Techniques for the experimental investigation of the protein corona

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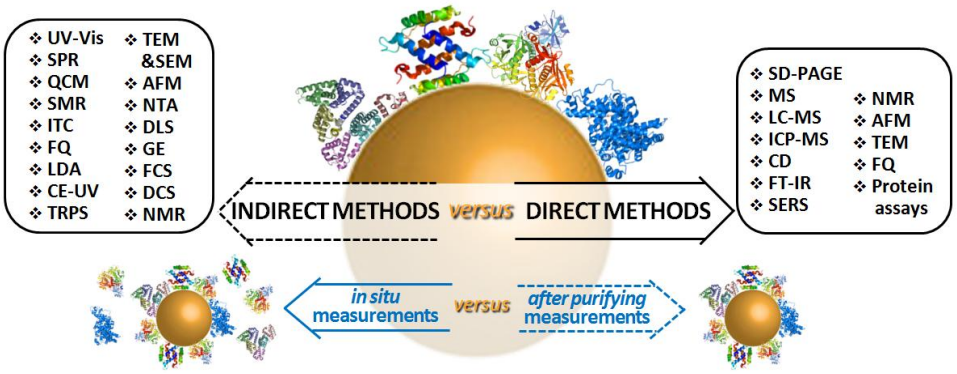
Abstract

Due to its enormous relevance the corona formation of adsorbed proteins around nanoparticles is widely investigated. A comparison of different experimental techniques is given. Direct measurements of proteins, such as typically performed with mass spectroscopy, will be compared with indirect analysis, in which instead information about the protein corona is gathered from changes in the properties of the nanoparticles. The type of measurement determines also whether before analysis purification from unbound excess proteins is necessary, which may change the equilibrium, or if measurements can be performed *in situ* without required purification. Pros and contras of the different methods will be discussed.



Highlights

- direct *versus* indirect analysis of the protein corona
- *in situ* measurements of protein corona *versus* measurements requiring purification
- characterization apart from mass spectroscopy

Introduction

Frequently colloidal nanoparticles (NPs) adsorb proteins (and other (bio-)molecules) spontaneously on their surface when they are placed in biological fluids, forming the so-called protein corona (Figure 1) [1,2]. This is a dynamic process [3,4] governed by the binding affinities and equilibrium constants of each type of protein to the respective NP surface, and variations in the protein composition of biological fluids [5]. The nature and composition of the corona can affect the fate, uptake, and performance of NPs in a biological context [6-8]. Indeed, it may provide NPs with additional colloidal stability and reduced toxicity [9,10], influence the cellular uptake of NPs [11] or their *in vivo* circulating times [12], as it may interfere with receptor-ligand interactions [13].

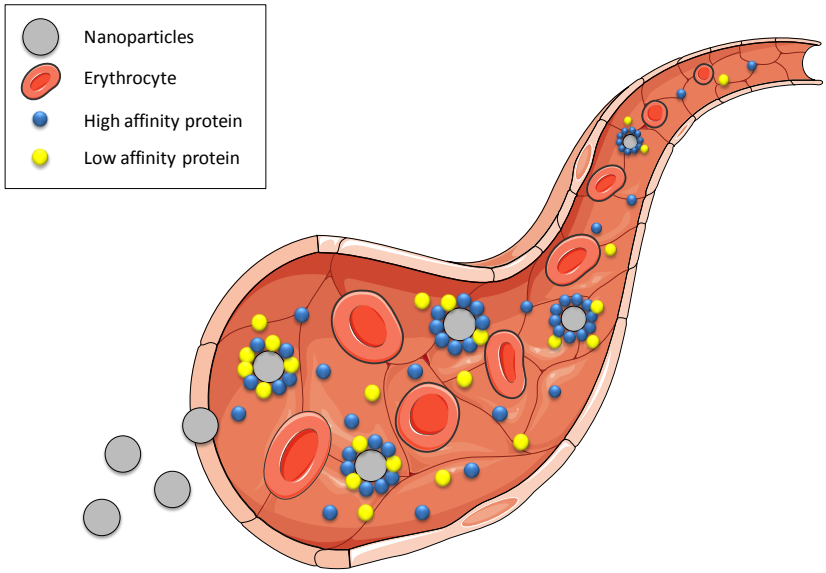


***Figure 1**. Representation of protein corona formation. Modified from Servier Medical Art.*

Hence, the protein corona is a subject of interest [14-17]. Concerning experimental analysis, the adsorbed proteins can be directly analyzed with mass spectroscopy, circular dichroism, etc, or indirectly via measuring modifications in the NPs properties, such as changes in size. In both cases, some techniques allow for detection of the protein corona *in situ*, while others require the removal of unbound proteins before measurements, which may however change equilibrium properties [15].

