adsorption and desorption kinetics. In a second step, structural adjustments occur at the interface that lead to persistent attachment of the protein to the NP surface. These adaptations are slow, especially if they involve major conformational rearrangements of the polypeptide chain (local or global denaturation), and they can only take place if the protein remains at the binding site for a significant fraction of time. Upon NP immersion in highly dilute protein solutions, only a few binding sites (or just a single one) on the NP may be appreciably populated for subsequent conformational changes to occur. Under these conditions, protein-protein interactions are absent and the loosely adsorbed protein may fully denature, flatten and spread over a large area [140], thereby establishing strong, irreversible interactions with the NP surface and also occluding binding sites for other proteins (Fig. 4). With increasing protein concentration, successively more binding sites become populated and permanently adsorb proteins, so the binding curve rises continuously according to a binding isotherm. Interactions of proteins with their simultaneously present neighbors become more important that can restrict structural changes and modify the overall binding strengths. This cooperativity affects the steepness of the binding curve. At high protein concentrations, the extent of protein spreading is severely restricted by neighboring proteins. We note that, in this regard, the adsorption of polypeptide chains shares analogies with the well-studied grafting of end-functionalized PEG chains to solid substrates [141,142]. Importantly, the final conformational state of the adsorbed proteins and, therefore, the structure of the protein corona depends on the balance between the protein’s internal stabilization energy on the one hand and the strengths of protein–NP and protein–protein interactions on the other hand. Indeed, the sharp agglomeration transition (Fig. 3a and b) suggests that dense grafting of proteins to NP surfaces (at high concentration) limits the extent of conformational change. Taken together, the “binding isotherms” observed for irreversible adsorption thus reflect the population of transient binding sites from which persistent adsorption occurs.

The dependence of the corona structure on the concentration of biomolecules in the fluid has important implications for engineering strategies of NPs for biomedical applications that utilize pre-adsorption of proteins for surface passivation or functionalization. If the adsorption layer forms at high protein concentration (e.g. in serum), dense grafting of the proteins will have a stabilizing effect, and the solvent-exposed protein shell is likely to retain native-like structure, and further proteins are not likely to adhere under physiological conditions due to their colloidal stability. If, however, a hard layer is formed at low protein concentration, polypeptide chains spread on the NP surface and expose hydrophobic moieties, and other proteins may possibly associate with this partially decorated hydrophobic surface.

4. Nanoparticle engineering for control of the protein corona

To successfully engineer NPs for biomedicine, researchers have to anticipate the ensuing interactions within the biological system, which modify the NP surface and determine its cellular uptake, intracellular trafficking, pharmacokinetics, biodistribution and toxicity [24]. NPs functionalized with targeting ligands such as antibodies, peptides, sugars and proteins might lose their targeting capability when a protein corona forms on their surface and occludes those ligands [143]. Moreover, the immune system features potent molecular tools that can modify surfaces that are recognized as non-self, and binding of opsonins (antibodies, complement components) to the corona can significantly enhance recognition and clearance by the mononuclear phagocyte system [144–146].

A straightforward strategy to cope with the complexity of NP interactions within the living system appears to be suppression of protein corona formation by using zwitterionic surface functionalization or dense decoration with hydrophilic polymers such as PEG brushes or carbohydrates [147]. The ‘stealth effect’ of the NPs toward the immune system gives rise to extended circulation times in the bloodstream that improves the efficacy of nanomedicines. Considerable efforts have been directed toward the design of such highly hydrophilic coatings with low affinity for any type of protein [148–151]. However, experiments have shown that complete resistance to protein adsorption seems to be difficult if not impossible to achieve [91]. Moreover, the additional decoration of these NPs with targeting moieties is likely to enhance immune recognition.

Alternatively, if protein adsorption cannot be avoided by any means, one may devise strategies to control the amounts, types and orientations of the proteins (and other biomolecules [152–155]) adsorbing from the biofluid [37,39,44,156–162]. Thus, the protein corona is not considered an obstacle, but rather an opportunity to achieve selective biological responses by specific preparation, e.g. by pre-adsorbing specific proteins or by using NP surface coatings that preferentially bind certain proteins from the biological environment. For example, pre-adsorption of opsonins as part of the corona can enhance recognition and clearance by phagocytes.

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**Fig. 4.** Mechanistic scheme of protein adsorption onto charge-stabilized, hydrophobic NPs. (I) Upon initial contact, the protein (represented by an oval with hydrophobic core (blue) and hydrophilic (red) shell) and the NP (assumed to have a hydrophobic surface (blue) and charged functionalized groups (red)) form a weakly and reversibly bound complex with fast protein adsorption and desorption. (II) The protein residing on the NP may change its conformation and (III) partially unfold to expose its hydrophobic core. Electronic repulsion between neighboring proteins may affect the affinity towards the NP. (IV) Strong hydrophobic interactions establish an irreversible complex. Bound water (hydration shells), which has to be removed when the NP-protein interface forms, has not been included in this scheme. Adapted from Ref. [72].
4.1. Opsonization of adsorbed proteins by the complement system

