Quantification of adsorbed human serum albumin at solid interfaces: a comparison between radioimmunoassay (RIA) and simple null ellipsometry

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Abstract

Radioimmunoassay (RIA) and null ellipsometry are two common methods to quantify adsorbed proteins. However, the accuracy of null ellipsometry with a constant protein refractive index \( n = 1.465, k = 0 \) at \( \lambda = 632.8 \) nm has this far not been explored. The present study compared the methods, and the degree of agreement between the simplified single wavelength null ellipsometry and RIA to quantify adsorbed proteins was explored on different surfaces. The quantification methods agreed well when Ångström smooth hydrophilic or hydrophobic silicon surfaces, and freshly radio-labelled proteins were used. Some discrepancies were noted when either rough surfaces or stored and aged labelled proteins were used. The differences decreased when the aged protein solution was equilibrated with freshly dissolved proteins at room temperature (RT) for a few hours prior to the surface incubations. Significant differences were also noted between the methods when albumin was adsorbed at it’s iso-electric point (pH 4.8).

Keywords: RIA; Radioimmunoassay; Ellipsometry; Human serum albumin; Adsorption; Comparison; Hydrophilic; Hydrophobic; Silicon

1. Introduction

Null ellipsometry has proven to be an excellent tool for the quantification of adsorbed proteins on optically reflecting surfaces. When combined with antibodies, this method can be used (cf. ELISA) to detect the antigenic expression of adsorbed proteins [1]. Ellipsometry can be used for single protein as well as serum and plasma experiments. In the latter case it is then possible to study e.g. the activation of different humoral cascades like activation of blood coagulation [2] and complement [3].

Another classical method to quantify proteins is radioimmunoassay (RIA). In the present study human serum albumin (HSA) was labelled with \(^{125}\)I \( (\tau_{1/2} = 60 \text{ days}) \) and used in single protein
adsorption experiments. The surface activity after the adsorption was a measure of the total amount of adsorbed labelled proteins. This in turn was translated into surface mass density.

It was noticed in a recent work with mixtures of labelled and unlabelled HSA that the $^{125}$I-labelled HSA preferentially bound to polystyrene and silicon rubber surfaces [4]. The reasons for this were not well understood, but may be due to changes in the protein conformation, hydrophobicity or charge during the iodination procedure or due to storage. Therefore, care has to be taken while making experiments with mixed protein solutions. This aspect was also taken into consideration in this work.

The present study is a comparison between the two quantitative methods, and primarily an attempt to reveal the validity of the assumption of a constant protein refractive index ($n = 1.465, k = 0$) at $\lambda = 632.8$ nm in null ellipsometry. This is an assumption commonly made in simplified ellipsometric models. Similar studies with different approaches have been performed previously [5–8], and ellipsometry has also been compared with other methods [9–12]. However, no unequivocal results were obtained regarding the protein refractive indices of the proteins, $n_f$, in these models. Thus, the goal was to study whether this very simple ellipsometric model yields an acceptable agreement with RIA. It also turned out, however, that the use of radio labelled proteins is far from straightforward and filled with caveats. Subsequently precautions must be taken using either method.

2. Experimental methods

2.1. Surface preparations

Double sided polished silicon wafers with surface roughness $S_q = 2.8$ Å were cut into 5 by 10 mm pieces. The samples were cleaned and made hydrophilic by washing in Milli-Q water with 30% $\text{H}_2\text{O}_2$ (Kebo Lab, Sweden), 25% $\text{NH}_2\text{OH}$ (Kebo Lab) in proportions 5:1:1 for 5–10 min at 80°C followed by Milli-Q H$_2$O with 30% $\text{H}_2\text{O}_2$, 37% HCl (Kebo Lab) in proportions 6:1:1 for 5–10 min at 80°C. The samples were extensively rinsed with Milli-Q water before and after each washing step.

In order to prepare hydrophobic surfaces, hydrophilic silicon was rinsed three times in ethanol (denatured 99.5%, Kemetyl, Sweden) and subsequently three times in trichloroethylene (Labassco, Sweden). The samples were then placed in a solution of 1% v/v dichlorodimethylsilane (DDS) in trichloroethylene for 5 min, rinsed three-fold in trichloroethylene, followed by three rinses in ethanol. The samples were stored in 99.5% ethanol until use.

Pieces of hydrophilic silicon were etched in 40% KOH (Kebo Lab) in order to increase their surface roughness to $S_q = 20$ Å. Subsequently these were made either hydrophilic or hydrophobic.

Pyrolytic carbon (PC) samples with surface roughness $S_q = 204$ Å, were a kind gift from Pacesetter AB, Sweden.