Direct methods

Direct methods directly analyze the proteins that are adsorbed on the NP surface (Figure 2). Besides the proteins bound to the NP also free proteins in solution will be present, which may interfere in the read-out. Thus unbound proteins have to be removed before measurements, which however leaves the purified NPs in non-equilibrium [15]. Loosely bound proteins, sometimes referred to as "soft corona" may be lost in the purification step, which would not be detected.

Adsorbed proteins can be directly visualized after negative staining with transmission electron microscopy (TEM) [14]. While in this way adsorbed proteins can be directly detected, and thus the presence of proteins on the NP surface can be verified, this method so far could not be applied for quantitative analysis. For improved analysis, in order to study the amount of adsorbed proteins and the composition of the protein corona, the proteins typically need to be desorbed from the purified NPs. For a mere quantification of the amount of adsorbed proteins standard biochemical protein quantification assays, such as Bradford or bicinchoninic acid assay [9,18] can be applied. In the case of inorganic NPs, these studies can be further completed by inductively coupled plasma mass spectrometry (ICP-MS) which allows for the calculation of the total content of sulfur coming from cysteine residues of the proteins, as well as for the total content of metal coming from the NPs [19]. In this way the ICP-MS data provide the amount of NPs, which together with the results from the protein quantification assays yield the number of proteins *per* NP [20].

Gel electrophoresis (GE) is a straightforward method to investigate the protein corona composition by using a protein molecular weight (M_w) standard marker to identify the different proteins by their apparent M_w. Frequently sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) systems are used, where proteins are denatured and disulfide bonds are partially digested prior to GE [21-24]. In case the protein corona has been obtained from a complex mixture of proteins, such as those present in serum or plasma, GE can only provide qualitative information regarding the corona composition. Hence, this technique is often complemented with MS based proteomics to determine the identity of proteins [25,26]. Selected bands from SDS-PAGE are recovered from the gel and analyzed by tandem mass spectrometry (MS/MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), or electrospray ionisation mass spectrometry (ESI-MS). By comparing the mass spectra of the recorded protein fragments with existing databases the composition of the protein corona can be obtained [27-29]. However, for a quantitative analysis yielding the absolute composition of proteins a chromatographic technique coupled with MS is required, such as liquid chromatography-mass spectrometry (LC-MS) [30,31]. Among these direct methods, TEM, GE, MS, and ICP-MS require purification from excess proteins and they are destructive. These techniques provide information about the identity of the adsorbed proteins, and in the case of LC-MS and ICP-MS it is also possible to quantify the amount of proteins; however, they cannot analyze the structure of adsorbed proteins.

Other methods can detect structural changes of proteins when adsorbed onto NP. The most common techniques are circular dichroism (CD) and Fourier transformed infrared spectrometry (FT-IR). In the case of CD it is possible to gain information about the secondary structure of proteins [32]. FT-IR is very sensitive to shifts and shape changes in vibrational bands corresponding to amide bonds, and it has also been used to monitor the binding of cysteine containing proteins from the corona to NPs over time [33]. Solution nuclear magnetic resonance (NMR) spectroscopy has scarcely been used to study the protein corona due to the high M_w of many proteins and the difficult interpretation of spectra when complex mixtures such as biological fluids are involved [34]. As free proteins in solution contribute to signal, purification of unbound protein is required. On the contrary, in surface enhanced Raman scattering (SERS) the Raman signal is amplified by several orders of magnitude in case the

Raman-active molecule (in this case proteins) are close to the surface of metal NPs. Thus, only proteins adsorbed to the NP surface contribute to the signal [35,36]. In summary, CD, FT-IR, NMR, and SERS are used for studying the protein corona with no need to digest the NP-protein complex. However, the sole information that one can obtain with the latter techniques is related to structural and conformational changes of the adsorbed proteins. With atomic force microscopy (AFM) proteins can be unfolded by application of mechanical force, and thus changes in structural elements can be assessed. We are however not aware of protein corona studies based on this.