Cells of the immune system continuously scan the organism for foreign materials in order to recognize pathogens and to remove them from the organism. Indeed, NPs become internalized by blood leukocytes and tissue macrophages upon intravenous injection. The complement system, an essential component of the innate immune system, consists of more than 30 plasma proteins and assists in the recognition of NPs by phagocytizing immune cells. C3, a protein present in human plasma at a concentration of ~5 mg/mL, plays a key role in this process. From the three complement activation pathways, only the alternative pathway is relevant here. It involves spontaneous hydrolytic cleavage of C3 into two fragments, C3a and C3b (more precisely, a C3b variant denoted C3b(H2O)), which occurs at low rate (0.005%/min) so that, at any given time, ~0.5% of C3 molecules are present in the fragmented form. C3b binds another protein, namely fragment Bb of complement factor B, from the serum to form the alternative pathway C3 convertase, which is stabilized by properdin, an essential protein factor of complement activation through the alternative pathway. This enzyme catalyzes hydrolysis of further C3 molecules into C3a and C3b, the latter of which contains a highly reactive thioester group through which it can covalently bind to nucleophilic groups, e.g. amines or hydroxyls, on the surface of an activating moiety. C3 and C3b are potent “eat me” signals (opsonins) for tissue macrophages and blood leukocytes; therefore, a protein-coated NP presenting C3 to complement receptors on leukocytes will enhance phagocytosis.

For drug delivery, hitchhiking of NPs in these cells to target sites could be a possible way to overcome biological barriers en route to the target. Alternatively, NP surfaces suppressing C3 or C3b could be a possible way to control binding of antibodies onto NPs. On the one hand, pre-coating with antibodies presenting their Fc fragment on the surface can enhance targeting of leukocytes via enhanced C3 opsonization. On the other hand, to create NPs that evade immune system recognition and, thereby, removal from the system via phagocytosis, binding of antibodies from bodily fluids to the biomolecular corona must be suppressed, for example, by protein-repellent surfaces (zwitterionic coatings or stealth polymers), or by pre-coating with dysopsonins.

4.2. Corona engineering to prevent immunoglobulin adsorption

About 20% of the blood proteome are Ig molecules; among these, IgG is the most prominent isotype. IgG levels depend on the health status of a patient and can be elevated due to infection, inflammation, autoimmunity or malignancy. Thus, intravenously injected NPs can encounter varying IgG concentrations in the circulation, which can potentially lead to varied biological responses. As IgG molecules adsorbed to NPs play a key role in the opsonization of NPs by the complement system, an important strategy to enhance the lifetime of NPs in the system is to engineer their surfaces so as to reduce IgG adsorption, thereby enhancing the probability that a drug delivering NP reaches its tumor target.

In their recent study of adsorption of human plasma proteins onto PEG- and poly(ethylene glycol) (PEE)-modified PS NPs, Schöttler et al. [39] found that these polymer coatings change the protein composition (with respect to aminated NPs) toward apolipoproteins, predominantly clusterin (apolipoprotein J). NP uptake by RAW264.7 macrophages was strongly suppressed if the NPs were first immersed in human plasma, indicating that an additional component from plasma was required to convey a stealth effect to the NP surfaces. Clusterin was identified as being mainly responsible for the strongly dysopsonizing effect.
To further elucidate the mechanism through which clusterin prevents macrophage uptake, Prozeller et al. [176] compared the protein corona formed by 1-h immersion of (non-covalently) PEGylated PS NPs and hydroxyethyl starch nanocapsules (HES-NCs) with and without prior coating with clusterin in fivefold diluted human plasma solution. Moreover, they studied the effect of IgG-enrichment (by a factor of 2) in the solution (Fig. 6a). The protein composition of the hard corona formed on the NP surfaces was analyzed via liquid LC-MS. For incubation with regular plasma, clusterin (38.8%) and apoA-I (19.4%) (PS-NPs), and clusterin (43.2%) and apoE (8.4%) (HES-NCs) were the two predominant proteins in the coronae. Remarkably, doubling of the IgG concentration led to an enormous increase of the IgG fraction in the corona by a factor of 40 for both NP types (Fig. 6b). Similar observations were made for other nanomaterials, suggesting a general effect. Moreover, the total mass of adsorbed protein almost doubled upon immersion in IgG-enriched plasma. Pre-coating with clusterin (10 min, ~250 nM in PBS) before immersion in the IgG-enriched plasma, however, again yielded a protein composition similar to the one obtained with regular plasma (Fig. 6b).

Comparison of the three different incubation procedures shows the crucial effect of clusterin in suppressing IgG in the protein corona. In normal plasma, IgG is much more abundant (~16 μM in fivefold diluted plasma) than clusterin (~250 nM). In the protein corona formed around these polymeric NPs, however, clusterin is enormously enriched with respect to IgG. Remarkably, the clusterin effect is not effective in twofold IgG-enriched plasma but can be largely restored by pre-incubation with clusterin. Apparently, the greater IgG concentration largely blocks clusterin access to the surface. This is an interesting effect; its strong concentration dependence is reminiscent of the sharp transition in the agglomeration experiments with hydrophobic PS NPs (see subsection 3.3.2.), which are also related to blocking of the surface by dense grafting at high protein concentration.