2.2. Radio labelling procedure

The Iodo-Bead iodination method (Pierce, USA) was used for labelling of human serum albumin (HSA, 96–99%, Sigma, Sweden) with sodium $^{125}$I-iodine. The beads were rinsed in phosphate-buffered saline (PBS) at pH 6.5. The sodium iodide was mixed in PBS, and equilibrated for 5 min. Subsequently HSA (1 mg/ml) in PBS was added, incubated and gently stirred for 7 min. The solution with labelled HSA (without Iodobeads) was filtrated in desalting gel colons (Pierce, USA) equilibrated with PBS. From each fraction a small volume was taken to control the activity. The two colons with the highest activity was pooled. The pooled solution was dialysed together with 50 mM KI. Dialysis tubings of pore size 3500 D were used. Small volumes were taken from the dialysate (1000 ml, PBS) and the activity was checked. The protein solution was dialysed until the activity fell below 5000 CPM for 1 ml of the dialysate. The protein concentration was determined by spectrophotometry (Shimadzu UV-1601PC, USA) at 280 nm. A series of solutions with known concentrations of unlabelled HSA were used for the calibration.
The protein size and charge were controlled with SDS-PAGE and IEF (PhastSystem, Pharmacia, Sweden) before and after the labelling. This was done in order to ensure that the proteins were pure and that the labelling caused minimal effect on the protein charge and stability. No changes due to the labelling procedure were noted (not shown).

The labelled proteins were aliquoted and stored at $-80^\circ$C until further use. The aliquots were thawed in a water bath at room temperature (RT).

2.3. AFM

A NanoScope IIIa (Digital Instruments, USA) atomic force microscope (AFM) was run in Tapping Mode™. The surface roughness parameters ($S_a$ and $S_q$) and surface enlargement ($\Delta A$) were calculated with the built-in software on images with scan sizes 1 and 10 $\mu$m after a third degree plane fit (see Table 1). Three samples of each kind were analysed.

2.4. Adsorption procedure

Labelled and non-labelled HSA at different concentrations were diluted in PBS with 0.1 M NaCl added, at pH 7.4 or 4.8 and equilibrated for 1 h, if not otherwise stated. All experiments were performed at RT. The silicon and PC samples were placed in PBS buffer a few minutes prior to the addition of the proteins. After incubation the samples were rinsed with Milli-Q water and blown dry with nitrogen. To a great extent this precaution helps to avoid deposition of salts and particles from the ambient.

2.5. Ellipsometry

After protein incubation, each silicon sample was rinsed with Milli-Q water and dried with gaseous nitrogen and measured at three locations in air. The PC samples were measured in situ in a glass cuvette using a Rudolph Research AutoEL III bench ellipsometer at RT.

Non-protein incubated surfaces of each kind were used as references. The thickness of the adsorbed protein films were calculated by the built-in software for air measurements and the algorithm by McCrackin [13] was used for in situ determinations. The surface protein concentration was calculated according to the method outlined by Stenberg [14] for air measurements, and de Feijter [15] for in situ measurements. The assumed refractive indices for protein and buffer were ($n_f = 1.465$, $k_f = 0$) at $\lambda = 632.8$ nm and ($n_{buffer} = 1.335$, $k_{buffer} = 0$), respectively.

2.6. Radioimmunoassay (RIA)

After the ellipsometric determinations of adsorbed amounts of HSA the samples were transferred to a gamma counter (Packard Cobra II, Canberra, USA), and the activity of $^{125}$I-HSA was measured during 10 min. Samples incubated in non-labelled HSA were used as background references. In order to correlate the gamma counter value with the amount of adsorbed protein, known volumes with known concentrations of labelled proteins were measured in the gamma counter.

Control experiments with the $^{125}$I dialysate showed no, or very low, adsorption of free $^{125}$I to the test surfaces. In order to test a possible uptake of free $^{125}$I by adsorbed and non-labelled HSA, such surfaces were incubated in the $^{125}$I-contain-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Surface roughness (nm) and surface enlargement (%) for the different surfaces used</th>
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<tbody>
<tr>
<td>Scan size ($\mu$m)</td>
<td>$S_a$ (nm)</td>
</tr>
<tr>
<td>Hydrophobic silicon</td>
<td>0.22</td>
</tr>
<tr>
<td>10</td>
<td>0.33</td>
</tr>
<tr>
<td>Hydrophobic silicon</td>
<td>0.31</td>
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<tr>
<td>10</td>
<td>0.70</td>
</tr>
<tr>
<td>Etched silicon</td>
<td>1.49</td>
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<tr>
<td>10</td>
<td>13.72</td>
</tr>
<tr>
<td>Pyrolytic carbon</td>
<td>16.48</td>
</tr>
<tr>
<td>10</td>
<td>18.67</td>
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ing dialysate. No uptake of free iodine by the unlabelled HSA was observed.