Fluorescence of proteins is another interesting parameter, since the absorption of proteins on NPs usually leads to quenching of their native fluorescence (*i.e.* proteins containing tryptophan, tyrosine and phenylalanine) [37,38]. Fluorescence quenching (FQ) studies upon formation of NP-protein complex can be carried out under equilibrium conditions and described by the Hill model. In case of non-equilibrium quenching (*i.e.* very low quencher concentrations), the Stern-Volmer equation is used [14,39]. Unfortunately, the FQ technique presents limitations like overestimation due to the inner filter effect (IFE) caused by light absorption or scattering. Moreover, for sufficient quenching the fluorescent parts of the proteins have to come close enough to the inorganic part of the NP surface and thus polymer shells around the NP cores may significantly reduce quenching.

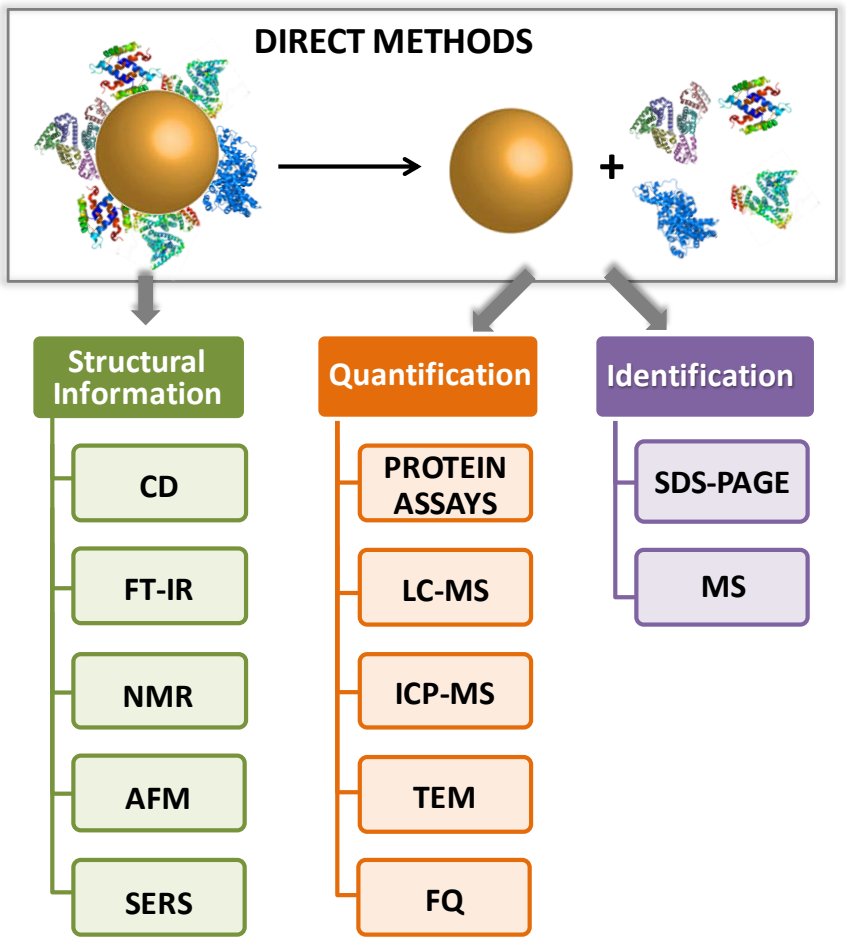


Figure 2. *Summary of direct methods.*

Indirect methods

There is a variety of analytical techniques that are frequent in the characterization of NPs, which are now being extended to indirectly investigate the protein corona. In this way, the protein corona is analyzed via measuring changes in the properties of the underlying NPs, such as changes in their size, charge, density, mass, absorbance, and fluorescence. These techniques are in the following classified according to the measured parameter, which is then correlated with the amount of adsorbed proteins (Figure 3).