Uptake into murine and human macrophages was markedly increased upon NP incubation with IgG-enriched plasma, demonstrating the opsonizing effect of IgG molecules [176]. It remains to be uncovered whether complement activation can also amplify the phagocytosis in response to these NPs, as discussed in the previous subsection.

In conclusion, modest variations in the IgG fraction of the biofluid have been shown to induce drastic changes of the protein corona composition. Clusterin enrichment in the protein corona may be a powerful means to reduce the influence of varying blood compositions between individuals on the outcome of nano-bio interactions. In protein corona engineering, this objective can be reached either by pre-coating with clusterin or by coating the NPs with surfactants that preferentially adsorb this protein [176].

4.3. Corona design by using specific protein binding

NP surface decoration with antibodies that recognize epitopes specific to (or at least more abundant on) target cells, or with ligands that are recognized by receptors overexpressed on target cells, is a conceptually simple strategy for engineering NPs for delivery of drugs to specific sites, e.g. tumors. Often, however, targeting moieties are not presented in the correct orientation at the surface or may become covered by proteins adsorbing onto the NP, so that targeting is dysfunctional [143]. For example, Tf is an important protein for iron homeostasis of a cell and abundant in blood. It binds to the Tf receptor on the cell membrane, which triggers its cellular uptake to deliver iron ions. Still, NP uptake by cells was strongly suppressed by Tf incubation [177], and the targeting specificity of Tf tethered to silica NPs was completely lost upon serum exposure due interference by the additional protein corona [143]. Therefore, great care has to be taken that targeting epitopes are presented on the NP surface (in the presence of a protein corona!) in such a way that their cognizant receptors can efficiently interact with them.

Tonigold et al. [159] explored how pre-adsorption of NPs with antibodies can be used to endow them with targeting moieties, ensuring their accessibility, correct orientation and flexibility to enable interactions with the cell surface in the presence of protein adsorption. They attached similar amounts of functional anti-CD63
antibodies to carboxylated PS NPs, either by simple physisorption or by covalent coupling via amines on the antibody surface with a random orientation. NPs with chemisorbed antibodies showed less internalization by monocyte-derived dendritic cells, suggesting that their randomly oriented antigen binding domains were only partially recognized by CD63 proteins in the cell membrane. Cellular uptake of NPs with physisorbed antibodies was unaffected by protein adsorption. The authors suggested that selective unfolding of the CH2 region of the antibody's Fc fragment can lead to the observed strong attachment to the NPs in an orientation presenting both antigen binding (Fab) fragments on the hydrophobic NP surface. From these results, we can conclude that the serum protein corona cannot be thicker than the extension of a Fab fragment (~7 nm), in agreement with the experiments discussed in subsection 3.3.1. The pre-adsorption strategies used by Tonigold et al. [159] may be simple yet effective. More sophisticated strategies for covalent attachment in specific orientations involve genetically engineered antibodies (single-chain Fv or Fab fragments) [178] or affibodies (Afbs), engineered antibody mimetics, as exemplified in the following subsection.

### 4.4. Corona design using engineered proteins

Molecular biology offers an elaborate toolbox, from which proteins can be tailor-made to form a protein shroud with precise design of the surface moieties. A simple design for this purpose is a fusion protein featuring two domains, one having a high affinity for functional groups on the NP surface, and the other one having a high affinity for targeting epitopes. If these proteins are pre-adsorbed under suitable conditions, they will form a shell of folded proteins that may even be recognized as "self" by the immune system.

Oh et al. [179] presented an example of how such an engineered protein corona around a NP can provide excellent targeting ability. Glutathione (GSH)-functionalized mesoporous silica nanoparticles (MSNs), referred to as GSH-MSNs, were coated with a fusion protein consisting of glutathione-S-transferase (GST) and an HER2-binding antibody (HER2-Afb). The GST domain binds GSH with high affinity and thus serves to attach the protein to the MSN. The HER2-Afb binds to the tyrosine kinase receptor HER2 with high affinity [180]. HER2 is a member of the human epidermal growth factor receptor family and is overexpressed in tumor cells of several types of cancer, including an aggressive form of breast cancer [181]. Therefore, this domain serves as a device to target cancer cells.

MSNs with the GST-HER2-Afb protein decoration (protein corona shield nanoparticles (PCSNs)) and GSH-MSNs and PEG-modified MSNs (PEG-MSNs) as controls were incubated for 1, 2, and 4 h in 55% fetal bovine serum (FBS). Then, unbound protein was removed by centrifugation, and the compositions of the hard protein coronae were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Compared to GSH-MSNs and PEG-MSNs, PCSNs showed ~15-fold lower adsorption of serum proteins, regardless of the incubation time. In the PCSN corona, the relative concentration of apolipoproteins was higher than in the control samples, while the amount of proteins involved in immune responses was lower, suggesting longer circulation times of the PCSNs.