2.7. Statistical methods

Since all samples were analysed by both quantification methods, a paired test was utilised. For each part of the present study the calculated surface concentrations on all samples and by both methods were included. Tests were made for the null hypothesis that the two methods yield no difference. In this case the reported significance levels indicate the probability that the alternative hypothesis (i.e. that there was a difference between the two methods) was erroneous. The abbreviations are * = 90%, ** = 95% and *** = 99% levels of significance, respectively. The asterisks were used in the figures when significant differences were observed.

The analysis of the agreement between the quantification methods was performed according to Bland and Altman [16–18]. The general idea is that two methods designed to measure the same type of quantity will always correlate. This is due to the fact that samples that cover a broad range of values will always show a high correlation between methods. When a paired study is performed, the plot of difference versus average of each pair will yield a better picture of a possible agreement between them.

3. Results

3.1. Surface roughness

The different samples were measured in Tapping Mode AFM at ambient conditions, using 1 and 10 µm scan sizes. The results are summarised in Table 1 and Fig. 1. The surfaces were evaluated in terms of $S_a$, $S_q$ and $\Delta A$. $S_a$ is the mean deviation from a theoretically flat surface, and $S_q$ the root mean square deviation from this surface. The

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Fig. 1. AFM images with scan size 1 µm and z-scale 100 nm. (a) hydrophilic silicon, (b) hydrophobic silicon, (c) KOH-etched silicon, (d) pyrolytic carbon.
surface enlargement, $\Delta A$, was in all cases too small to explain any differences between the methods and was not taken into account when the surface protein density was calculated. Hydrophilic and hydrophobic silicon showed similar topographies. After KOH etching of silicon an increase in all roughness parameters was noted. The higher values at the larger scan size were due to introduction of a waviness at the 10 $\mu$m scale by the etching. The pyrolytic carbon was much rougher than the silicon samples, as is also evident in Fig. 1.

3.2. Time course adsorption study

Hydrophobic and hydrophilic silicon samples were incubated for 1, 3 and 6 h in 0.1 mg/ml solutions of labelled HSA, see Fig. 2.

After 1 and 6 h of incubation of the hydrophobic silicon and after 1 h of hydrophilic silicon, significant differences were observed between the methods. No other differences were found. All differences between the methods were below 10% on hydrophilic and below 20% on hydrophobic samples. Thus, a very good methods agreement was observed on hydrophilic silicon. The hydrophobic silicon samples showed slightly different results. This may be due to a larger extent of denaturation of proteins, that may lead to changes in the protein refractive index, or different protein packing structures.

3.3. Dilutions of $^{125}$I-labelled HSA in unlabelled HSA

Labelled HSA was mixed with unlabelled HSA, and with a constant final concentration of 0.1 mg/ml. The calculated adsorbed amounts were similar by both methods when freshly labelled HSA, not older than 1 week was used, see Fig. 3. The RIA method indicated slightly lower values than ellipsometry on hydrophilic silicon. Small differences were noted on hydrophobic silicon, although overall a good agreement was observed between the methods.

3.4. Adsorption at different pH

Hydrophilic and hydrophobic silicon were incubated in 0.1 mg/ml solutions of $^{125}$I-labelled HSA for 1 h at pH 7.4 and close to the iso-electric point (pI) of HSA, pH 4.8. At the iso-electric point HSA possesses zero net charge and minimal volume, and Coulombic interactions between the proteins are then minimised. This led to increased adsorption on both hydrophilic and hydrophobic
silicon, see Fig. 4. The point of zero charge (pzc) of hydrophilic silicon is close to pH 2, and the surface was therefore negatively charged at both pH values. The ellipsometry and RIA values agree well at pH 7.4 (<10% difference) on both hydrophilic and hydrophobic silicon surfaces.

3.5. Etched silicon surfaces

The hydrophilic silicon became rougher after the KOH etching ($S_q = 20 \text{ Å}$). Ellipsometry now overestimated the adsorbed amounts on both hydrophilic and hydrophobic silicon surfaces compared to RIA, see Fig. 5. However, the differences were not statistically significant.

