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Dissociation coefficients of protein adsorption to nanoparticles as quantitative metrics for description of the protein corona: a comparison of experimental techniques and methodological relevance

Abstract

Protein adsorption to nanoparticles is described as a chemical reaction in which proteins attach to binding sites on the nanoparticle surface. This process can be described with a dissociation coefficient, which tells how many proteins are adsorbed per nanoparticle in dependence of the protein concentration. Different techniques to experimentally determine dissociation coefficients of protein adsorption to nanoparticles are reviewed. Results of more than 130 experiments in which dissociation coefficients have been determined are compared. Data show that different methods, nanoparticle systems, and proteins can lead to significantly different dissociation coefficients. However, we observed a clear tendency of smaller dissociation coefficients upon less negative towards more positive zeta potentials of the nanoparticles. The zeta potential thus is a key parameter influencing protein adsorption to the surface of nanoparticles. Our analysis highlights the importance of the characterization of the parameters governing protein-nanoparticle interaction for quantitative evaluation and objective literature comparison.

Introduction

Proteins in solution may adsorb to surfaces. This phenomenon has been investigated with a long history for extended (and in general planar) surfaces. One example in this direction are intended protein coatings of cell culture substrates to improve cell growth such as fibronectin, laminin, polyornithine, *etc.* [1-4]. Even when cell culture substrates are not coated intentionally, proteins from serum-containing media adsorb to the surface and thus provide coating. Formation of such protein films can be for example measured with ellipsometry [5]. With nanotechnological tools such coatings can nowadays even be directly visualized, for example by scratching of the surface with the tip of an atomic force microscope (AFM) [6]. Protein coatings can drastically change the physicochemical properties of surfaces [7]. Hereby already early work has demonstrated that protein coatings are not a static entity, but may undergo continuously adsorption and desorption, in which originally adsorbed proteins desorb and other adsorb. This is nowadays described in the context of the Vroman effect [8, 9]. The order in which proteins may adsorb to a surface has been quantified for many examples. For example human fibrinogen (FIB) binds stronger than human serum albumin [10].

From the physicochemical point of view adsorption and desorption can be interpreted as a chemical reaction

in which the educts are proteins P in solution and the free surface S, and the product is a protein-surface complex $P_n S$ in the form of a protein-layer bound to the surface. Such chemical reaction can be quantified in terms of a dissociation equilibrium coefficient or apparent dissociation coefficient

$$K_D = \frac{c^n(P) \cdot c(S)}{c(P_n S)} \quad (\text{Eq. 2})$$

which describes in dependence of the protein concentration $c(P)$ *etc.* whether in equilibrium the reaction will be shifted towards free or adsorbed proteins [11]. According to the Hill model [12] in equilibrium N of $N_{\max}$ binding sites of the surface for proteins will be occupied.

$$\frac{N}{N_{\max}} = \frac{1}{1 + \left(\frac{K_D}{c(P)} \right)^n} \quad (\text{Eq. 3})$$

Hereby

$$K'_D = (K_D)^{1/n} \quad (\text{Eq. 4})$$

is the protein concentration at which half of the surface is saturated with proteins. In this way also K'_D can be used in a similar way as K_D as a quantifier for protein adsorption to surfaces. In the context of Eq. 3 n is the Hill coefficient. Dissociation coefficients are thus a convenient way to quantify protein adsorption of different surfaces.

While being textbook knowledge for planar surfaces, these concepts have gained new interest in the context of colloidal nanoparticles (NPs). In case NPs are dispersed in protein-containing media, also for this geometry protein adsorption may occur, which is termed the formation of a protein corona [13, 14]. In the last decade it has been demonstrated experimentally that the protein corona plays a crucial role in the interaction of the NPs with cells. As the outermost part of the NP the protein corona largely determines the “biological identity” of a NP [15, 16]. Adsorption of proteins (and likely other macromolecules) from the host changes the actual physico-chemical nature of the NPs, which can change general features of the nanosurface “seen” by soluble and cellular biological actors, like surface charge or hydrophobicity. Dependence of the corona formation on several NP parameters such as size [17-20], charge [21, 22], shape [23], surface chemistry [24], *etc.* and on external parameters such as pH [25], temperature [26, 27] *etc.* has been investigated. It is also known that the protein corona is not a static entity, but undergoes dynamic changes during the lifetime of a NP from the first contact with extracellular medium until having reached the final location inside cells [28-30].

However, besides changing the physico-chemical properties of NPs the protein corona may also directly interfere with biological signal cascades. Indeed, virtually any biological macromolecule is “full” with information, encoded by its structural determinants, substantially dictating its ability to bind to other molecules, free in solution or expressed on cells. In this respect, it is to be remembered that often protein-mediated binding to other molecules leads to the activation of normally inactive self-amplifying cascades of biological and pathological paramount relevance, like, for example, the coagulation [31] and the complement cascades [32]. Another related aspect to be closely monitored when considering new nanoparticle properties induced by the protein corona, is the interaction with phagocytic cells [33-35]. Indeed, there may be induction of an improved or a reduced ability to evade, or obstruct, the clearance of the formed nanoentity by macrophages placed in organs like the liver and the spleen. This is of utmost importance, since the intensity of these phenomena influences the blood circulation half-life and hence the efficacy of a nanotheranostic formulation.

Despite joint efforts by many research groups to investigate the protein corona there are still some important questions. This involves for example the geometry of the protein corona. While in some studies formation of monolayers are claimed, others report thick coronas and multiple shell formation [36]. One general problem for unravelling the remaining secrets is that though numerous studies exist, many of them are hard to compare, as there is a lack of quantitative parameters which could be used as metrics for direct comparison.

In Figure 1 transmission electron microscopy (TEM) data are presented, in which the protein corona adsorbed to NPs is visualized with negative staining. These data correspond nicely with the monolayer hypothesis, as shown in Table 1.

Figure 1: TEM images of MnFe_2O_4 NPs with CoFe_2O_4 shell ($\text{MnFe}_2\text{O}_4/\text{CoFe}_2\text{O}_4$) a) NPs as synthesized in chloroform. b) NPs after transfer to water with a polymer shell (PMA) [37], imaged with negative staining [24]. c) NPs after transfer to water with a polymer shell (PMA) and addition of a PEG layer ($M_w = 10$ kDa), imaged with negative staining. d) NPs after transfer to water with a polymer shell (PMA) and addition of bovine serum albumin (BSA) [37], imaged with negative staining. Note that here BSA was linked chemically to the NP surface with EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) chemistry. However, the BSA corona upon adsorption was shown to be the same using gel electrophoresis, cf. Figure 2. The core radius of the NPs as dissolved in chloroform was $r_c = 6.9 \pm 0.7$ nm. The diameter of the NPs as visualized with the negative staining was b) $r_h(0) = 9.6 \pm 0.4$ nm, c) 12.4 ± 1.0 nm, and d) 13.8 ± 1.5 nm, as determined from images of > 300 NPs. The thickness of the polymer shell (PMA) thus is $9.6 \text{ nm} - 6.9 \text{ nm} = 2.7 \text{ nm}$. The thickness of the PEG shell added to the polymer coating is $12.4 \text{ nm} - 9.6 \text{ nm} = 2.8 \text{ nm}$. And the thickness of the layer of adsorbed BSA molecules on top of the polymer shell is $\Delta r_h = 13.8 \text{ nm} - 9.6 \text{ nm} = 4.2 \text{ nm}$.

Similar to planar surfaces, also in case of NPs the strength with which proteins bind to the surface of NPs can be quantified by determining the protein concentration $K'_D = (K_D)^{1/n}$ at which half of the NP surface is saturated with proteins [11]. K'_D values thus could be used as quantifier to compare protein corona formation for different NPs and different proteins under equilibrium conditions. K'_D values regarding the protein corona have been determined by several groups. The aim of this review article is to describe the different methods with which these values have been experimentally obtained and to compare the results obtained for different NPs and proteins.