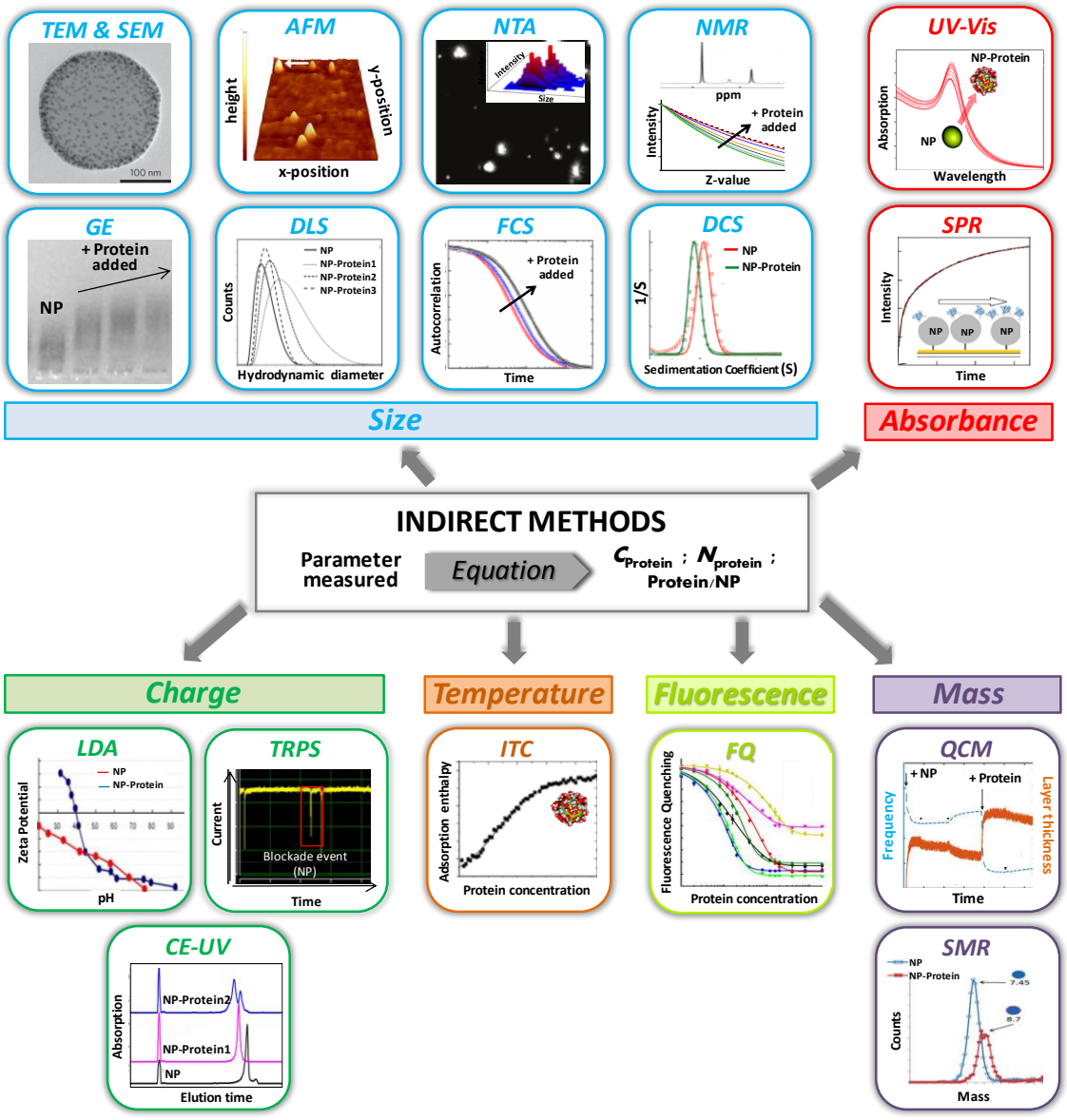


Figure 3. *Summary of indirect methods.*

There are several techniques to measure increase in the size of NPs upon adsorption of proteins. Techniques involve TEM, scanning electron microscopy (SEM), AFM, nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), fluorescence correlation spectroscopy (FCS), NMR, differential centrifugal sedimentation (DCS), and GE. Assuming that the NPs are not agglomerated, these techniques allow for determining the thickness of the protein shell by comparison of the hydrodynamic diameter of NPs before and after the corona formation. Increase in size upon adsorption of proteins to NPs can be quantified by TEM after negative staining [14], SEM, or AFM [40]. This however typically requires interfering pretreatments before an image can be made.