To evaluate whether the PCSNs can evade internalization by phagocytic cells, the murine macrophage-like cell line RAW264.7 was exposed to DiI-loaded PCSNs and PEG-MSNs for 6 h. NPs were pre-incubated with 55% FBS for 4 h. Confocal microscopy showed more than 10-fold lower cellular uptake of PCSNs compared to PEG-MSNs, demonstrating the stealth effect of the protein shield (Fig. 7a). To examine cell-specific targeting via the HER2-afibody, HER2-receptor-overexpressing SK-BR3 cancer cells and receptor-free HEK293T cells were exposed to DiI-loaded PCNs for 4 h. Uptake was only observed for the SK-BR3 target cells, indicating that the targeting functionality was retained.

To test the PCSNs on an in-vivo tumor model, PCNs and PEG-MSNs as control were injected into the tail vein of nude mice bearing SK-BR3 cell xenografts. Already 30 min after injection, in-vivo live fluorescence imaging revealed a 1.8-fold higher emission intensity in tumor sites of the PCSN-treated mice compared to mice treated with PEG-MSNs. The PCSN emission persisted for 24 h, while the signal of the PEG-MSNs gradually decreased. In addition, the PCSN fluorescence signal in the tumor was seven-fold higher than that in reticuloendothelial organs, while there was no difference for the control particles (Fig. 7b). Therefore, the engineered protein corona enabled the NPs to evade the immune system due to the reduced protein adsorption and to accumulate in the target...
tumor. As the strategy used in this work is very versatile, we anticipate that it will be extended to other affinity tags and antibody constructs in the future.

5. *In-vivo* experiments to study corona formation

Recent years have seen a vast number of *in-vitro* studies aimed at characterizing protein corona formation on NPs, often using cell-culture media, serum, plasma or whole blood. We note that the source of the biofluid (e.g. mouse or human) and even the process to isolate a biofluid for *in-vitro* studies (e.g. blood with or without ethylene diamine tetra-acetic acid (EDTA), serum or plasma) may cause decisive differences in the composition of the protein corona around NPs [15,182,183]. The expectation within the field is that these huge compilations of data will eventually lead to a profound mechanistic understanding of protein adsorption that allows researchers to predict from *in-vitro* studies what happens in the real biological environment. This lack of *in-vitro* methods to predict *in-vivo* consequences has been identified as one of the key obstacles towards efficient translation of biomedical NPs from the bench to the clinic [184]. The problem is rooted in the bewildering complexity that an organism offers to internalized NPs.

To illustrate the complexity, there are different ways in which NPs may enter the human body, via injection into the bloodstream, via inhalation through the lungs, through contact with the skin, or through the gastrointestinal tract after ingestion. These entry routes

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**Fig. 7.** (a) The engineered protein corona shield (PCS) retains the NC targeting ability by inhibiting blood protein adsorption. (b) Fluorescence images of organs and tumors 48 h after intravenous injection of PEG-MSNs and PCSNs and biodistribution of injected NCs in animals with SK-BR3 tumor xenografts (obtained from fluorescence intensity analysis). Adapted with permission from Ref. [179].
offer distinctly different environments to the NPs, with different amounts and types of biomolecules, different pH and ionic strengths [185]. Upon exposure to biofluids, NPs will immediately be coated with proteins and, on its journey through the body, the corona will evolve due to its own slow dynamics and the ever-changing physiological environment, e.g., when a NP leaves the capillary to transgress the blood-brain barrier [186,187]. NPs in the body experience dynamic flow, which introduced shear forces that are typically not present in in-vitro experiments [174,188,189]. This dynamic nature of the environment must be taken into account to be able to reliably predict the outcome of NP-protein interactions [190]. Even the health status of an individual may modulate the protein corona ('personalized protein corona' [191]) and the efficacy of nanomedicines [192], as already discussed in subsection 4.2.

A crucial first step toward the prediction of in-vivo corona formation is to characterize them and to compare them with those formed in vitro. For example, NPs are injected into the tail vein of a mouse and subsequently recovered from blood withdrawn by cardiac puncture at specific time points after administration [193,194]. During the following ex-situ proteomics analysis, only persistently binding proteins will be retained; loosely bound biomolecules will be lost, although they may be important for the in-vivo fate of the NPs. Thus, there is a need for techniques enabling in-situ analysis of the protein composition [195], as is also the case for in-vitro studies. At this point in time, in-vivo studies are still scarce. In the following, we discuss recent progress in the in-vivo application of NPs in the context of protein adsorption.