Description of experimental techniques

Fluorescence correlation spectroscopy (FCS) and dynamic light scattering (DLS)

Protein adsorption to NPs can be directly observed by measuring changes in hydrodynamic diameter d_h . The more proteins adsorb on the surface of one NP, the bigger the NP-protein complex and thus the hydrodynamic diameter of the NP becomes. Hydrodynamic diameters can be determined *via* measurements of the diffusion coefficient of NPs, related by the Stokes-Einstein equation. Diffusion coefficients can be measured *via* observation of NPs diffusing in and out of a test volume from the autocorrelation function of signal fluctuation. Signal of the NPs can be obtained by light scattering (DLS) or by fluorescence of the NPs (FCS) [38, 39]. The resulting data set $d_h(c(P))$ of hydrodynamic diameters determined at different protein concentrations $c(P)$ can be fitted with the Hill model, yielding the protein concentration in which half of the NP surface is saturated with protein K'_D , the maximum number N_{max} of proteins which can be bound per NP, and the Hill coefficient n [11]. The group of Nienhaus *et al.* has used such FCS measurements to determine protein adsorption of several proteins to different NP surfaces [21, 24, 26, 40-45]. Notably FCS measurements can be performed *in situ*, *i.e.* unbound proteins do not have to be separated, as fluorescence originates only from fluorescent NPs. In case of DLS similar measurements are complicated by light scattering also from free proteins. In general such measurements are also limited to small NPs, as size changes of big NPs upon adsorption by proteins would be too low to be detected with reasonable experimental error.

Detection of free or NP-bound proteins

There are several biochemical methods for the characterization of the protein corona which are based on the rapid separation of unbound proteins and the NP-protein complexes by centrifugation and the subsequent measurement of the free protein left in the supernatant or of the proteins in the purified NP-protein complexes by any sensitive enough methods (absorbance, fluorescence or colorimetric protein assays). Combining flow field-flow fractionation (F4) with centrifugation, as an open channel separation technique, has proved to be very valuable for the dissociation-based screening of NP-protein interaction [46]. F4 separates compounds based on their size and shape and has already been employed to study antibodies

binding affinity to receptors and to cleanup nonspecifically bound proteins from the surface of microbeads that are used in affinity pull-down assay [47, 48]. Most recently, centrifugation coupled with F4 technique was utilized to purify the protein corona formed on superparamagnetic iron oxide nanoparticles (SPIONs) after incubation with ImmunoglobulinG (IgG)/ albumin-depleted human serum [49]. Optimal fitting of free protein concentration according to the one-site equilibrium equation, being the NPs molar concentration known, allows for obtaining K_D and N_{max} values. This has been recently demonstrated for the interaction of human Histidine Rich Glycoprotein (HRG), human Kininogen 1 (Kin 1) and FIB with SiO_2 -NPs [35]. However such method, being based on the measurement of what is left free, is limited by the accuracy of the protein determination assays and by the affinity and binding capability of the NPs to be analyzed. Low affinity (high K_D) and/or low capacity (low N_{max}) binding may introduce a too high experimental error to get reasonable quantitative information. For example, the affinity of HSA for SiO_2 -NPs could be just extrapolated ($> 1 \mu M$) and the N_{max} was not defined [35]. Alternatively, the amount of NP-bound protein can be determined after nanoparticle washings with any suitable method, as above, and plotted *versus* free protein (deduced) by fitting data with the Hill model [50]. In such approach, compared to the measurement of the free protein, even binding characterized by low affinity or capacity can be measured accurately since the measure is not subtractive. However, since further steps are required to eliminate any un-bound residual proteins, this procedure may suffer from some degree of dissociation of NPs-bound proteins, during the dilution in ligand free media, used for washings. In fact, this is a critical aspect of both approaches, *i.e.* due to the progressive concentration of NPs during the pelleting procedure, the equilibrium between NPs and solutes are altered to some extent [11], so that the final value of the obtained K_D may be underestimated. Another aspect to be considered is that there may be temperature changes of the system during the procedure. In particular, the temperature kept during the incubation of NPs with proteins (which can be set as desired) may be required to be changed during the following centrifugation steps, usually performed at temperatures between 0-4 °C, in order to avoid overheating of the rotors. This technical aspect may affect K_D values as well, since the kinetical constants characterizing the NP–protein conjugates may be temperature dependent [26]. A strong point of this methodology is that it is robust, label-free and needs few theoretic assumptions.

Surface plasmon resonance (SPR)

Real-time SPR is another label-free technique usable, where K_D can be deduced by the analysis of the kinetics of association and dissociation of the ligands from the complex (K_D can be indeed properly defined as the ratio between the dissociation K_{off} constant and the association K_{in} constant in defined temperature conditions). This technique also has the advantage of detecting phenomena inducing alteration of the structure (denaturation) of bound proteins, which influence the thermodynamics of the interaction and that must be considered for the final K_D determination. However, since this methodology implies the chemical coupling of NPs or proteins to the solid support (cheap) of the SPR device a major problem might be the interference of the chemical nature of this supporting matrix with protein-NP interaction. Canoa *et al.* [51] applied SPR to obtain the affinity

values of FIB and HSA for TiO_2 , CeO_2 , Al_2O_3 NPs and found that, in most cases, they were not grossly influenced by the different supports. However, there were exceptions (HSA interaction with Al_2O_3 -NPs) in which the choice of the support was found to be very critical. This case indicates that different supporting matrixes should be screened and carefully selected to have reliable results.

Isothermal titration calorimetry (ITC)

ITC is a very informative label-free method that allows for obtaining both affinity and maximal binding capacity of a certain ligand to NPs, by the detection of the generated, or adsorbed, heat. The measured thermal exchanges may be due to the formation of protein-NPs complexes but also to possible protein denaturation events that also contribute in determining K_D values.

(Affinity) Capillary Electrophoresis (ACE, CE) and related methods

In few cases the ACE technique was used to assess NP-protein interaction. In this approach the velocity of NPs in an applied electric field [52] is a parameter related to the mean NP charge [53, 54], if other hydrodynamic features are not modified. As in capillary electrophoresis no matrix as in gel electrophoresis is used increase in hydrodynamic diameters has less effect than in case of gel electrophoresis, in which conjugates of biological molecules and NPs may be sorted predominantly by size [55-58]. The charge change due to protein binding to NPs hence modifies their electrophoretic mobility, which in this way reflects the amount of bound proteins. Hence the relative changes in the retention of the NP-protein conjugates as a function of the protein dose can be a parameter for protein binding to NPs and can be fitted with appropriate mathematical models, like the Hill model [59], in order to obtain K_D and n as fit parameters. This method has several advantages. It is label-free and, being non-optical, does not suffer for alterations such as the IFE (Inner Filter Effect) that may be acting in fluorescence quenching (FQ) experiments. There is also no purification from unbound proteins requires, and thus ACS can be performed *in situ*. However, it has been shown that ACE may not work if positive NPs are to be tested, possibly due to interfering interaction with the capillary material (silica), and is sensitivity to the formation of aggregated NP/protein complexes which affects hydrodynamic diameters. In case a solid matrix is used during electrophoresis such as in gel electrophoresis, then NP-protein conjugates can be also sorted by size [57, 58], cf. Figure 2.

Figure 2: MnFe_2O_4 NPs with CoFe_2O_4 shell ($\text{MnFe}_2\text{O}_4/\text{CoFe}_2\text{O}_4$) NPs [37] were coated with the PMA polymer shell. The NPs were incubated with a) water, b) EDC, c) EDC and BSA (TEM image shown in Figure 1), and d) BSA. After applying an electric field the negatively charged NPs migrated from the wells towards the plus pole. Due to the size increase by BSA the BSA containing samples are retarded on the gel.

