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Methods for the quantification of particle-bound protein – Application to reagents for lateral-flow immunoassays (LFIA)

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Protein immobilization for the functionalization of particles is used in various applications, including biosensors, lateral-flow immunoassays (LFIA), bead-based assays, and others. Common methods for the quantification of bound protein are measuring protein in the supernatant before and after coating and calculating the difference. This popular approach has the potential for a significant overestimation of the amount of immobilized protein since layers not directly bound to the surface (soft protein corona) are usually lost during washing and handling. Only the layer directly bound to the surface (hard corona) can be used in subsequent assays. A simplified amino acid analysis method based on acidic hydrolysis and RP-HPLC-FLD of tyrosine and phenylalanine (aromatic amino acid analysis, AAAA) is proposed to directly quantify protein bound to the surface of gold nano- and latex microparticles. The results are compared with indirect methods such as colorimetric protein assays, such as Bradford, bicinchoninic acid (BCA), as well as AAAA of the supernatant. For both particle types, these indirect quantification techniques show a protein overestimation of up to 1700% compared to the direct AAAA measurements. In addition, protein coating on latex particles was performed both passively through adsorption and covalently through EDC/sulfo-NHS chemistry. Our results showed no difference between the immobilization methodologies. This finding suggests that usual protein determination methods are no unambiguous proof of a covalent conjugation on particles or beads.

Introduction

Quantification of the protein content in a solution is a well-explored field, and a variety of methods are available for such tasks. In contrast, the quantification of surface-bound proteins is not trivial. This discrepancy in difficulty leads to workarounds using protein quantification techniques for proteins in solution (e.g., BCA assays, Bradford assays, UV/vis spectroscopy) to examine protein coatings. The easiest way is to determine the protein concentration in the supernatant of a protein solution before and after the coating had been performed. The difference in concentration is assumed to be equivalent to the protein "bound" to the surface.[1]

Proteins are known to associate with surfaces of particles in distinct layers.[2-4] The protein coating thickness increases with the excess of protein used for coating. This represents an enrichment of protein on the surface of the particle. The layer directly associated with the surface (hard protein corona) generally is strongly bound up to the point of being practically irreversibly immobilized. In standard immunoassay formats like enzyme-linked immunosorbent assays (ELISA), a protein-coated surface (polystyrene) may undergo multiple washing steps, and the protein reagents remain immobilized to a large extent. Outer layers (soft protein corona) are based on protein-protein interactions and may slowly leach from the surface or are quickly removed during buffer exchanges or washing steps with surfactants (Fig. 1).[3-7] For this reason, non-crosslinked multilayers are not useful for heterogeneous immunoassays in most cases.

Simple supernatant-based quantification techniques do not sufficiently account for the loss of protein from the soft corona of a coated particle. Therefore, their results may greatly overestimate the amount of the relevant, firmly surface-bound protein. This potential for overestimation increases as the protein concentration for coating is increased. Furthermore, if only a small proportion of the total protein in solution is bound to the particles, the difference in protein concentrations in the

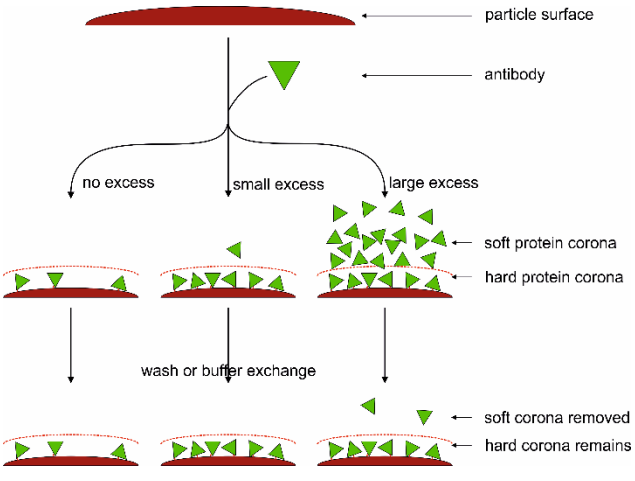

Figure 1: Protein layers in regimes of a low amount of protein relative to a potential monolayer, a small excess in which practically only the monolayer is formed, and a large excess in which more protein associates loosely with the monolayer but is removed easily during handling.

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supernatants before and after coupling may be relatively small. The statistical error of this value is comprised of the sum of two individual measurement errors. Hence the resulting error may be large relative to the result. In the case of a large excess, the difference might be even undetectable.