5.1. Comparing in-vitro vs. in-vivo corona formation

Kostarelos and coworkers [196] found significant differences in the relative abundances of proteins in the coronae formed on PEGylated liposome NPs (diameter ~ 120 nm, with and without attached antibodies for targeting) that were either incubated for 10 min in CD-1 mouse plasma or recovered from CD-1 mice 10 min after intravenous injection, with a greater variety of protein species in the in-vivo corona. In follow-up work focusing on the time evolution of the corona [197], the total amount of adsorbed proteins (determined by a colorimetric assay) remained constant over 3 h, whereas the relative amounts of different proteins changed considerably, reflecting the dynamic nature of the protein corona (Fig. 8).

Wang et al. [198] compared the uptake of dextran-coated SPIO NWs in vitro (by peritoneal macrophages) and in vivo (by leukocytes in mice). They reported that SPIO NWs that were crosslinked with epichlorohydrin to reduce opsonization by C3 showed little uptake by mouse peritoneal macrophages in vitro. Additional functionalization with PEG antibodies had essentially no effect on the in vitro uptake but increased the level of complement-dependent in-vivo uptake by leukocytes in mice. They also showed that using fresh mouse lepirdzin plasma instead of serum improved the correlation between the in-vitro and in-vivo outcome.

As protein corona formation may affect the targeting ability of engineered NPs (see section 4.3.), Zhang et al. [160] incubated PS NPs functionalized with Tf receptor targeting ligands (DT7 and LT7 peptides, TF) for 1 h in human plasma and, for comparison, collected plasma samples from mice 10 min after intravenous injection of the NPs. Uptake experiments in HepG2 cells overexpressing TF receptors showed that the targeting capacity was lost after incubation with plasma in vitro, whereas it was partially retained after in-vivo corona formation.

5.2. In vivo corona formation

Corbo et al. [199] compared the temporal evolution of the protein corona around leukosomes and control liposomes (~170 nm diameter each), and also their uptake by macrophages. Leukosomes are liposomes with leukocyte membrane proteins, including IgG receptors, coagulation factors, and complement proteins, introduced in the liposomal lipid bilayer to obtain NPs mimicking the activity of natural leukocytes. Both types of NPs were injected into the tail vein of healthy mice; blood was withdrawn by cardiac puncture 10 min and 1 h after injection and the proteins adsorbed to the liposomes were identified by MS. Overall, leukosomes adsorbed fewer protein types than liposomes. One of those, clusterin, was exclusively found in the leukosomal corona. Interestingly, the protein corona formed in vivo increased the uptake of liposomes by immune cells but decreased the uptake of leukosomes. The authors suggested that the interaction of IgGs and other corona proteins with the receptors on the leukosomes prevented them from binding to their receptors on the macrophages.

Recently, Hadjidemetriou et al. [200] presented a proof-of-concept clinical study on in-vivo corona formation onto PEGylated liposomal NPs in humans, which had been infused intravenously into patients with recurrent ovarian carcinoma. Protein-coated liposomes were subsequently recovered from the blood, separated from blood components, and the adsorbed proteins were qualitatively and quantitatively analyzed. The most abundant protein classes were IgGs, lipoproteins, and complement proteins, in agreement with an earlier study on mice [197]. Moreover, more protein species were identified in the in-vivo corona than in its counterpart ex-vivo corona. Interestingly, the most abundant serum protein, albumin, was essentially absent from the in-vivo corona. Instead, low-abundance, low-molecular weight proteins were present. In a subsequent study, liposomal NPs were used as tools to fingerprint the blood proteome [201]. PEGylated liposomes were intravenously administered to subcutaneous melanoma-bearing and lung carcinoma xenograft-bearing mice. Recovery and purification of the corona-coated liposomes from the blood circulation of the two mice models enabled the detection of tumor-released and thus tumor-specific proteins of low abundance, which were not noticeable in plasma samples obtained by cardiac puncture of healthy and tumor-bearing mice (w/o prior NP injection) because they were masked by highly abundant plasma proteins such as albumin. The liposomal NPs even allowed murine host response proteins and human inoculated tumor-released proteins of minor abundance to be detected concurrently in the xenograft model.

Temperature-sensitive liposomes (TSLs) are sophisticated drug carriers that release their cargo in response to the higher temperature inside the tumor interstitium or the vasculature/blood [202]. Al-Ahmady et al. [203] investigated the effects of protein corona formation on their release activity. They administered the TSLs via intravenous injection into the tail vein of CD-1 mice and recovered those 10 min later from the blood. Afterwards, the drug release kinetics was determined ex situ, both in full plasma and after separation of unbound and weakly bound proteins. This study showed that the in-vivo corona had a dramatic impact on the drug release profile, the extent of which varied with the TSL lipid composition and the protein content of the environment. Therefore, it is crucially important to take these effects into account in the engineering of TSLs and the applied temperature protocols.

6. Conclusions and perspectives

The road toward routine fabrication of nanomaterials for diagnostic or therapeutic applications is a long and arduous one. Currently, only a few types of NPs have been approved by the US Food and Drug Administration (FDA), mostly for cancer therapy, and a few more are in the approval pipeline [204]. The essentially
Inevitable biomolecular adlayer largely uncouples the surface properties of the as-prepared NPs from those that are relevant in the biological setting, making predictions of functional behavior in the real biological environment exceedingly challenging. In recent years, a wealth of studies has been devoted to the exploration of the protein corona, and we are slowly and steadily learning more about its complexity and the implications for biological responses, including safety and toxicity issues and, importantly, efficacy of nanomedicines. Although significant progress has been made over the last decade, our understanding of the structure, composition and time evolution of the corona around NPs at the molecular scale is still at an early stage. This raises questions about the strategies that may ensure swift progress in the field.