Fluorescence quenching (FQ)

Upon formation of protein-NP complex P_nS (here the substrate S is the NP) in case of intrinsically fluorescent NPs the NP fluorescence, or in case of fluorescent proteins (*i.e.* proteins containing tryptophan, tyrosine and phenylalanine) the protein fluorescence can be quenched. Quenching under equilibrium conditions can be described by the Hill model, leading to K_D and n as fit parameters [60]. In case of very low quencher concentrations (*i.e.* in the case fluorescence of the proteins and the NPs is quenched the NPs and the proteins are the quenchers, respectively) nonequilibrium quenching this can be described by the Stern-Volmer equation [61, 62]. In this approach the quenching efficiency depends on the degree of shielding of the fluorophores by the quenchers, and thus reveals the relative accessibility of quenchers to the fluorophores, which is done by a diffusion model [60, 63, 64]. The Stern-Volmer constant K_{SV} can be considered to be reciprocal to the dissociation constant $K_D \approx 1/K_{SV}$, though actually K_D refers to equilibrium and $1/K_{SV}$ to nonequilibrium conditions. The Stern-Volmer model is based on a diffusion model and only applies for the case that all fluorophores have equal access to the quenchers, *i.e.* there are no cooperative or noncooperative effects. In this case of $n = 1$ (*i.e.* noncooperative binding) the Hill and the Stern-Volmer model can be formally merged as shown in the following. The initially added NPs with concentration $c_0(\text{S})$ divide after addition to the proteins in free NPs without attached proteins with concentration $c(\text{S})$ and in the NPs with adsorbed proteins with concentration $c(\text{P}_n\text{S})$:

$$c_0(\text{S}) = c(\text{S}) + c(\text{P}_n\text{S}) \quad (\text{Eq. 5})$$

In case the proteins are the quenchers for the NP fluorescence the ratio I_0/I of the NP fluorescence without and the NP fluorescence with proteins added is described by the Stern-Volmer equation.

$$I_0/I = 1 + K_{SV} \cdot c(P) \quad (\text{Eq. 6})$$

As the fluorescence intensity I of the NPs is proportional to the NP concentration $c(S)$ Eq. 6 can be rewritten to

$$c_0(S)/c(S) = I_0/I = 1 + K_{SV} \cdot c(P) \quad (\text{Eq. 6'})$$

Insertion of Eq. 5 leads to

$$\frac{c(S) + c(P_n S)}{c(S)} = 1 + K_{SV} \cdot c(P) \Rightarrow K_{SV} = \frac{c(P_n S)}{c(S) \cdot c(P)} = 1/K_D \quad (\text{in Eq. 2 with } n = 1) \quad (\text{Eq. 7})$$

While in Table 1 the $1/K_{SV}$ values (from the Stern-Vollmer model) are enlisted as K_D values (from the Hill model), one has to be aware that this is only true in case $n = 1$ is assumed, quencher concentrations need to be low, and equilibrium and nonequilibrium conditions are mixed. In general FQ may be also overestimated due to the Inner Filter Effect (IFE) caused by light absorption or scattering [52]. Also not all NPs may quench fluorescence of adsorbed proteins with the same efficiency. For sufficient quenching the fluorescent parts of the proteins have to come close enough to the inorganic part of the NP surface (such as Au or Ag surfaces) [65] and thus polymer shells *etc.* around the NP cores may significantly reduce quenching. In case of NP (*i.e.* QD) quenching one needs to make sure that protein solutions due not contain other molecules as some ions, as those also may quench the fluorescence of QDs [66].

Results

Results obtained from more than 130 different datasets with different NPs, proteins, and methods are summarized in Table 1. This table is representative for one general problem in literature concerning investigation of the protein corona. Most authors are using very different systems and thus it is hard to compare data. In this review we focus on K_D values, which represent absolute numbers and thus should be comparable. However, due to the different NP materials, modifications, and different detection methods it is even hard to find strong correlations in the set of K_D

data presented in Table 1. In general K_D values as obtained with FQ as method are smaller than the ones as measured with different techniques. In fact, for obtaining this table we simply set $K_{SV} = 1/K_D$ (cf. Eq. 7), despite the fact that in general both values are not equivalent and this approximation can be used only for certain conditions. Thus, values as obtained with FQ will not be involved in the analysis as given below. A second problem hampering direct comparison of different studies is lack of NP characterization. In several studies experimental determination of the colloidal properties, as in particular state of dispersion (*i.e.* analysis of potential NP agglomeration) and about basis parameters such as the zeta potential are missing. In those studies it is hard to relate the protein-binding properties of the NPs to their physicochemical parameters.