Gold nanoparticles (AuNPs) represent a popular option for designing lateral-flow immunoassays (LFIAs). Basic particle syntheses are relatively simple, and various techniques exist to shape them as desired[8]; they are commercially available in many different sizes and with different surface coatings. Gold nanoparticles are reasonably well behaved in terms of stability, monodispersity, suspendability, and bind protein readily, e.g., through strong sulfur-gold interactions.[9] Gold particles precipitate easily under high ionic strength conditions.[10] A protein coating is known to stabilize gold particles in suspension.[11] This change in behavior may be used to monitor the protein coating process by observing the color of such suspended colloidal gold: If sodium chloride solution is added to the AuNP suspension, non-stabilized AuNPs precipitate while protein-coated particles stay still in suspension (Fig. 2). Using varying amounts of protein for the coating process, followed by sedimentation with sodium chloride and measurement of UV/vis absorbance, serves as an indicator for the amount of protein needed to achieve a complete coating under the chosen conditions. This is not necessarily equivalent to the amount of protein firmly bound to the particles. However, this method is simple and often used for AuNP characterization because the instrumentation needed is readily available in most labs.

More recently, polystyrene particles (latex particles, LP) have grown in popularity in LFIA. They are even better behaved than AuNPs, offer easily modifiable surfaces, may be dyed, and have a substantially higher protein binding capacity per mass unit. In addition, they are not susceptible to precipitation with sodium chloride, which makes them easier to use, e.g., with buffers, such as phosphate-buffered saline (PBS).

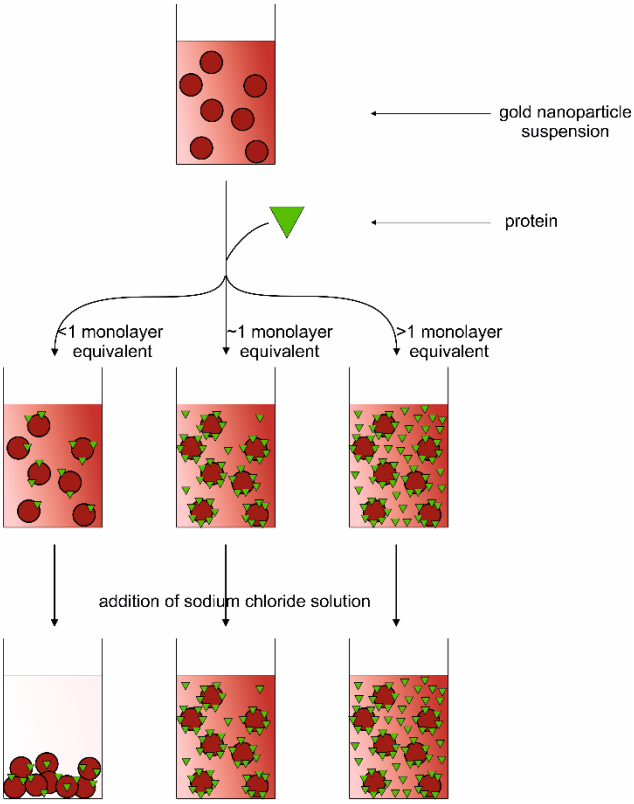

Figure 2: Gold-sodium chloride precipitation assay used to estimate the amount of protein needed to coat gold nanoparticles.

For the reasons discussed, methods for the direct quantification of protein on surfaces and particles may be highly preferable. Radiolabeling of protein and subsequent quantification of the surface-bound fraction was widely used, but requires working with radioisotopes, for instance, I^{125}.[12-15] Other, relatively expensive techniques such as hydrolysis and subsequent amino acid analysis or inductively coupled plasma mass spectrometry of sulfur in proteins have also been utilized for this purpose.[2, 16]