3.6. Adsorption to pyrolytic carbon

Pyrolytic carbon is a material that is used in biomedical applications such as implantable electrodes, i.e. for pacemakers. It is a rough material ($S_q = 204 \text{ Å}$) that scatters most of the incident light. The adsorbed amounts of protein were measured in situ because of the ill-defined optical properties. Drastic quantitative differences were now found between ellipsometry and RIA. The ellipsometric data indicated too low an adsorption, only 18 and 19% at pH 4.8 and 7.4, respectively, of the obtained RIA values, see Fig. 6. In other words, the indicated adsorbed amount was five times larger by RIA than by ellipsometry. At both of these pHs there exists a 99% (*** signifi-
Fig. 7. RIA-determinations of adsorbed amounts of HSA. 

\[ ^{125}\text{I}}\text{-HSA was diluted in unlabelled HSA at pH 7.4 with a final concentration of 0.1 mg/ml. The incubation time was 1 h at RT. (a) hydrophilic silicon incubated in freshly labelled HSA immediately after thawing and after 5 h of equilibration at RT. (b) same as (a), but with labelled HSA freeze-stored for 2 months. (c) freshly labelled and 2 months freeze-stored labelled HSA adsorbed to hydrophobic silicon. Neat-incubation in labelled HSA only, \( n = 12 \) except for the equilibrated in (a) and (b) where \( n = 24 \).

3.7. The effects of storage on \(^{125}\text{I}}\text{-HSA}

Fig. 7(a–b) show the RIA determined amounts of HSA on hydrophilic silicon when \(^{125}\text{I}}\text{-HSA was mixed and diluted in non-labelled HSA, before and after storage of the \(^{125}\text{I}}\text{-HSA for 2 months in a freezer (−80°C). In Fig. 7(a) freshly labelled HSA (＜1 week) was adsorbed onto hydrophilic silicon immediately after thawing and mixing, after 5 h of equilibration at RT. Fig. 7(b) shows the same as Fig. 7(a), but with the labelled HSA freeze-stored for 2 months. Fig. 7(c) shows a comparison between adsorption of freshly labelled and freeze-stored (2 months) labelled HSA without equilibration time onto hydrophobic silicon. In Fig. 7(a) the two curves almost coincide, indicating no change in the adsorption behaviour after <1 week of freeze-storage of \(^{125}\text{I}}\text{-HSA. In Fig. 7(b) we observe that the non-equilibrated solution deposited more proteins than the equilibrated onto hydrophilic silicon. The equilibrated solution in Fig. 7(b) shows values similar to those in Fig. 7(a). The tendency of a higher adsorption of \(^{125}\text{I}}\text{-HSA to hydrophobic silicon after protein freeze-storage and without a proper relaxation time (renaturation) is shown in Fig. 7(c) for freshly labelled and 2 months freeze-stored HSA. Interestingly, the RIA quantifications became similar to the ellipsometric when the proteins were allowed to equilibrate in solution for at least 4 h (data not shown).

3.8. Statistical analysis of agreement between single wavelength null ellipsometry and RIA

The paired values originating from respective quantification methods were pooled from the time study and dilution experiments (see Section 3.2 and Section 3.3) with freshly labelled HSA adsorbed onto flat hydrophilic and hydrophobic silicon at pH 7.4. For each pair of values the difference within each pair was plotted against the average of the pair, see Fig. 8.

The degree of agreement (bias and error) was evaluated with a paired t-test. For hydrophilic silicon, the bias and standard errors of the means were 19.4 and 3.4 and for hydrophobic silicon 4.6 and 6.7 ng/cm\(^2\) respectively. Thus, a significant \( (p ≪ 0.001) \) bias was observed for hydrophilic silicon, but not for hydrophobic silicon \( (z = 0.6) \). The bias of 19.4 ng/cm\(^2\) corresponds 1.6 Å and 4.6 ng/cm\(^2\) to 0.4 Å thickness of a protein layer, i.e. the adsorption biases are within the noise level of the methods.
The repeatability is an important aspect when methods are compared. The standard deviation of ellipsometry was $< 4 \text{ ng/cm}^2$ over the entire adsorption range, and was not amplitude-dependent. For the RIA method, however, an increase in the variation is expected as the values increase. The reason for this is that RIA data follow a Poisson-type of distribution, and hence the standard deviation is estimated as the square root of the mean. This may be a major reason for the observed higher standard deviation in the difference between the two methods on hydrophobic when compared to hydrophilic silicon.