#	protein	NP core	NP coating/ modification	r_s^a [nm]	$r_h(0)$ [nm]	ζ [mV]	buffer	T [°C]	method	K_D [μ M]	$K_D' = (K_D)^{1/n}$ [μ M]	N_{max}	n	Δr_h [nm]	report
1	HSA	FePt	PMA	1.6 ± 0.2	5.6 ± 0.2	-44 ± 3	PBS	RT	FCS	3.1^*	5.1 ± 1.3	27 ± 4	0.7 ± 0.1	3.3 ± 0.3	[40]
2	HSA	FePt	PMA	1.6 ± 0.2	6.0 ± 0.1	-44 ± 3	PBS	RT	FCS	7.9^*	9.9 ± 4.7	27 ± 3	0.9 ± 0.2	3.3 ± 0.3	[42]
3	HSA	FePt	PMA	1.6 ± 0.2	5.4 ± 0.1	-44 ± 3	PBS	RT	FCS	2.7^*	4.1 ± 0.8	15.2 ± 0.8	0.7 ± 0.1	2.6 ± 0.1	[24]
4	HSA	Au	P(-)	2.3 ± 0.6	7.9 ± 0.2	-39.8 ± 10	PBS	RT	FCS	1.4^*	1.5 ± 0.8	32 ± 4	0.8 ± 0.3	2.5 ± 0.3	[21]
5	HSA	Au	P(+)	2.3 ± 0.6	5.1 ± 0.1	$+9.7 \pm 8.9$	PBS	RT	FCS	1^*	1.0 ± 0.3	35 ± 3	0.8 ± 0.3	4.3 ± 0.4	[21]
6	HSA	CdSe/ZnS	PAA	6×12	7.9 ± 0.3	-	PBS	RT	FCS	18^*	37 ± 12	44 ± 8	0.8 ± 0.2	3.2 ± 0.4	[40]
7	HSA	CdSe/ZnS	PAA	5.0	9.3 ± 0.2	-27 ± 2	PBS	RT	FCS	13^*	24.8 ± 3.8	-	0.8 ± 0.1	3.2 ± 0.7	[43]
8	HSA	CdSe/ZnS	DPA	5.0	6.1 ± 0.1	-27 ± 2	PBS	RT	FCS	0.3^*	0.31 ± 0.06	-	1.0 ± 0.2	2.8 ± 0.4	[43]
9	HSA	CdSe/ZnS	DHLA	5.0	4.8 ± 0.2	-31 ± 4	PBS	RT	FCS	3.5^*	6 ± 3	-	0.7 ± 0.1	3.2 ± 0.8	[43]
10	HSA	CdSe/ZnS	cysteamine	-	6.3 ± 0.4	$+29 \pm 4$	H ₂ O	RT	FCS	0.004^*	0.025 ± 0.007	-	1.5 ± 0.4	4.4 ± 0.4	[43]
11	HSA	Fe ₃ O ₄	PMA	4.0 ± 0.6	6.8 ± 0.2	-55 ± 3	PBS	RT	FCS	1.3^*	1.5 ± 0.5	33.0 ± 4.0	0.6 ± 0.2	3.6 ± 0.5	[24]
12	HSA	Au	DHLA	-	2.9 ± 0.1	-37 ± 3	PBS	RT	FCS	0.1^*	0.08 ± 0.02	-	0.8 ± 0.1	-	[67]
13	HSA	Ag	DHLA	0.7 ± 0.2	1.1 ± 0.2	-51 ± 1	PBS	RT	FCS	-	0.37 ± 0.2	-	-	-	[68]
14	HSA	FePt	PMA-0.75kDa PEG	1.6 ± 0.2	6.5 ± 0.1	-17.8 ± 0.5	PBS	RT	FCS	1.9^*	2.0 ± 1.0	9.0 ± 1.0	0.9 ± 0.3	1.4 ± 0.2	[24]
15	HSA	FePt	PMA-5kDa PEG	1.6 ± 0.2	7.9 ± 0.2	-28 ± 1	PBS	RT	FCS	1^*	1.0 ± 0.4	14.0 ± 2.0	1.5 ± 0.8	1.5 ± 0.3	[24]
16	HSA	FePt	PMA-10kDa PEG	1.6 ± 0.2	9.7 ± 0.2	-14.3 ± 0.4	PBS	RT	FCS	1.4^*	1.4 ± 0.7	21.0 ± 3.0	1.1 ± 0.8	1.5 ± 0.3	[24]
17	HSA	FePt	PMA-glucose	1.6 ± 0.2	5.4 ± 0.1	-25 ± 3	PBS	RT	FCS	1.9^*	2.3 ± 0.3	18.3 ± 0.7	0.8 ± 0.1	3.0 ± 0.1	[24]
18	HSA	FePt	PMA	1.7 ± 0.3	5.5 ± 0.3	-45 ± 2	PBS	13	FCS	4^*	10 ± 4	31 ± 5	0.6 ± 0.1	3.7 ± 0.4	[26]
19	HSA	FePt	PMA	1.7 ± 0.3	6.0 ± 0.1	-45 ± 2	PBS	23	FCS	5.2^*	6.3 ± 2.2	30 ± 3	0.9 ± 0.2	3.3 ± 0.2	[26]
20	HSA	FePt	PMA	1.7 ± 0.3	6.0 ± 0.1	-45 ± 2	PBS	43	FCS	0.9^*	0.8 ± 0.4	23 ± 2	0.7 ± 0.2	2.8 ± 0.2	[26]
21	HSA	Au	-	8	-	-	-	RT	FQ	18	-	-	-	-	[69]
22	HSA	SiO ₂	-	12	-	-18.8	RPMI 1640	37	free protein	> 1	-	-	-	-	[35]
23	HSA	TiO ₂	-	1.8	-	$+47.0 \pm 1.0$	PBST	25	SPR	> 0.066 $< 0.094^{\dagger}$	-	-	-	-	[51]
24	HSA	CeO ₂	-	6.5	-	$+26.5 \pm 0.5$	PBST	25	SPR	> 0.025 $< 0.045^{\dagger}$	-	-	-	-	[51]
25	HSA	Al ₂ O ₃	-	-	-	$+20.3 \pm 0.2$	PBST	25	SPR	$2.5 \pm 0.5^{\dagger}$	-	-	-	-	[51]
26	HSA	HES	-	-	-275 ± 26	-34 ± 3	H ₂ O	25	ITC	1.25	-	0.114	-	-	[70]
27	HSA	HES	-COOH	-	-200 ± 20	-34 ± 3	H ₂ O	25	ITC	2.5	-	0.156	-	-	[70]
28	HSA	HES	-NH ₂	-	-256 ± 26	-27 ± 3	H ₂ O	25	ITC	2	-	0.184	-	-	[70]
29	HSA	CdSe/ZnS	DHLA	2.6 ± 0.4	5	-25 ± 2	PBS	RT	FCS	6.4 ± 3.6	-	9.5 ± 0.8	0.8 ± 0.1	3.3 ± 0.6	[71]
30	HSA	CdSe/ZnS	MHPC	2.6 ± 0.4	6.8 ± 0.9	-9.3 ± 0.5	PBS	RT	FCS	400 ± 70	-	15 ± 2	0.7 ± 0.2	3.3	[71]
31	HSA	Fe ₃ O ₄	PAA	3.85 ± 0.45	24.2 ± 4.1	-46.2	PBS	RT	CE	1.4 ± 0.11	-	-	1	-	[72]
32	HSA	Fe ₃ O ₄	PAA	4.05 ± 0.5	29 ± 5.9	-43.8	PBS	RT	CE	5.1 ± 0.18	-	-	1.1	-	[72]
33	HSA	Au	Asp	8 ± 2	10.5 ± 1	-31 ± 3	PBS	25	FQ	$0.000032^{\dagger}$	-	-	-	4.5 ± 0.5	[73]
34	HSA	Au	Gly	20 ± 4	23.5 ± 0.5	-32 ± 1	PBS	25	FQ	$0.00067^{\dagger}$	-	-	-	5.5 ± 1	[73]
35	HSA	Au	Lys	5.5 ± 2	7.5 ± 1.5	-26 ± 1	PBS	25	FQ	$0.00071^{\dagger}$	-	-	-	4.5 ± 3	[73]
36	HSA	Au	Ser	8 ± 1	7.5 ± 0.5	-34 ± 2	PBS	25	FQ	$0.00091^{\dagger}$	-	-	-	3 ± 0.5	[73]
37	HSA	Au	Leu	12.5 ± 3.5	13.5 ± 1	-35 ± 3	PBS	25	FQ	$0.00012^{\dagger}$	-	-	-	4.5 ± 1.5	[73]
38	HSAuc	CdSe/ZnS	DHLA	5.0	4.8 ± 0.2	-31.2 ± 0.8	PBS	RT	FCS	4.6^*	19 ± 8	89 ± 8	0.52 ± 0.04	8.1 ± 0.6	[44]
39	HSAuc	Au	DHLA	0.7 ± 0.15	3.2 ± 0.1	-37 ± 3	PBS	RT	FCS	0.1^*	0.034 ± 0.008	12.5 ± 0.6	0.7 ± 0.1	1.9 ± 0.2	[67]
40	HSAam	CdSe/ZnS	DHLA	5.0	5.6 ± 0.1	-31.2 ± 0.8	PBS	RT	FCS	40.8^*	22 ± 3	31 ± 1	1.2 ± 0.1	4.6 ± 0.1	[44]
41	HSAam	Au	DHLA	0.7 ± 0.15	3.2 ± 0.1	-37 ± 3	PBS	RT	FCS	1.1^*	1.2 ± 0.4	5.6 ± 0.4	0.7 ± 0.1	2.4 ± 0.2	[67]
42	BSA	starch	-	20-25	-	-	-	20	FQ	0.0053	-	-	-	-	[74]