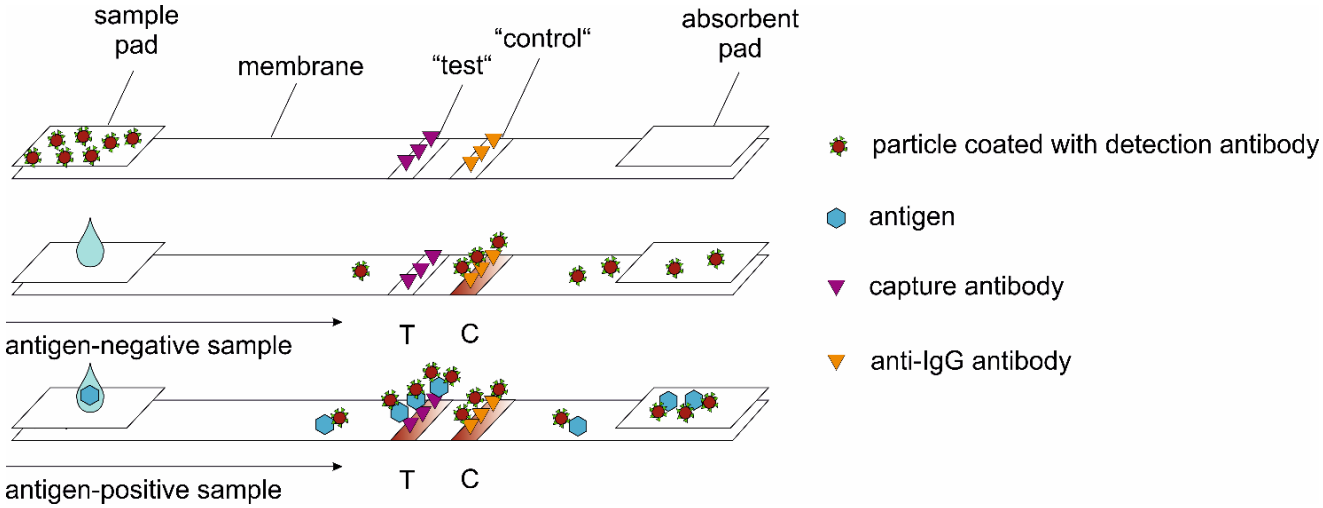

Figure 3: General setup of a lateral-flow immunoassay (LFIA); The sample fluid carries the antibody-particle conjugate to test and control lines of the strip. The control line (C) of the strip acts as a positive control for the assay. It turns colored, regardless of the antigen is present in the sample, by having the antigen coated onto that zone. The test line (T) turns only colored if the antigen-particle complex is captured by the antibody coated onto it and antibody-nanoparticle conjugates adhere to the captured antigen (sandwich immunoassay).

These methods tend to require laborious sample preparation, expensive instrumentation and come with their own issues. Further, the BCA assay also has been employed for direct protein quantification.[17, 18] This approach requires the desorption of the protein from the surface before the assay is performed. Its applicability has only been shown for reversibly adsorbed protein – covalently bound protein may be outside its reach.

The use of reagent proteins in general and particularly antibodies as coatings for nano- and microparticles has led to the widespread use of nanoparticles in immunoassays. Lateral-flow assays use the binding property of the antibody to specific antigens to bind and trap colored particles on specific lines on a membrane, thus rendering them visible (Fig. 3). The widespread use of such immunoassays in commercial and clinical applications necessitates implementing quality control measures.[19-28] This includes the characterization of the particles themselves as well as their coating since both influence the outcome of the assay.

In the present work, a derivatization-free variant of amino acid analysis based on fluorescence detection of aromatic amino acids (aromatic amino acid analysis, AAAA) is used to quantify surface-bound human antibodies directly and indirectly in a time- and cost-effective manner when compared to previously described techniques.[2, 29] It represents an improved version of a technique first described with UV-detection in literature.[30, 31] The results were compared to common supernatant-based, indirect approaches involving BCA and Bradford assays, as well as the precipitation of gold nanoparticles using sodium chloride. The direct AAAA methodology involves substantially faster hydrolysis of only one hour compared to traditional amino acid analysis. It also retains its specificity for protein-specific hydrolysis products and is applicable to a variety of particle types, as exemplified using this method on coated AuNPs and LMPs. The total analysis time of AAAA is around 2.5 hours.

Materials and Methods

Purification of human IgG

Centrifuge filter cartridges (Merck KGaA, Darmstadt, Germany; Amicon Ultra 0.5, 10 kDa MWCO) were conditioned with 450 µL of ultrapure water (13800 g, 5 min). First, 20 µL of polyclonal IgG solution (Jackson ImmunoResearch, ChromPure Human IgG, whole molecule, 009-000-003, 11.5 mg/mL, determined with BCA-assay calibrated with NIST 927e, Bovine Serum Albumin) were added to 80 µL of ultrapure water. The cartridge was centrifuged at 13800 g for 5 min. Then, the permeate was discarded, more water (350 µL) was added, and the filter cartridge was centrifuged at 13800 g for 5 min again. This step was repeated two more times, after which the cartridge was inverted, and the retentate was collected in a separate vial (1000 g, 5 min). The volume of retentate was adjusted to 400 µL with ultrapure water. The protein concentration of the diluted retentate was determined via BCA assay using the IgG stock solution as calibration standard (see “Bradford and BCA assays of supernatants”).

Sodium chloride precipitation of IgG-coated gold nanoparticles

10 µL of an AuNP suspension (nanoComposix, San Diego, CA, USA, BioReady Gold Nanospheres, bare, citrate, 40 nm, OD20) was transferred into wells of a non-binding microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany, 655901) and diluted with 190 µL of water, 1 mM phosphate (pH 7.4), 1 mM borate (pH 9.4) or 1 mM carbonate buffers (pH 10.0). A series of IgG dilutions ranging from 1.6 to 1150 µg/mL was prepared, and 10 µL of the diluted IgG solutions were added to the wells. The suspensions were incubated for 1 h at 750 rpm, and subsequently, 20 µL of sodium chloride solution (10 wt%) was added. Absorbance was measured at 520 nm after incubating the suspensions for 10 min at 750 rpm in an Epoch 2 microplate spectrophotometer (BioTek, Winooski, VT, USA).