In our view, it is important to deepen our fundamental understanding of the detailed processes involved in corona formation. As we have emphasized in this work, even elementary issues (monolayer/multilayer, softness/hardness) are still under debate. We believe that the enormous complexity associated with the processes at the nano-bio interface is still not sufficiently appreciated by many researchers in the field. Proteins are typically viewed (and schematically depicted) as compact, globular moieties when in fact they may turn into grossly denatured polypeptide chains under the influence of only moderate perturbative forces exerted by the NP surface. A deep understanding of the protein corona implies that we require knowledge of the optimal (free-energy minimized) architecture of polypeptide chains interacting with the NP surface (and their neighboring chains on the NP surface). To this end, different processes need to be scrutinized at the molecular level, including protein adsorption and desorption, conformational changes, surface migration, also in the presence of other and different polypeptides, polypeptide competition resulting in exchange (Vroman effect), and so on. Such studies are demanding, and conventional experimental techniques can yield only limited insight (see section 3). Novel single molecule/particle-based methods have the potential to enable entirely new views on the protein adsorption problem, and especially techniques that enable

![Fig. 8. In-vivo investigation of the protein corona as a function of time. (a) Work flow: Liposomes were intravenously administered via tail vein injection into CD-1 mice and recovered by cardiac puncture 10 min, 1 h and 3 h after injection. After separating the plasma from the recovered blood by centrifugation, the protein-coated liposomes were separated from unbound proteins. The compositions of the protein coronae formed at the different time points were qualitatively and quantitatively characterized and compared. (b) Total amount of proteins adsorbed on liposomes recovered 10 min, 1 h and 3 h after injection (average and standard error from three independent experiments). (c) Venn diagram showing the numbers of unique proteins identified in the in vivo corona at the different time points and their respective overlap. Adapted from Ref. [197].](image)
in-situ, time-resolved studies of protein corona formation would be invaluable. Furthermore, molecular-level computer simulations will be most helpful for advancing the field, and we believe that such activities will intensify in the upcoming years. This involves the development of specific computational tools for this purpose, which should be guided by — and calibrated with — experimental studies on well-defined model systems. To summarize, the structural exploration of the protein corona at the molecular level will continue to be a fascinating area of research with important implications for nanobiomedicine.

In the coming years, the efforts by many labs all over the world to design and synthesize new NPs will continue and presumably even intensify. It should be appreciated that nanomaterials for biomedical applications must possess highly defined properties and batch-to-batch reproducibility. This, in turn, requires reliable methods and procedures for the assessment of their physicochemical properties. For NP as well as protein corona characterizations, it would be useful if standardized experimental conditions could be defined that facilitate comparison of data from different labs. For technical reasons, the analysis of biomolecular adsorption onto NPs has mainly focused on proteins due to the availability of powerful proteomics methods but need to be extended to other types of biomolecules as well.

An enormous amount of data regarding protein corona formation on a wide variety of NPs under a wide variety of conditions has been amassed. To keep track of all these experimental data and to further utilize them for computational analysis, databases need to be assembled that include physicochemical characterizations of both the NPs and the protein corona. Importantly, these data are only useful if procedural parameters are also reported in great detail, so that experiments can be reproduced or compared with others. Indeed, the varying standards of reporting in scientific publications in the field have been criticized, and guidelines for minimum information reporting have been proposed to ensure that all relevant procedural details have been made available [205,206]. On the basis of precise and highly reproducible data, the complex relations between NP properties, reaction conditions and the resulting protein corona composition and properties can be analyzed using computational models to yield qualitative and quantitative structure–activity relationships (SARs) [207,208]. Perhaps, machine learning algorithms, trained with extensive and reliable data compilations, may enable predictions of the biological response without even understanding the underlying processes [209].

Researchers are becoming increasingly aware that in-vitro studies of protein corona formation and the assessment of biological responses in cell cultures may have deficiencies that prevent predictions of the behavior of protein coated NPs in the real biological environment. Therefore, studies employing complicated in-vitro environments will intensify, including more complex cell cultures, e.g. organoids, and flow reactors that simulate, e.g., the blood circulation. Nevertheless, in-vivo testing of engineered NPs using animal models will remain an essential step toward closing the translational gap in nanobiomedicine.

In conclusion, to understand biomolecular adsorption onto nanoparticles in sufficient depth so that biological outcomes can be predicted, much progress is still needed. Enormous challenges lie ahead on this road, but the ultimate goal, i.e. gaining the ability to design highly specific and effective, perhaps even patient-specific tools for nanomedicine, is certainly worth the effort.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments
This work was supported by the Helmholtz Association, Program ‘Science and Technology of Nanosystems’.