43	BSA	starch	-	20-25	-	-	-	30	FQ	0.0055	-	-	-	-	[74]
44	BSA	starch	-	20-25	-	-	-	37	FQ	0.0062	-	-	-	-	[74]
45	BSA	Au	-	1.25 ± 0.25	-	-	PB	25	ITC	1.3	-	1.5	-	-	[75]
46	BSA	Au	CTAB	4 × 10	-	-	PB	25	ITC	0.085	-	2	-	-	[75]
47	BSA	MnFe ₂ O ₄ /CoFe ₂ O ₄	PMA	6.9 ± 0.7	9.6 ± 0.4	-31.8 ± 1.3	vacuum/H ₂ O	RT	TEM	-	-	-	-	4.2	this study
48	BSA	ZnO	-	3.75	-	-	H ₂ O	9	FQ	0.000000066	-	-	-	-	[76]
49	BSA	ZnO	-	3.75	-	-	H ₂ O	25	FQ	0.0000001	-	-	-	-	[76]
50	BSA	ZnO	-	3.75	-	-	H ₂ O	37	FQ	0.0006	-	-	-	-	[76]
51	BSA	Cu	-	3.75	-	-	H ₂ O	9	FQ	0.2	-	-	-	-	[77]
52	BSA	Cu	-	3.75	-	-	H ₂ O	25	FQ	0.08	-	-	-	-	[77]
53	BSA	Cu	-	3.75	-	-	H ₂ O	37	FQ	0.05	-	-	-	-	[77]
54	BSA	Cu	-	3.75	-	-	H ₂ O	42	FQ	0.015	-	-	-	-	[77]
55	BSA	Ag	-	-	31	-	H ₂ O	4	FQ	0.00006	-	-	-	-	[78]
56	BSA	Ag	-	-	31	-	H ₂ O	28	FQ	0.000000055	-	-	-	-	[78]
57	BSA	Ag	-	-	31	-	H ₂ O	37	FQ	0.000000066	-	-	-	-	[78]
58	BSA	Ag	-	-	31	-	H ₂ O	42	FQ	0.000000009	-	-	-	-	[78]
59	BSA	Au	citrate	25.6 ± 3.4	26 ± 1.3	-49 ± 8	H ₂ O	RT	FCS	256 ± 50	-	295 ± 30	0.4 ± 0.1	4.6 ± 2.4	[79]
60	BSA	Au	citrate	25.5 ± 3.5	26 ± 2	-50	H ₂ O	RT	FCS	600	-	-	0.6	5 ± 2	[80]
61	BSA	Au	-	7.5 ± 0.5	-	-	-	RT	FQ	27	-	-	0.95	-	[69]
62	BSA	Au	PAA	10.8 ± 1.3	-	-70	H ₂ O	4	FQ	0.00033	-	-	0.93	-	[52]
63	BSA	Au	PAH	10.8 ± 1.3	-	+49	H ₂ O	4	FQ	0.00058	-	-	0.57	-	[52]
64	BSA	Au	PEG	10.8 ± 1.3	-	-5	H ₂ O	4	FQ	0.00036	-	-	1.36	-	[52]
65	BSA	Au	PAA	8 × 27	-	-70	H ₂ O	4	FQ	0.000105	-	-	1.3	-	[52]
66	BSA	Au	PAH	8 × 27	-	+49	H ₂ O	4	FQ	0.000068	-	-	1.2	-	[52]
67	BSA	Au	PEG	8 × 27	-	-5	H ₂ O	4	FQ	0.0002	-	-	1.3	-	[52]
68	BSA	Au	PAH	8 × 27	-	+49	H ₂ O	4	FQ**	0.0041	-	-	-	-	[52]
69	BSA	Au	PAA	8 × 27	-	-70	MOPS	4	FQ	0.000103	-	-	-	-	[52]
70	BSA	Au	PAH	8 × 27	-	+49	MOPS	4	FQ	0.000042	-	-	-	-	[52]
71	BSA	Au	PEG	8 × 27	-	-5	MOPS	4	FQ	0.000072	-	-	-	-	[52]
72	BSA	Au	PAA	10.5 × 171	-	-70	H ₂ O	4	FQ	0.000082	-	-	1.1	-	[52]
73	BSA	Au	PAH	21 × 171	-	+49	H ₂ O	4	FQ	0.000036	-	-	0.7	-	[52]
74	BSA	Au	PEG	21 × 171	-	-5	H ₂ O	4	FQ	0.00012	-	-	1.2	-	[52]
75	BSA	Au	PAA	8 × 27	-	-70	MOPS	RT	ACE	13	-	-	2.3	-	[52]
76	BSA	Au	PEG	8 × 27	-	-5	MOPS	RT	ACE	65	-	-	2.3	-	[52]
77	BSA	Au	MUS	2.3 ± 0.6	5.4 ± 0.1	-	PBS	22	FQ	0.0005 ⁵	-	-	-	~ 7	[64]
78	BSA	Au	MUS/brOT	2.4 ± 0.5	6.3 ± 1.2	-	PBS	22	FQ	0.00074 ⁵	-	-	-	~ 7	[64]
79	BSA	Au	MUS/OT	2 ± 0.4	4.7 ± 0.5	-	PBS	22	FQ	0.011 ⁵	-	-	-	~ 8.5	[64]
80	BSA	Au	MPA/brOT	2.9 ± 0.2	5.3 ± 0.2	-	PBS	22	FQ	0.005 ⁵	-	-	-	~ 5	[64]
81	BSA	Au	MPA/OT	2.9 ± 0.2	5 ± 0.3	-	PBS	22	FQ	0.013 ⁵	-	-	-	~ 7	[64]
82	BSA	Au	Asp	8 ± 2	10.5 ± 1	-31 ± 3	PBS	25	FQ	0.00029 ⁵	-	-	-	4 ± 0.5	[73]
83	BSA	Au	Gly	20 ± 4	23.5 ± 0.5	-32 ± 1	PBS	25	FQ	0.000024 ⁵	-	-	-	5.5 ± 0.5	[73]
84	BSA	Au	Leu	12.5 ± 3.5	13.5 ± 1	-35 ± 3	PBS	25	FQ	0.00015 ⁵	-	-	-	4 ± 1.5	[73]
85	BSA	Au	Lys	5.5 ± 2	7.5 ± 1.5	-26 ± 1	PBS	25	FQ	0.00071 ⁵	-	-	-	2.5 ± 1.5	[73]
86	BSA	Au	Ser	8 ± 1	7.5 ± 0.5	-34 ± 2	PBS	25	FQ	0.00071 ⁵	-	-	-	3.5 ± 0.5	[73]
87	BSA	Fe ₃ O ₄	PAA	4	23.8	-26.55	BBS	RT	ACE	7.9 ± 0.21	-	-	3.3 ± 0.3	0.8	[59]
88	BSA	Fe ₃ O ₄	PAA	5	-	-	BBS	RT	ACE	0.19 ± 0.0073	-	-	2.4 ± 0.3	-	[59]
89	BSA	Au	Citrate	2.5	11.3	-	BBS	RT	ACE	0.23 ± 0.019	-	-	2.3 ± 0.4	-	[59]
90	BSA	Au	Citrate	5	18.9	-	BBS	RT	ACE	0.095 ± 0.0068	-	-	1.2 ± 0.1	-	[59]
91	BSA	PS	COOH	30	32.1 ± 0.7	-39.8 ± 3.9	HEPES	25	ITC	4.17	-	-	-	6 ± 0.7	[81]
92	BSA	PS	COOH	30	32.1 ± 0.7	-39.8 ± 3.9	PBS	RT	FQ	0.00056 ⁵	-	-	-	6 ± 0.7	[81]
93	BSA	PS	NH ₂	29	31.7 ± 0.6	40.3 ± 4	HEPES	25	ITC	25	-	-	-	31.7 ± 0.6	[81]
94	BSA	PS	NH ₂	29	31.7 ± 0.6	40.3 ± 4	PBS	RT	FQ	0.0013 ⁵	-	-	-	31.7 ± 0.6	[81]
95	BSA	PPy	PVAm	67 ± 12	93 ± 21.5	13.5	PBS	RT	CE	0.115 ± 0.005	-	-	1.355 ± 0.078	10	[82]
96	BSA	CdSe/ZnS	GSH	-	-	-	BBS	RT	CE	8.8	-	-	2.6	-	[83]
97	FIB	FePt	PMA	1.7 ± 0.3	5.8 ± 0.6	-44.1 ± 0.3	PBS	RT	FCS	0.1 ⁵	0.03 ± 0.01	65 ± 5	0.7 ± 0.1	20.0 ± 1.0	[24]
98	FIB	FePt	PMA-10kDa	1.7 ± 0.3	9.8 ± 0.5	-14.3 ± 0.4	PBS	RT	FCS	0.5 ⁵	0.14 ± 0.004	18 ± 4	0.4 ± 0.1	8.1 ± 1.6	[24]
99	FIB	SiO ₂	PEG	12	-	-18.8	RPMI 1640	37	free protein	0.028 ± 0.012	-	27 ± 3	-	-	[35]
100	FIB	TiO ₂	-	1.8	-	47.0 ± 1.0	PBST	25	SPR	> 0.00074 < 0.000206 ⁵	-	-	-	-	[51]
101	FIB	CeO ₂	-	6.5	-	26.5 ± 0.5	PBST	25	SPR	> 0.000037 < 0.000047 ⁵	-	-	-	-	[51]
102	FIB	Al ₂ O ₃	-	7	-	20.3 ± 0.2	PBST	25	SPR	> 0.010.9	-	-	-	-	[51]