Passive coating of gold nanoparticles

A 2 mM borate buffer (pH 9.5) was prepared. IgG was transferred into it following the 10 kDa centrifuge filter cartridge method described above. A total of 125 µL of IgG solution was used in five filter cartridges, and the retentates were pooled after exchanging the solvent with borate buffer. The final volume of the pooled retentates was adjusted to 1 mL with 2 mM borate buffer. Three 1 mL aliquots of AuNP suspension (OD 20) were diluted with 993, 967 or 837 µL of borate buffer (2 mM, pH 9.5), respectively, after which 7, 33, or 163 µL of purified IgG in borate buffer were added (1.3 mg/mL). The mixtures were incubated at 4 °C, 40 h. A fourth 1 mL aliquot of gold nanoparticle suspension using 1 mL of borate buffer (2 mM, pH 9.4) and no protein solution was carried along as a negative control.

Passive and covalent coating of latex particles

100 µL of latex particle suspension (Merck Estapor Microspheres, carboxyl-modified, K1-030 Blue) (10 wt%) were mixed with 1 mL of MES buffer (50 mM, pH 6) and centrifuged (18000 g, 7 min) afterward. The supernatant was removed and replaced with fresh MES buffer. This was repeated two more times. The particles are then centrifuged, suspended in 1 mL of MES buffer, and incubated (4 °C, 40 h) with 163 µL of IgG solution (1.3 mg/mL, in 2 mM borate buffer pH 9.5) and 837 µL of borate buffer (2 mM, pH 9.5). 100 µL of latex particle suspension (10 wt%) was conditioned, and the particles were transferred into 1 mL of MES buffer (50 mM, pH 6) as outlined before. Sulfo-NHS (200 mM) and EDC solutions (200 mM) were prepared in MES buffer, and 240 and 24 µL, respectively, were added to the latex particle suspension immediately after dissolution. The suspension was mixed and incubated (750 rpm, 30 min). The particles were centrifuged, the supernatant removed, and 1 mL of MES buffer was added. 163 µL of IgG solution (1.3 mg/mL, in 2 mM borate buffer pH 9.5) and 837 µL

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of borate buffer (2 mM, pH 9.5) were added. The mixture was incubated (RT, 750 rpm, 2.5 h; then 4 °C, 40 h). A negative control using 100 µL of a latex particle suspension, transferred into 1 mL of MES buffer and 1 mL of borate buffer (2 mM, pH 9.5), was carried along.

Separation of particles and supernatants for subsequent work

Both AuNP and latex particle suspensions were centrifuged (31,000 g, 15 min), the supernatants were collected, and the particles were resuspended in borate buffer (2 mM, pH 9.5, 1 mL). To wash the particles, this is repeated thrice, and the supernatants of these washing steps were collected for control experiments.

Bradford and BCA assays of supernatants

Bradford and BCA kits (Pierce/Thermo Fisher Scientific, MA, USA; 23236 and 23225, respectively) were used for this work. A serial dilution ranging from 0 to 115 µg/mL was prepared from an IgG stock solution (11.5 mg/mL) with borate buffer (2 mM, pH 9.5). BCA working reagent was prepared by adding 0.5 mL of copper sulfate reagent to 25 mL of BCA reagent. Bradford reagent was used as provided in the kit. 25 µL of the sample or standard solutions were pipetted into wells of non-binding microtiter plates. 200 µL of Bradford reagent or BCA working reagent were added. The plate in which the Bradford assay was performed was incubated for ten minutes at room temperature (750 rpm). The plate with the BCA assay was mixed and incubated for 90 minutes at 37 °C. Absorbances were then measured at 562 and 595 nm for BCA and Bradford assays, respectively, on a microplate spectrophotometer.