References
[1] R.A.J. Latour, Biomaterials: protein–surface interactions, in: Encyclopedia of Biomaterials and Biomedical Engineering, Taylor & Francis, New York, 2013, p. 270, https://doi.org/10.1016/B978-1-105-04158-5.
[2] R. Fazal, C. Alexiou, R.A. Alvarez-Puebla, F. Alves, A.M. Andrews, S. Ashraf, et al., Diverse applications of nanomedicine, ACS Nano 11 (2017) 2313–2381, https://doi.org/10.1021/acsnano.6b06040.
[3] X. Han, K. Xu, O. Taratala, K. Farsad, Applications of nanoparticles in biomedical imaging, Nanoscale 11 (2019) 799–819, https://doi.org/10.1039/cfln07760.
[4] Y.H. Lin, K. Nienhaus, G.J. Nienhaus, Nanoparticle probes for super-resolution fluorescence microscopy, ChemNanoMat 4 (2018) 253–264, https://doi.org/10.1002/cnma.201700375.
[5] J. Kim, P. Chhouk, J. Hsu, H.I. Litt, V.A. Ferrari, R. Popovtzer, et al., Use of nanoparticle contrast agents for cell tracking with computed tomography, Bioconjug. Chem. 28 (2017) 1581–1597, https://doi.org/10.1021/acs.bioconjchem.7b00596.
[6] J.E. Lemaster, J.V. Jokerst, What is new in nanoparticle-based photoacoustic imaging? Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 9 (2017) e1404, https://doi.org/10.1002/wnmn.1404.
[7] L. Shang, J. Xu, G.J. Nienhaus, Recent advances in synthesizing metal nanocluster-based nanocomposites for application in sensing, imaging and catalysis, Nano Today 28 (2019) 100767, https://doi.org/10.1016/j.nantod.2019.100767.
[8] C.D. Spicer, C. Jumeaux, B. Gupta, M.M. Stevens, Peptide and protein nanoparticle conjugates: versatile platforms for biomedical applications, Chem. Soc. Rev. 47 (2018) 3574–3620, https://doi.org/10.1039/C8CS00777E.
[9] J. Shi, P.W. Kantoff, R. Wooster, O.C. Farokhzad, Cancer nanomedicine: progress, challenges and opportunities, Nat. Rev. Cancer 17 (2017) 20–37, https://doi.org/10.1038/nrc.2016.108.
[10] E.C. Wang, A.Z. Wang, Nanoparticles and their applications in cell and molecular biology, Integr. Biol. 6 (2014) 9–26, https://doi.org/10.1039/c3ib01465k.
[11] T. Cederwall, I. Lynch, S. Lindman, T. Berggard, E. Thulin, H. Nilsson, et al., Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 2050–2055, https://doi.org/10.1073/pnas.0608582104.
[12] M.P. Monopoli, C. Åberg, A. Salvati, K.A. Dawson, Biomolecular coronas provide the biological identity of nanosized materials, Nat. Nanotechnol. 7 (2012) 779–786, https://doi.org/10.1038/nnano.2012.207.
[13] A.E. Nel, L. Madler, D. Velegol, T. Xia, E.M.V. Hoek, P. Somasundaran, et al., Understanding biophysicochemical interactions at the nano-bio interface, Nat. Mater. 8 (2009) 543–557, https://doi.org/10.1038/nmat2444.
[14] M.P. Monopoli, D. Walczyk, A. Campbell, G. Elia, I. Lynch, F.B. Bombelli, et al., Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles, J. Am. Chem. Soc. 133 (2011) 2525–2534, https://doi.org/10.1021/ja110753h.
[15] G. Caracciolo, D. Pozzi, A. Capriotti, C. Cavaliere, S. Povesana, G. Barbera, et al., The liposome–protein corona in mice and humans and its implications for in vivo delivery, J. Mater. Chem. B 2 (2014) 7419–7428, https://doi.org/10.1039/c4tb0136f.
[16] V. Mirshafee, R. Kim, M. Mahmoudi, M.L. Kraft, The Importance of selecting a proper biological milieu for protein corona analysis in vitro: human plasma versus human serum, Int. J. Biochem. Cell Biol. 75 (2016) 188–195, https://doi.org/10.1016/j.biocel.2015.11.015.
[17] N.J. Braun, M.C. DelBosse, S.M. Hussain, K.K. Comfort, Modification of the protein corona-nanoparticle complex by physical factors, Mat. Sci. Eng. C, Mater. 64 (2016) 34–42, https://doi.org/10.1016/j.msec.2016.03.059.
[18] D. Pozzi, G. Caracciolo, L. Digiacomo, V. Colapichioni, S. Palchetti, A.L. Capriotti, et al., The biomolecular corona of nanoparticles in circulating biological media, Nanoscale 7 (2015) 13958–13966, https://doi.org/10.1039/c5nr03701h.
[19] S. Tenzer, D. Doctor, J. Kuharev, A. Musyanovych, V. Fetz, R. Hecht, et al., Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology, Nat. Nanotechnol. 8 (2013) 772–781, https://doi.org/10.1038/nnano.2013.181.
[20] M. Mahmoudi, A.M. Abdelmonem, S. Behzadi, J.H. Clement, S. Dutz, M.R. Ejtehadi, et al., Temperature: the “ignored” factor at the nanobiointerface, ACS Nano 7 (2013) 6555–6562, https://doi.org/10.1021/nn305337c.
[21] L.A. Lane, X. Qian, A.M. Smith, S. Nie, Physical chemistry of nanomedicine: understanding the complex behaviors of nanoparticles in vivo, Annu. Rev. Phys. Chem. 66 (2015) 521–547, https://doi.org/10.1146/annurev-physchem-040113-103718.
M. Hoe, M. Elsabahy, K.L. Wooley, Strategies toward well-defined nanoparticles inspired by nature: chemistry versus versatility, J. Polym. Sci. A 50 (2012) 1869–1880, https://doi.org/10.1002/pola.25555.