103	FIB	γ -Fe ₂ O ₃	-	5	-	-	-	25	FQ	0.045	-	-	-	-	[84]
104	FIB	γ -Fe ₂ O ₃	-	5	-	-	-	30	FQ	0.09	-	-	-	-	[84]
105	FIB	γ -Fe ₂ O ₃	-	5	-	-	-	37	FQ	0.14	-	-	-	-	[84]
106	FIB	Au	MUA	2.8	14.4 ± 0.3	-40.8 ± 1.51	H ₂ O	25	bound protein	-	2.25	1	2.31	-	[50]
107	FIB	Au	MUA	7.1	23.9 ± 0.3	-41.2 ± 1.31	H ₂ O	25	bound protein	-	1.3	2	1.2	-	[50]
108	FIB	Au	MUA	15.7	46 ± 3	-46.2 ± 2.78	H ₂ O	25	bound protein	-	3.5	128	0.75	-	[50]
109	FIB	Au	MUA	32.5	90 ± 1	-40.7 ± 2.84	H ₂ O	25	bound protein	-	4.3	1124	1.1	-	[50]
110	apo-Tf	Fe ₃ O ₄	OA/AMP	5	7.45 ± 2.75	-40.4 ± 0.3	PBS	37	CE	0.233 ± 0.1	-	-	-	-	[49]
111	apo-Tf	FePt	PMA	1.6 ± 0.2	5.1 ± 0.2	-44 ± 3	PBS	RT	FCS	254'	26 ± 6	23 ± 3	1.7 ± 0.2	7.0 ± 0.4	[41]
112	apo-Tf	FePt	PMA	1.7 ± 0.3	5.0 ± 0.2	-45 ± 2	PBS	22	FCS	7'	16 ± 6	40 ± 6	0.7 ± 0.1	9.3 ± 0.7	[26]
113	apo-Tf	FePt	PMA	1.7 ± 0.3	5.1 ± 0.2	-45 ± 2	PBS	9	FCS	4.7'	13 ± 4	47 ± 7	0.6 ± 0.1	10.0 ± 0.8	[26]
114	apo-Tf	FePt	PMA	1.7 ± 0.3	5.3 ± 0.1	-45 ± 2	PBS	43	FCS	3.1'	5 ± 1	17 ± 2	0.7 ± 0.1	5.7 ± 0.4	[26]
115	apo-A-I	FePt	PMA	1.6 ± 0.2	6.0 ± 0.1	-44 ± 3	PBS	RT	FCS	140'	140 ± 60	52 ± 10	1.0 ± 0.3	4.8 ± 1.4	[42]
116	apo-A-I	HES	-	-	275 ± 26	-34 ± 3	H ₂ O	25	ITC	0.003	-	10 ± 4	-	-	[70]
117	apo-A-I	HES	-COOH	-	200 ± 20	-34 ± 3	H ₂ O	25	ITC	0.0053	-	6 ± 3	-	-	[70]
118	apo-A-I	HES	-NH ₂	-	256 ± 26	-27 ± 3	H ₂ O	25	ITC	0.185	-	8 ± 6	-	-	[70]
119	apo-A-I	Fe ₃ O ₄	OA/AMP	5	7.45 ± 2.75	-40.4 ± 0.3	PBS	37	CE	0.155 ± 0.001	-	-	-	-	[49]
120	apoE3	CdSe/ZnS	DHLA	2.6 ± 0.4	5	-25 ± 2	PBS	RT	FCS	0.06 ± 0.01	-	19 ± 1	1.7 ± 0.2	8.2 ± 0.3	[71]
121	apoE3	CdSe/ZnS	MHPC	2.6 ± 0.4	6.8 ± 0.9	-9.3 ± 0.5	PBS	RT	FCS	430 ± 120	-	28 ± 1	1.5 ± 0.2	8.2	[71]
122	apoE4	FePt	PMA	1.6 ± 0.2	6.0 ± 0.1	-44 ± 3	PBS	RT	FCS	0.004'	0.021 ± 0.003	65 ± 3	1.4 ± 0.2	5.7 ± 0.2	[42]
123	β -casein	Fe ₃ O ₄	PAA	3.85 ± 0.45	24.2 ± 4.1	-46.2	PBS	RT	CE	0.23 ± 0.0085	-	-	2.7	-	[72]
124	β -casein	Fe ₃ O ₄	PAA	4.05 ± 0.5	29 ± 5.9	-43.8	PBS	RT	CE	4.6 ± 0.093	-	-	1.3	-	[72]
125	Calmodulin	Fe ₃ O ₄	PAA	3.85 ± 0.45	24.2 ± 4.1	-46.2	PBS	RT	CE	0.28 ± 0.024	-	-	0.4	-	[72]
126	Calmodulin	Fe ₃ O ₄	PAA	4.05 ± 0.5	29 ± 5.9	-43.8	PBS	RT	CE	0.015 ± 0.0006	-	-	2.1	-	[72]
127	Myoglobin	Fe ₃ O ₄	PAA	3.85 ± 0.45	24.2 ± 4.1	-46.2	PBS	RT	CE	1.8 ± 0.11	-	-	1.3	-	[72]
128	Myoglobin	Fe ₃ O ₄	PAA	4.05 ± 0.5	29 ± 5.9	-43.8	PBS	RT	CE	4 ± 0.27	-	-	1.4	-	[72]
129	Cytochrome C	Fe ₃ O ₄	PAA	3.85 ± 0.45	24.2 ± 4.1	-46.2	PBS	RT	CE	18 ± 4.8	-	-	1.9	-	[72]
130	Cytochrome C	Fe ₃ O ₄	PAA	4.05 ± 0.5	29 ± 5.9	-43.8	PBS	RT	CE	2.9 ± 0.091	-	-	1.1	-	[72]
131	C3	CdSe/ZnS	DHLA	2.6 ± 0.4	5	-25 ± 2	PBS	RT	FCS	0.015 ± 0.001	-	8.9 ± 0.4	2.1 ± 0.2	14.6 ± 0.2	[71]
132	C3	CdSe/ZnS	MHPC	2.6 ± 0.4	6.8 ± 0.9	-9.3 ± 0.5	PBS	RT	FCS	201 ± 30	-	14 ± 2	1.3 ± 0.1	14.6	[71]
133	AlAT	Fe ₃ O ₄	OA/AMP	5	7.45 ± 2.75	-40.4 ± 0.3	PBS	37	CE	1.01 ± 0.1	-	-	-	-	[49]
134	HaG	Fe ₃ O ₄	OA/AMP	5	7.45 ± 2.75	-40.4 ± 0.3	PBS	37	CE	0.612 ± 0.074	-	-	-	-	[49]
135	HRG	SiO ₂	-	12	-	-18.8	RPMI 1640	37	free protein	0.0024 ± 0.0011	-	30 ± 2	-	-	[35]
136	Kin 1	SiO ₂	-	12	-	-18.8	RPMI 1640	37	free protein	0.0046 ± 0.0013	-	47 ± 2	-	-	[35]