AAAA of protein in supernatants

L-Cysteine was dissolved in concentrated hydrobromic acid (48%) in a concentration of 1 mg per 60 µL of acid. 10 µL of sample suspension or solution and cysteine-HBr (60 µL) were mixed in a 300 µL, single-use crimp neck vial (BGB Analytik Vertrieb GmbH, Rheinfelden, Germany, 0800035 vial, 080302 cap). The vials were crimped shut and heated to 150 °C for 1 hour. Once cooled to room temperature, the hydrolysates were diluted 1:5 with ultrapure water, centrifuged (31,000 g, 15 min), and 200 µL of the supernatant was transferred into HPLC vials with suitable inserts. A commercial amino acid standard (Sigma, AAS18, 2.5 mM, in 0.1 N hydrochloric acid) was diluted with ultrapure water to give concentrations ranging from 0 to 20 µM, and the diluted calibration standards were transferred into HPLC vials. Samples and standards were injected in full loop mode (50 µL sample loop, 15 µL flush volume) and underwent chromatographic separation within 4.5 minutes. The HPLC instrument was a Knauer Azura P 6.1L pump with a high-pressure gradient valve, a 3950 autosampler, CT 2.1 column thermostat, and Shimadzu RF-20Axs fluorescence detector with a semi-micro flow cell. An Agilent AdvanceBio Peptide Mapping column (150 mm x 2.1 mm x 2.7 µm) with a 5 mm guard column was used. The column temperature was set to 40 °C. The mobile phases consisted of ultrapure water (A) and acetonitrile (B), both with 0.2% (v/v) of trifluoroacetic acid. The flow was set to 0.4 mL/min, the gradient was set to 10% B at 0 min, 40% B at 1.2 min, 90% B at 1.3 min, 90% B at 3 min, 10% B at 3.2 min, and 10% B at 4.5 min. Fluorescence was measured at 272 nm excitation/303 nm emission until 1.96 min and 260 nm excitation/280 nm emission afterward for L-Tyr and L-Phe, respectively. In addition to the supernatants, IgG solution was hydrolyzed to measure the ratios of Tyr and Phe to the intact protein.

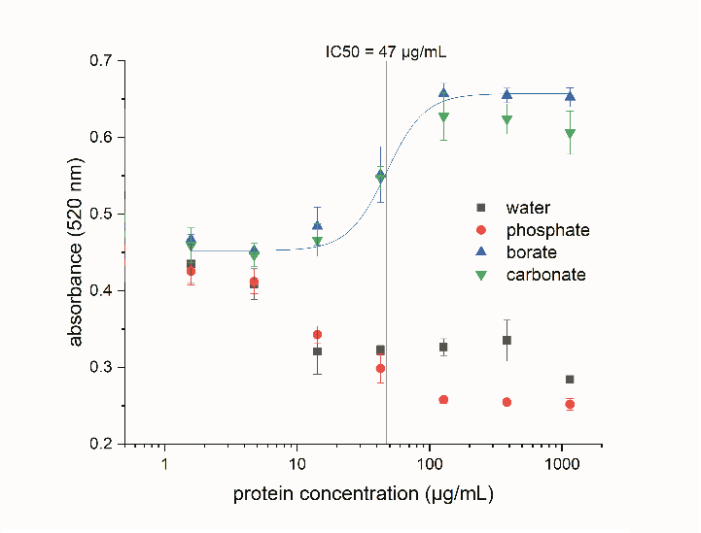

Figure 4: Sodium chloride precipitation curves using water, phosphate (pH 7.4), borate (pH 9.4) and carbonate buffers (10.0); all buffers were 1 mM.

AAAA of protein on particles

Pelleted particles were suspended in as little ultrapure water as possible on the last wash, typically around 30 µL. The volume was measured, after which 10 µL of suspension were mixed with Cysteine-HBr (60 µL) in single-use hydrolysis vials. The hydrolysis and HPLC procedures were as described above.

Results and Discussion

Sodium chloride precipitation as a method for the evaluation of coating conditions

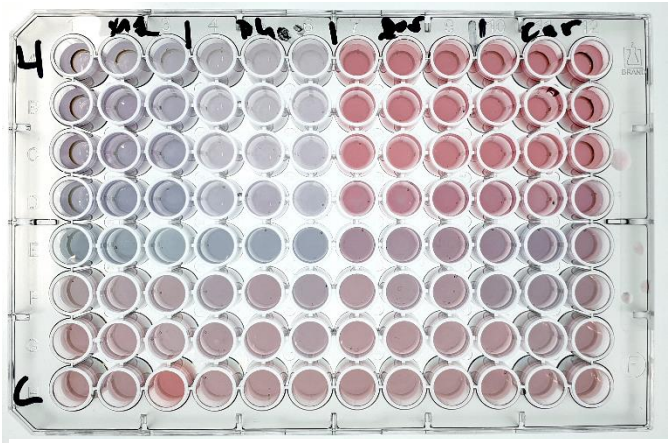

Figure 5: Sodium chloride precipitation experiment; a red color indicates AuNPs staying in suspension after coating with protein and incubation with sodium chloride solution; protein concentration is highest at the top and zero at the bottom. All experiments were performed in triplicate.

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Table 1: Bound human IgG, quantified from supernatant concentration by difference method.