Upon adsorption of proteins also the Brownian motion of NPs slows down, due to the increase in mass. This can be quantified by NTA, in which the movement of individual NPs is traced by light-scattering [41]. However, for small NPs DLS and FCS are better suited, in particular for obtaining absolute data about the hydrodynamic diameter of NP-protein complexes. Diffusion of NPs in and out of a test volume is recorded, and fluctuations in the read-out signal are fitted with an autocorrelation function, from which the diffusion coefficient of the NP-protein complexes can be derived [42,43]. Hydrodynamic diameters can then be determined from the diffusion coefficients of NPs by the Stokes-Einstein equation. Concerning the principle of analysis, DLS and FCS are highly related, though DLS measures fluctuations in scattered light, while FCS measures fluctuations in the fluorescent emitted from the NPs. Both techniques in general fail for big NPs (> 100 nm), as the relative increase in size after the adsorption of proteins will be so low that it will fall within the experimental error of measurement. Due to its availability, DLS is a frequently used method for the systematic study of NP-protein interactions [7,44,45]. However, DLS is hard to apply when NPs are in the size range of proteins (≤ 10 nm), as unbound proteins may interfere. This can be circumvented by separation of excess proteins, which however results in desorption of originally bound proteins to solution towards formation of a new equilibrium [15]. Alternatively, DDLS has been used for measurements of the protein corona after incubation with complex matrices such as cell medium because the unwanted signals from unbound proteins are completely suppressed [47]. This technique relies on the optical anisotropy of many NPs owing to their inhomogeneous internal polycrystalline structure and not perfectly spherical shape. Upon scattering, the localized surface plasmon resonant (LSPR) coupled with such optical anisotropy results in a depolarized speckle pattern, whose temporal fluctuations yield precise information on the NP size. In contrast, the depolarized scattering from the biological matrix is virtually invisible.

FCS has the advantage that being based on fluorescence labelling of the NP the signal originates only from the fluorescent NPs, and therefore measurements can be performed *in situ* without the need of removing unbound proteins [48], thus also allowing for kinetic studies [49]. Dual-focus FCS (2fFCS) is an improved alternative that includes an absolute calibration standard and, therefore, allows for improved high-precision NP size measurements [50]. FCS has been applied to a large range of different proteins and NP surfaces. As quantitative parameter from such measurements (one type of protein, one type of NP surface) the dissociation coefficient of the NP-protein interaction can be determined [15]. For example, a recent FCS study on the adsorption of plasma proteins (HSA, ApoE3, and C3) to lipid-coated

quantum dots showed very low affinity, *i.e.* orders of magnitude weaker than for quantum dots coated with dihydrolipoic acid [52]. Interestingly, FCS has also been applied in complex mixtures such as human blood serum, and the results pointed out that surprisingly the corona formed from serum also consists essentially of a monolayer[29]. As size is the detected parameter, no information about the composition of the protein corona is possible. However, as the overall size of NP-protein conjugates depends on the geometry in which the proteins are adsorbed to the surface of NPs, in principle also some information about the conformation of adsorbed proteins may be deduced [48,53]. In addition, diffusion and thus hydrodynamic diameters may also be measured by non-optical techniques such as NMR [54].

Changes in size can also be inferred by NP movement in matrices. In GE charged NPs migrate driven by the applied electric field through the pores of the gel. Upon presence of adsorbed proteins, due to an increase in size (neglecting changes in charge) the NP-protein conjugates are retarded on the gel [14]. Also passing pores of a gel driven by gravity or pressure, such as with size exclusion chromatography (SEC) allows for sorting of NPs by size [55]. Differential centrifugal sedimentation (DCS) measures the time of sedimentation of NPs through a density gradient (normally a sucrose gradient) exposed to a centrifugal force [56,57]. The sedimentation time is a function of the NPs' size and density, and knowing the mean density of NPs, their hydrodynamic diameter can be determined. DCS has been widely used to measure the amount of proteins attached to NPs but this technique provokes a significant perturbation of the NP-protein complex, and usually underestimates protein shell thicknesses.