Table 1: Protein adsorption quantified for different NPs, buffers, temperatures T (RT = room temperature), and methods. As NP materials FePt, Au, Ag, Cu, CdSe/ZnS, SiO₂, TiO₂, CeO₂, Al₂O₃, ZnO, γ -Fe₂O₃, MnFe₂O₄/CoFe₂O₄ core/shell hybrids [37], starch, HES (hydroxyethyl starch), PPy (polypyrrol), and PS (polystyrene) were investigated. NP surface coatings involve OT (1-octanethiol), brOT (branched version of OT, 3,7 dimethyloctane 1-thiol), MPA (mercaptopropionic acid), MUA (11-mercaptopundecanoic acid), MUS (11-mercapto-1-undecanesulfonate), OA (oleic acid), DPA (D-penicillamine), DHLA (dihydrolipoic acid), GSH (glutathione), CTAB (cetyltrimethylammonium bromide), MHPC (myristoyl hydroxyl-phosphatidylcholine), Asp (aspartate), Gly (glycine), Leu (leucine), Lys (lysine), Ser (serine), PMA (modified poly (isobutylene alt- maleic anhydride), where 75% of the anhydride rings were reacted with dodecylamine) [85], P(-) (PMAPOS-stat-PLMA) [21, 86], P(+) (PTMAEMA-stat-PLMA) [21, 86], AMP (amphiphilic polymer), PAA (polyacrylic acid), PAH (poly-allylamine hydrochloride), PEG (polyethylene glycol), and PVAm (Poly(vinyl-amine)). In some cases the same materials or the coatings were further chemically modified to add carboxylate (- COOH) or amine (-NH₂) groups. A large set of proteins was investigated, including HSA (human serum albumin), HSAsuc (succinylated HSA), HSAam

(aminated HSA), BSA (bovine serum albumin), FIB (human fibrinogen), apo-Tf (apo-transferrin), apoA-I (apolipoprotein A-I), apoE3 (apolipoprotein E3), apoE4 (apolipoprotein E4), HRG (human Histidine Rich Glycoprotein), Kin 1 (human Kininogen 1), A1AT (Alpha-1-antitrypsin), HaG (Haptoglobin), and C3 (complement component 3). Incubation of NPs with proteins was carried out in different media, i.e. H₂O, PB (100 mM phosphate buffer), PBS (phosphate buffered saline, pH = 7.4), PBST (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 0.005% Tween 20, pH 7.4), RPMI 1640 (GIBCO brl, code number 11875-085), HEPES (20 mM HEPES buffer, pH 7.4), and MOPS (5 mM 3-Morpholinopropane-1-sulfonic acid buffer, pH 7). The radius r_c of the (spherical) inorganic cores of the NPs was determined by transmission electron microscopy (TEM). For the FePt NPs reported here [40-42], the r_c data are taken from Lehmann et al. [87]. In the case of the CdSe/ZnS NPs, according to the provider (Invitrogen), these NPs are not spherical but ellipsoids with 6 nm and 12 nm axes, and thus in case of rod-shaped NPs the radii of the short and long axes are given[#]. SiO₂ NPs of amorphous silica were synthesized and characterized as described previously by Fedeli et al [35]. The protein corona was quantified with different methods. In case of FCS (fluorescence correlation spectroscopy) $r_h(0)$ and $r_h(N_{max})$ are the hydrodynamic radii of the NPs without adsorbed proteins and upon saturation of the NP surface with proteins, respectively, as determined by FCS using the Hill model. Thus $\Delta r_h = r_h(N_{max}) - r_h(0)$ is the thickness of the protein corona. The Hill coefficient n controls the steepness of the binding curve, N_{max} is the maximum number of proteins adsorbing onto a single NP, and K'_D represents the concentration of protein molecules at half coverage. FCS measurements are described in a series of publications from the Nienhaus group [40-42]. *In case of FCS experiments the measured data are K'_D and n , and K_D was calculated from these two parameters. Measurement of free protein or NP-bound protein concentrations are based on removal of NP-protein complexes by centrifugation and fitting of data using a one-site equilibrium equation or Hill model equation to obtain K_D and N_{max} values [35], or K'_D values [50]. For SPR (surface plasmon resonance) data [51] K_D values can be calculated from the binding and dissociation kinetics, obtained using increasing protein ligand concentrations in PBST, and fitting the binding or dissociation curves with Langmuir or bivalent analyte models. [§]NPs were coupled to alginate (GLC), NeutrAvidin (NLC) or bare gold (BGD) supports and the two extreme values of the range are here indicated. ^{§§}In the case of HSA-Al₂O₃ binding K_D could not be determined using NLC and BGD supports, likely for interfering phenomena, and high density GLC supports (GLCH) were employed in two different experiments (the two obtained values are reported). In case of TEM imaging of the negative-stained HSA corona the $r_h(0)$ and $r_h(N_{max})$ values were determined by TEM, and the zeta-potential in H₂O. [§] K_D values were assumed as $K_D = 1/K_{SV}$. ^{***}FQ data were experimentally corrected for Inner Filter Effect by using BSA-presaturated NPs.

First we analyzed different K_D values of proteins. In order to achieve comparable conditions we limited this analysis to highly negatively charged NPs at room temperature, excluding data obtained with FQ. The results are displayed in Figure 3. While for some proteins there is only one of few values available some tendencies in the K_D values in-between different protein species can be observed. In agreement with the classical Vroman

data K_D values for FIB are in general orders of magnitude lower than that for HSA. FIB thus is a stronger binder to negatively charged NP surfaces than HSA. Still surprisingly there are enormous differences between data obtained in different studies.

Figure 3. K_D values from Table 1 for different proteins. In order to get comparable conditions only NPs with -COOH groups on their surface (citrate, DPA, DHLA, PMA, PAA) with zeta potential $\zeta < -25$ mV, at room temperature (RT, 22 °C, 25 °C) were considered. Data obtained with FQ measurements are excluded from this graph.

Next we attempted to analysis dependencies on physicochemical parameters of NPs. As albumins (HSA and BSA) are the most studied proteins, in Table 1 we first screened these data for potential dependencies of the K_D values with physicochemical NP properties. As most evident parameter we identified the zeta potential ζ . In Figure 4 the K_D values are plotted for both bovine and human albumins, assumed to behave similarly, versus the zeta potential of the respective NPs. In spite of scattering of data, reasonable considering the many other variables involved (materials, coatings, size and methodologies) there is a clear trend for lower K_D values towards less negative and more positive zeta potentials.

Figure 4. K_D values from Table 1 relative to albumin-NP binding plotted versus the zeta potential of the NPs. Values obtained using NPs made with the indicated materials are represented irrespectively of size, shape, or further coating and chemical modification, and are uniquely expressed as a function of their zeta potential. Obviously reported K_D values not implemented with determined zeta potential of the NPs in the study could not be used for this analysis. The dashed line-delimited area groups all data that were obtained with FQ, while points outside this area correspond to all other methodologies (cf. Table 1).

However, as anticipated above in commenting Table 1, this graphical representation allows to appreciate better how, while the majority of methodologies seem to generate data in the same rough direction, data obtained with FQ show a peculiar and divergent distribution. Not only the absolute values of K_D are orders of magnitudes lower than those determined with other approaches, in some cases within the same study and exactly with the same NPs [81], but also no clear changes were reported as a function of the zeta potential. Although IFE, if not properly evaluated, may be responsible for K_D underestimations, this is not enough to account for the observed divergence with values from alternative methods. This

analysis demonstrates that data obtained with FQ experiments are significantly different from those obtained with other methods, and suggests caution in assuming that K_D can be deduced from K_{sv} . In Figure 5 the K_D values characterizing HSA/BSA interaction with NPs, omitting FQ derived ones for the reasons above motivated, are analyzed for different NP materials. This allowed to confirm that the trend above evidences of an affinity improvement of albumin for positively charged NPs, independently on the material or the coating chemistry, confirming the importance of the z potential in governing albumin-NP association. However in the case of the three organic NPs that could be analyzed (HSE, PS and PPy) no clear trend could be seen. In fact, in the only homogeneous comparison possible (PS) the charge switch from negative to positive charge of the NP, obtained by $-COOH$ or $-NH_2$ groups coupling, determined a sensible increase of the K_D , indicating an affinity decrease, opposite to the trend observed for other NP types. On the other hand, positive PPy NPs coated with a cationic polymer bind BSA with an affinity apparently one-two order of magnitude stronger than those seen in the other organic NPs here compared, in line with data obtained with other NPs. Hence, due to the paucity of data and to the conflicting results, no conclusion can be drawn and more quantitative data are necessary.