Sample	AuNP [µg]	IgG used for coating [µg]	Bound IgG, estimated by concentration difference in supernatant [µg]		
			Bradford	BCA	AAAA from Tyr
Large excess	850	212	120 ± 10	74 ± 7	59 ± 21
Small excess	850	43	37 ± 3	29 ± 2	20 ± 14
No excess	850	9	6.1 ± 0.1	6.2 ± 0.1	4.0 ± 5.5

In this study, four solutions and buffers were tested: Pure water (pH 7.0), as well as phosphate (pH 7.4), borate buffer (pH 9.4), and carbonate buffers (pH 10.2). All buffers were prepared at a concentration of 1 mM to avoid the precipitation of the gold particles. Antibodies are frequently supplied in phosphate-buffered saline with biocides like sodium azide for better stability, as was the case here. Therefore, a buffer exchange to pure water was performed via 3 kDa centrifuge filters. The concentration of the buffer exchanged antibody solution was then measured with a BCA assay using the antibody stock solution and the supplier's concentration as a reference. The sodium chloride precipitation (Fig. 4 and 5) showed the expected behavior for borate and carbonate buffers: the higher the protein concentration, the higher the absorbance from the protein-coated AuNPs remaining in suspension after the addition of sodium chloride solution. In water and phosphate buffer, the gold particles exhibited the opposite behavior. For subsequent experiments, 1 mM borate buffer was chosen.

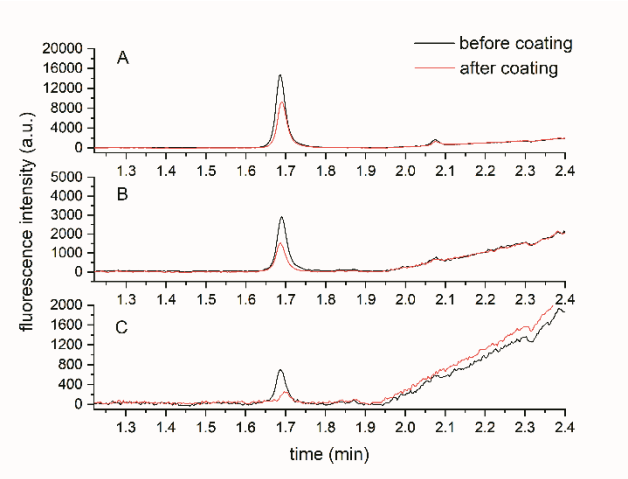

Figure 7: Chromatograms of supernatant hydrolysates, taken before and after coating from large (A), small (B) and no excess (C) samples

Quantification of AuNP-bound protein via supernatant analysis

Based on the surface area of the employed spherical 40 nm gold nanoparticles and the size of antibodies, it can be estimated that the mass of a monolayer of protein is only a few percent of the mass of the gold. An antibody sized at 6x12x14 nm is treated as a cuboid with its rectangular surfaces for this estimation. A side-on orientation (12x14 nm) of the Y-shaped antibody yields an antibody monolayer mass equivalent to 1.2% of the gold nanoparticle. An end-on orientation (6x12nm) yields the maximum theoretical monolayer mass and is equivalent to 2.7% of the mass of the gold nanoparticle. The IgG amount for coating was chosen accordingly at 1, 5, and 25% of the mass of gold, respectively. The lowest mass represents a no excess scenario in which all available protein might theoretically bind to the gold surface. 5% is estimated to be a small excess with a significant portion of the protein binding to the gold. 25% represents a large excess regime, where a fully saturated monolayer with an accompanying loosely bound soft corona and additional protein in the supernatant can be expected. Traditionally, a significantly larger excess may be employed for the coating of nanoparticles.

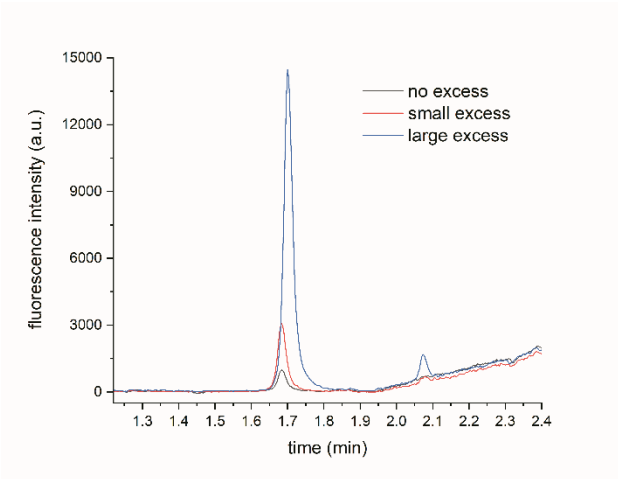

Figure 6: Chromatograms of supernatant hydrolysate samples taken before the coating of all three different IgG concentrations used; Tyr signal at approx. 1.7 min, Phe signal at 2.08 min

Indirect measurements quantify the amount of surface-bound protein by measuring the concentration in solution before and after the coating process. This allows for the use of simple colorimetric assays such as Bradford and BCA assays or UV(280 nm) absorbance. Amino acid analysis and any other advanced method may also be performed with the supernatants. Aromatic amino acid analysis (AAAA) of Tyrosine (Tyr) and Phenylalanine (Phe), as proposed in the literature as a simple substitute for AAA, has been further improved for this work and is employed here.