Laser Doppler anemometry (LDA), tunable resistive pulse sensing (TRPS), and capillary electrophoresis (CE), rely on changes in surface charge of the NPs. With LDA NPs are illuminated with a laser beam and migrate toward the oppositely charged electrode of an applied electrical field, and from the drift velocity the ζ-potential is determined [58,59]. Adsorption of proteins may vary the ζ-potential depending on the sign of the charge of the initial NPs, the identity of the proteins, their respective isoelectric point (pI) and the pH of the medium. Analysis may provide the amount of adsorbed proteins [7,21]. The ζ-potential can also be measured using tunable resistive pulse sensing (TRPS) [60,61]. This technique measures surface charge of individual NPs by mapping the “blockade” signals in electric current through the pore of a membrane when NPs pass through it. The amplitude of the blockade signal increases upon the adsorption of proteins, and the obtained NP surface charge can be correlated with the protein layer thickness. This method has been successfully applied to monitor the protein corona formation on NPs placed into serum and/or plasma [61]. Related to charge measurements, CE [62] imposes little impact to NP-protein complexes and operates in an open-column format without any packing materials [14,63]. CE is based on measuring the electrophoretic mobility of the NPs in an applied electric field, which is related to the NP charge. This approach allows for quantifying affinities between individual proteins and NPs. The relative changes in the retention of the NP-protein conjugates as a function of the protein concentration can be a parameter for protein binding to NPs and can be fitted with appropriate mathematical models, such as the Hill model [15] for obtaining K_D [64]. Although the detection sensitivity is not very high for conventional CE with UV detection, it increases strongly with laser induced fluorescence (LIF) or MS detection.

Changes in NP fluorescence (e.g. in quantum dots) are also indicative of the formation of a protein corona, since the adsorption of proteins on NPs usually leads to quenching (but in some times to enhancement) of the NP fluorescence. Elsewise these NP-based FQ studies are similar to protein-based FQ studies, whereby only the roles of fluorophores and quenchers are permutated.

UV-Vis absorption spectroscopy is widely used with plasmonic NPs because incubation of NPs with proteins usually leads to a red-shift and widening of the plasmon peak. Recently, a promising "chemical nose" method has been developed for the quantification of protein mixtures and proteins within human serum by using Au NPs [65]. Unique fingerprints were obtained after the interaction of two NPs with combinations of six proteins, allowing for the simultaneous quantification of several proteins in a sample with the aid of principal component analysis (PCA). In case the NPs are not plasmonic, they also can be bound to a surface plasmon resonant (SPR) device. The binding of proteins to NPs can be detected as a change in the angular position of the SPR peak. Multiplexed SPR using microchips with multiple microfluidic channels has been used for the simultaneous analysis of the interaction of Au NPs with several plasma components [66]. Also second harmonic light scattering (SHLS) has been proposed as a powerful technique for the quantification of weakly interacting proteins, which cannot be easily detected by conventional techniques [67]. The SHLS efficiency is significantly enhanced when the fundamental or harmonic wavelengths are tuned close to their localized surface plasmon resonance. Thermodynamic parameters such as the free energy of adsorption and the number of protein molecules adsorbed on the NP surface are extracted from the measured SHLS signal decay by using a modified Langmuir model.

Quartz crystal microbalance (QCM), and suspended microchannel resonator (SMR) measure mass changes due to protein corona formation. For QCM the NPs need to be fixed on top of an oscillating quartz surface, which then records increase in mass as proteins adsorb [40]. Similarly, SMR measures the mass of a NP suspension flushed through a microchannel inside the resonator [40]. A strong point of these techniques is their high sensitivity, which is in the range of femtogram to attogram. However, frequency changes, and therefore mass changes, are associated with total mass loading (including water and ions) as well as viscoelastic contributions from the adsorbing protein, which complicates quantitative analysis.

Isothermal titration calorimetry (ITC) is used to determine thermodynamic parameters of the protein corona formation such as binding affinity constant, stoichiometry, and enthalpy change [68,69]. ITC measures the time-dependent evolution of heat of a NP solution upon injections of an aliquot of protein, and the integration over time gives the incremental heat as function of the molar ratio between proteins and NPs. However, thermal exchanges may be due to either the formation of NP-protein complexes or protein denaturation events. Besides, ITC requires high sample concentrations, which is a limitation.

Conclusions

There is not "one" technique for quantitative analysis of the protein corona, but many experimental techniques are used. Pronounced differences have been frequently found, depending on the type of characterization technique applied. This suggests that the use of complementary characterization techniques is crucial to analyze different aspects of the protein corona and to get a better understanding of this process. This comprises important questions, for example whether in fact there is a "soft corona" and a "hard corona", or whether proteins adsorb in mono- or multilayers. Different results in these questions often originate from the use of different experimental techniques. Thus, only combination of several techniques will get the full picture.

Acknowledgments

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