Figure 5. Data from Figure 4 divided into different subgroups. All K_D values of Figure 4 were split into four groups: FePt and CdSe/ZnS NPs; metal NPs (Au and Ag); oxides and organic NPs. The surface coating or chemical modification of the bare material is indicated near to the respective data point. “bare” indicates the pristine material surface. The yellow line guides the eye to follow the trend of data, without analytical rationale behind the shape of the curve.

The same analysis as for albumins (HSA, BSA) was also done for FIB, *cf.* Figure 6. Although the data set for this protein is smaller, and negative and positive NPs are all chemically different, the graph clearly suggests that, as for albumins, there is a tendency to lower K_D values for FIB for more positive NPs. In this case, quantitative homogeneous data would greatly help to confirm the trend. With all the caveats emerged, the available data indicate that zeta-potential of the NPs thus seems to be a very influential parameter.

Figure 6. Fibrinogen K_D values from Table 1 plotted versus the zeta potential of the NPs. The surface coating or chemical modification of the bare material, when present, is indicated near to any data point. “bare” indicates the pristine material surface. These data are taken from several studies [24, 35, 50, 51]. The yellow line acts merely as a guide to the eye to follow the trend of data, without analytical rationale behind the shape of the curve.

In Table 1 there are also some sets of data in which the effect of PEGylation within one series of NPs is investigated [24, 52]. Surprisingly, in the case of albumin, K_D values are weakly influenced by PEGylation. Data plotted in Figure 6 are relative to a set of data in which the same particles (FePt) coated only with PMA or with PMA plus a further layer of PEG with increasing molecular weights are compared [24]. Interestingly, while for BSA K_D and $N_{\max}$ values only slightly changed, for FIB K_D increased 5 times and $N_{\max}$ decreased sensibly in PEGylated NPs compared to the PMA-coated. Considering that the isoelectric points of albumin and FIB are similar (4.7 and 5.5 respectively), and that hence both proteins are negatively charged in the experimental condition used (pH around 7), this information suggests that zeta potential and PEG polymer coating are not the unique factors governing the K_D and the binding capacity of a NPs to proteins, *cf.* Figure 7. The kind of protein, and its specific biochemical and structural properties are also a very important factors. Also temperature dependence has been investigated in several studies. However, comparison of these data does not suggest any trend, *cf.* Figure 8.

Figure 7. K_D values from Table 1 plotted versus the degree of PEGylation of the NPs. The PEGylation is described via the molecular mass M_{PEG} of the attached PEG molecules. No attached PEG is referred to as $M_{PEG} = 0$. These data are taken from several studies [24, 52]. From the data set of

Boulos et al. compared the data with PAA (i.e. $M_{PEG} = 0$) and with 5 kDa PEG [52]. The lines connecting the data points act merely as a guide to the eye to follow the trend of data, without analytical rationale behind the shape of the curve.

Figure 8. K_D values from Table 1 plotted versus the temperature upon incubation of the NPs with proteins. These data are taken from several studies [26, 74, 76, 78, 84]. The material forming the NPs and the binding protein are indicated. Coatings are within brackets. The lines connecting the data points act merely as a guide to the eye to follow the trend of data, without analytical rationale behind the shape of the curve.

Conclusions

For the use of NPs towards biological applications the characterizations of the proteins selectively adsorbed to proposed NP formulations is a recognized central aspect. Thanks to increasingly sophisticated proteomic and bio-informatics approaches, the list of specific biofluid proteins (especially from serum or plasma) found on various NPs is becoming more and more long and detailed. However, it is still to be remembered that the dynamic association and dissociation of a protein to and from the NP surface, is the first essential phenomenon governing the formation of the NPs' protein corona, and that its quantitative characterization is critically important. The dissociation constant (K_D) is a useful and intuitive thermodynamic parameter related to the efficacy, or affinity, of a given molecule to bind to NPs. In fact, the K'_D value ($K_D = K'_D$ for $n = 1$), the reciprocal of the association constant (K'_A), corresponds to the concentration of a certain protein ligand resulting in half saturation (50%) of the NPs maximal binding capacity (N_{max}), in the absence of other potential binders which may compete for attachment to the NP surface. Hence, the lower the K_D , the higher the affinity and, in the end, the lower the concentrations of a solute necessary to significantly cover the NPs surface. Clearly, for a systematic studies K_D values need to be determined with single purified protein ligands, while, within the context of complex protein mixtures, the actual concentrations of the potential protein binders and their reciprocal competitions for the NP surface will lead to the final binding extent of any ligand and hence to the actual corona composition. Nevertheless, the determination of both K_D and N_{max} values characterizing all the main corona-forming proteins may be an obliged step to understand and even simulate, with appropriate models, the corona composition at equilibrium and possibly to predict its evolution in changing physiological environments. Affinity determination is strongly desirable also because it would allow for objective and quantitative comparisons of the performance and features of different NPs and corona components, studied in different investigations.

With this perspective, in this review we have done a detailed literature survey, screening for those studies in which the interaction of proteins with NPs was approached in a quantitative way and wherefrom K_D values could be evinced and compared. In doing this we also described the different available methodologies that were applied, evaluating their strong points or their critical aspects and limitations. Data analysis and comparison indicated that, in spite of many years effort, only for few proteins like albumin and fibrinogen larger sets of data have been produced, while in other cases, the information is more limited and sometime sporadic. This is understandable, due to the extreme variability of the NPs developed or studied by the various groups. In some other cases, like for HRG, the protein was only recently identified as a functionally important actor in the corona of a single NP type (SiO_2 -NPs). At the same time, considering that NPs proteoma are available in several cases, our analysis indicates that we are only beginning to approach protein-NP interactions with a quantitative attitude.

With the above limitations, data comparison allowed us to draw some conclusions. First, the zeta-potential, which is related to the surface charge of the NPs, was found to be a strongly influential parameter determining protein binding. In fact, a general trend of increased affinity, or decreased K_D , in the case of moderately negative or positive NPs could be seen. Importantly, however, in agreement with some specific experiments present in one study, our vast comparison confirmed that among all possible methods used to obtain K_D , the Fluorescence Quenching approach (FQ) strongly underestimates K_D values and deviates from the above found trend.

A final consideration deserves the classical distinction between hard and soft NP coronas. The hard corona is popularly defined as the layer of bound proteins irreversibly or strongly bound to the NP while, in opposition, the soft corona is assumed to be formed by reversibly or weakly bound proteins. However, one can image a whole spectrum of affinities for different pairs of NP and proteins, as clearly emerged from our survey, with K_D values gradually ranging from mM to pM. This fact makes it difficult and also arbitrary to establish a clear-cut border between hard and soft binding. K_D values, being a continuous parameter, and being the ratio between the kinetics dissociation constant and the kinetics association constants, may be a more appropriate and less subjective way to define and to compare the strength and the reversibility of different NP-ligand complexes.

In the same way reversible *versus* irreversible binding may be the reason for differences in K_D values as obtained with the different experimental methods. Here the time scale on which measurements take place in relation to the time needed to reach equilibrium is of importance [43]. Reversible *versus* irreversible binding has only been scarcely discussed in literature. Treuel *et al.* have demonstrated that in case of ligand capped negatively charged NPs, *i.e.* NPs with a very thin shell rich in -COOH, protein binding is full reversible [44]. They refer this to the fact that in this case protein binding is fully controlled by electrostatic forces. In buffer the charge of the NP surface and the proteins is screened, and thus only local electrostatic interaction plays a role. In this way adhesion is driven by positive patches on the protein surface that bind to the negatively charged NP surface. Binding thus is dominated by Coulomb energy. This was demonstrated by modifying the charge of HSA by chemical modification, followed by determining K_D constants of the modified proteins [43, 44]. In contrast, for NPs coated with an amphiphilic polymer binding is also influenced by entropy [26, 88], which may be due to the hydrophobic parts in the inside of the polymer shell. This in turn may lead to some irreversible binding of proteins [43].

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