The results of performing Bradford and BCA assays as well as AAAA on supernatants of gold coating experiments have shown

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Table 2: Determination of bound IgG by hydrolysis of coated gold nanoparticles

Sample	AuNP mass [µg]	IgG used for coating [µg]	Bound IgG determined by hydrolysis of the protein layer [µg]	
			AAAA, from Tyr	AAAA, from Phe
Large excess	850	212	7.0 ± 0.9	7.6 ± 1.0
Small excess	850	43	3.4 ± 0.2	3.6 ± 0.9
No excess	850	9	0.35 ± 0.15	0.10 ± 0.10

similar trends for all three methods. Both colorimetric assays produce results of low deviation. AAAA struggles with sensitivity at low concentrations used in the no excess scenario (Fig. 6 and 7).

Direct quantification of particle-bound protein via AAAA

Coated and carefully washed particles can be subjected to direct hydrolysis, followed by chromatography. Washing the particles removes most of the remaining protein from the supernatant. After pelleting, the supernatant was removed, and the particles were resuspended. This concentrated the particles and the bound protein, facilitating more precise quantification, as shown in Fig. 8. The original hypothesis that practically all protein would be bound to the particles in such a situation could not be confirmed under our conditions. Experimental data suggest that in a no excess regime, only very incomplete coatings are achieved. Comparing the values presented in Tables 1 and 2, direct quantification of the protein on the particles leads to much lower results than indirect quantification. This follows the assumption that washing and handling of the particles remove loosely bound protein layers. The large excess situation shows, consistent with the soft particle corona hypothesis, the largest discrepancy as approximately 90% of the protein is lost from the particles during the washing steps.

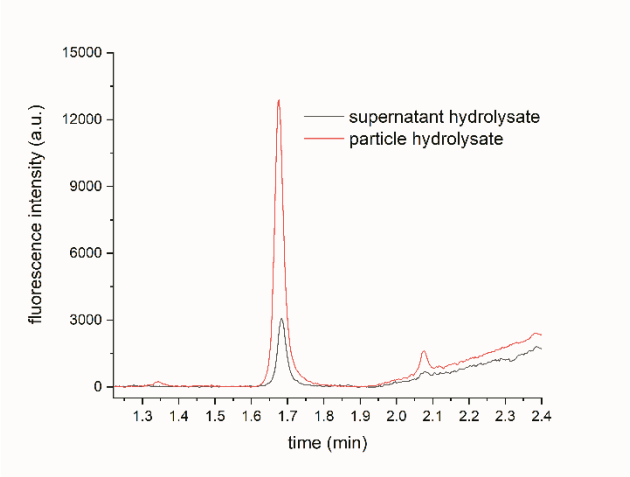

Figure 8: Comparison of chromatograms of large excess supernatant hydrolysate sample, taken before coating, and particle hydrolysate sample; increase in intensity observed in particle hydrolysate indicates protein enrichment in sample.

Monolayer mass equivalents in relation to the various methodologies

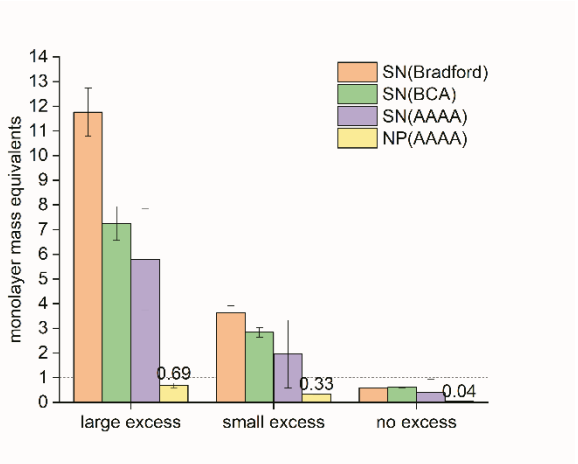

Figure 9: Measured monolayer mass equivalents in relation to the method being utilized and IgG amount used for coating on gold nanoparticles. One monolayer (side-on) mass equivalent corresponds to an IgG mass equal to 1.2% of the mass of gold; Particle (AAAA) values were obtained from direct hydrolysis and AAAA of particles (yellow), all others are based on supernatant (SN) measurements using the respective methods; all AAAA values are based on Tyr. The dashed line shows the equivalent of a single monolayer.