B.L. Bank, P. Fattahi, J.L. Brown, Polymeric nanoparticles: the future of nanomedicine, Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 8 (2016) 271–289, https://doi.org/10.1002/won.1396.

A. George, P.A. Shah, P.S. Shrivastav, Natural biodegradable polymers based nanof ormulations for drug delivery: a review, Int. J. Pharm. 561 (2019) 244–264, https://doi.org/10.1016/j.ijpharm.2019.03.011.

A.D. Bangham, M.M. Smith, J.C. Warren, Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol. 13 (1964) 238–252, https://doi.org/10.1016/0022-2836(65)90093-6.

G. Gregoriadis, The carrier potential of liposomes in biology and medicine (second of two parts), Nat. Engl. J. Med. 295 (1976) 765–770, https://doi.org/10.1056/NEJM197603292950806.

G. Gregoriadis, The carrier potential of liposomes in biology and medicine (first of two parts), Nat. Engl. J. Med. 295 (1976) 704–710, https://doi.org/10.1056/NEJM197603292950801.

D.B. Kenske, P.R. Cullis, Liposomal nanomedicines, Expert Opin. Drug Deliv. 5 (2008) 25–44, https://doi.org/10.1517/17425247.5.1.25.

A. Fournier, S. Zaug, D. DeMichele, Frequency-dependent dielectric behavior of an emulsion of hard silica nanoparticles, J. Phys. Condens. Matter 14 (2002) 5655–5665, https://doi.org/10.1088/0953-8984/14/23/312.

A. Bekdemir, S. Liu, F. Stellacci, On the effect of ligand shell heterogeneity on nanoparticle/protein binding thermodynamics, Colloids Surf., B 174 (2019) 367–373, https://doi.org/10.1016/j.colsurfb.2018.11.027.

D. Langevin, O. Lozano, A. Salvati, V. Kestens, M. Monopoli, E. Raapstad, et al., Inter-laboratory comparison of nanoparticle size measurements using dynamic light scattering and differential centrifugal sedimentation, NanoImpact 10 (2018) 97–107, https://doi.org/10.1002/nipa.2017.12.004.

G.U. Nienhaus, J.D. Muller, B.H. McMahon, H. Frauenfelder, Exploring the conformational energy landscape of proteins, Physica D 107 (1997) 297–311, https://doi.org/10.1016/S0166-2202(97)00097-3.

T.E. Creighton, Protein folding, Biochem. J. 270 (1990) 1, https://doi.org/10.1042/bj2700001.

T. Arai, W. Norde, The behavior of some model proteins at solid liquid interfaces. I. Adsorption from single protein solutions, J. Colloid Interface Sci. 51 (1975) 356–365, https://doi.org/10.1016/0021-9797(75)90167-5.

M. Shoja, C.K. Hall, Protein adsorption on nanoparticles: model development using computer simulation, J. Phys. Condens. Matter 28 (11) (2016) 414019, https://doi.org/10.1088/0953-8984/28/41/414019.

P. Delcanale, B. Miret-Ontiveros, M. Arista-Romero, S. Pujals, L. Albertazzi, L. Baweja, Computer simulations for understanding nanoparticle-agglomeration. Small (12 pages) (2019) 1900974, https://doi.org/10.1002/smmi.201900974.

G. Gregoriadis, The carrier potential of liposomes in biology and medicine (first of two parts), Nat. Engl. J. Med. 295 (1976) 704–710, https://doi.org/10.1056/NEJM197603292950801.

D.B. Fenske, P.R. Cullis, Liposomal nanomedicines, Expert Opin. Drug Deliv. 5 (2008) 25–44, https://doi.org/10.1517/17425247.5.1.25.

A. Fournier, S. Zaug, D. DeMichele, Frequency-dependent dielectric behavior of an emulsion of hard silica nanoparticles, J. Phys. Condens. Matter 14 (2002) 5655–5665, https://doi.org/10.1088/0953-8984/14/23/312.