Fig. 1 shows the three scenarios targeted in this work: One in which the protein binds to the AuNPs in a low density, one in which a significant proportion of protein binds to the AuNPs, and one in which only a small proportion of protein binds to the AuNPs due to the large excess applied.

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Table 3: IgG bound to latex particles (LP)

Sample	LP mass [µg]	IgG mass used for coating [µg]	Bound IgG mass by supernatant [µg]			Bound IgG mass by particle hydrolysate [µg]	
			Bradford	BCA	AAAA (Tyr)	AAAA, calculated from Tyr	AAAA, calculated from Phe
Adsorption	100	212	246 ± 6	212 ± 8	179 ± 8	30 ± 3	30 ± 2
Covalent	100	212	248 ± 4	186 ± 8	179 ± 8	29 ± 2	30 ± 2

In Fig. 9, the values found in both direct and indirect measurements were converted into monolayer mass equivalents, i.e., how many monolayers could be obtained with this amount of protein mass.

An end-on orientation of the IgG molecules would lead to a mass ratio of 2.7% of protein related to gold. A more realistic side-on orientation would achieve around 1.2% of protein binding. Even with a large excess of 25 times this amount, only ca. 70% of such a single monolayer could be achieved. This might be caused by a non-oriented binding pattern on the surface, which leads to some smaller gold areas not being large enough to bind another IgG molecule.

Quantification of latex particle-bound protein via direct and indirect methods

Latex nanoparticles enjoy wide applicability in the field of lateral flow immunoassays. This means that their coating behavior needs to be characterized. Unlike gold, precipitation with sodium chloride is not an issue due to the particles' relative stability in high ionic strength solutions. Furthermore, due to their relatively low density, their binding capacity per particle mass unit is higher than AuNPs. Usually, a blocking step is required with a protein like bovine serum albumin (BSA) after coating with antibodies. Due to their higher binding capacity, it is not unusual that antibodies can be quantitatively bound to these particles, and the supernatant does not contain significant amounts of IgG anymore.

Common coupling protocols suggest the use of 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and (Sulfo-)N-hydroxysuccinimide (NHS) chemistry for the covalent conjugation of protein to carboxylated latex particles. However, due to the higher adsorptive capacity, it may happen that covalent coupling does not yield a larger amount of firmly bound protein. For this discussion, the amount of bound protein was measured both indirectly and directly with the described methods, differentiating between covalent coupling and passive adsorption of protein.

Table 3 shows the amounts of IgG bound to latex particles (LP). With one exception, no significant difference between covalent coupling and passive adsorption can be found for the supernatant results. It has been published in the literature, that NHS and sulfo-NHS are known to interfere with BCA assays, thus limiting its applicability in this particular scenario.[32, 33] Bradford leads to the highest values around 250 µg; it exceeds the nominal amount of protein used. In all cases, residual protein in the supernatant after coupling is close to the LoD, indicating that the particles seem to have a high binding capacity for proteins.

Direct particle measurements show excellent agreement with each other. Experiments with standard reference materials (NIST 927e, bovine serum albumin in solution) show a recovery of approximately 96 and 97% for Tyr and Phe, respectively. The direct measurement produces substantially lower results for the firmly bound protein. Unexpectedly, the values for adsorptive and covalent attachment are practically the same even after the particles were subjected to repeated centrifugation and resuspension in a fresh buffer. While this may suggest that covalent coupling is not necessary, further examinations regarding the functionality of the particles should be performed. Notably, because of the nature of the direct method, these particles were not blocked with another protein after the IgG coating step. If blocking is necessary, an aliquot of the particles needs to be examined before the blocking step is performed.

Conclusions

Quantification of surface-bound protein is not trivial. Simple methods that rely on differences in supernatant protein concentration seem to be not adequate to monitor the coating process. Protein desorption during the washing and handling of the coated nanoparticles is likely to occur due to a large proportion of protein not being bound directly to the particle but rather loosely associated with the protein on the particle (soft protein corona). Hence, the calculation of the difference in concentration of protein in the supernatant to determine the amount of surface-bound protein seems to be prone to highly misleading results, if not the protein consumption, but the firmly bound fraction needs to be quantified. These methods should be employed with caution. In contrast, direct methods, such as the use of radiolabeled proteins, aromatic amino acid analysis (AAAA), or traditional amino acid analysis (AAA), are preferable to quantify protein immobilized on nano- and microparticles as well as on other surfaces.

Author Contributions

Teodor Tchipilov: conceptualization, methodology, investigation, formal analysis, writing – original draft, writing – review, and editing. Anna Raysyan: conceptualization, methodology, writing – review, and editing. Michael Weller: conceptualization, project administration, supervision, writing – review and editing.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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