4. Discussion

HSA is a globular protein with an effective diameter of $\approx 55 \text{ Å}$ [19]. If one assumes that the adsorption does not change its conformation, a close packed monolayer of HSA corresponding to $\approx 400 \text{ ng/cm}^2$ is formed. In Fig. 2, however, the maximum adsorbed amount on hydrophobic silicon was $\approx 250 \text{ ng/cm}^2$. Assuming that a monolayer was formed on the hydrophobic surface it is possible that the protein conformation was changed during the adsorption process and/or electrostatic repulsion occurred between the adsorbed proteins, which counteracted the formation of a close packed layer. Another explanation to this finding may be the jamming limit modelled for hard objects within the random sequential theory; thus, a confluent monolayer cannot be formed. A protein layer with an average protein surface density of 250 and 50 ng/cm$^2$ corresponds to jamming limits of $\approx 0.63$ and $0.13$, respectively.

It is generally believed that protein solutions should be used as fresh as possible. In our long time freeze stored protein study we found that it is advantageous to equilibrate the protein solution for some time, even overnight, before undertaking the experiments. The decrease in adsorbed amount of proteins from solution with increased equilibrium time was probably due to renaturation of the freeze stored and partially denatured protein.

4.1. Ellipsometry

Ellipsometry is a method where the state of polarisation of light, shifts after reflection. In real terms the ellipsometric angles $\Delta$ and $\Psi$ are determined. By using different models, with different assumptions about the optical properties of the surface, it is possible to calculate the thickness of the adsorbed protein film on flat surfaces from these angles ($\Delta$ and $\Psi$). The de Feijter [15], Cuypers [5] or Stenberg [14] formulas are then often used to estimate the amount of adsorbed mass per unit area. The former is used for in situ measurements in fluid and the latter for measurements in air. The de Feijter formula contains the increment of the protein refractive index as a function of protein concentration ($dn/dc$). This is normally determined with an Abbe refractometer under white light, and is an average over the visible wavelength range. The value of $dn/dc$ probably is dispersive as is the refractive index.

The calculated amount of adsorbed proteins depends in all models upon several model assumptions such as low surface roughness, and a fairly constant protein refractive index that is independent of different surface characteristics. In the worst case, the assumptions may result in misleading ellipsometric models and erroneous calculations. These models are presently not well calibrated by other methods.
Null ellipsometry with a single wavelength of light is often used e.g. in biomaterials research. This implies that assumptions on the refractive index \( n_i \) of the adsorbed proteins have to be made. Often \( n_i \) is set to \( \approx 1.5 \), i.e. \( k_i = 0 \); it is assumed that no adsorption of light takes place. This may cause problems, especially for thin films when the calculated refractive index and the thickness of the adsorbed layer are correlated.

In the present study the protein layer refractive index was kept constant \( n_i = 1.465 \), i.e. the same as for silicon dioxide at \( \lambda = 632.8 \) nm, 1.96 eV. This is close to the reported value for ovalbumin [20], between 1.4 and 1.5 in the visible range of light. Smith [10] indicated that the methods used by Stenberg [14] are relevant even at sub-mono-layer surface coverages. One of the aims of this study was to check the validity of this simplest possible ellipsometric model in relation to RIA.

The uncertainty in the small changes in the ellipsometric angle \( \Psi \), during measurements, makes it difficult to accurately calculate thin protein film thickness’. On the other hand, by using the McCrackin algorithm [13] the method gives a reciprocal correlation between thickness and refractive index, and a noisy signal in both values. However, due to this reciprocity, the calculated amount of adsorbed protein per unit area obtains a lowered noise level [20]. The refractive index of the adsorbed protein layer may hypothetically depend on the type of protein, its actual conformation and orientation, buffer, ionic strength, pH etc. The effective refractive index may therefore vary slightly between different experimental set-ups, although it is unknown to what extent. The details of this were outside the scope of this study.

The pyrolytic carbon had a comparatively rough surface. Therefore, a space overlap may be formed between the adsorbed protein layer and the substrate. The effective refractive index in the transition region will then be higher than that of the pure protein layer. This in turn may cause an underestimation of the protein layer thickness, leading to an underestimation of the surface protein concentration. In other words, the assumption used for the Fresnel equations (and the de Feijter formula) of a mathematically flat inter-

face is no longer valid here. Unfortunately it is not well explored as to how sensitive the Fresnel equations are to variations in surface roughness, although performed simulations indicate that rougher surfaces will yield differing thickness’ [21]. This was confirmed in a study where ellipsometry was performed on a series of roughened silicon surfaces [12]. In the present study ellipsometry clearly underestimated the adsorbed amount of protein on pyrolytic carbon compared to RIA.

An attempt was made to calculate the effective complex refractive index \( N = n_i + ik_i \) for the adsorbed protein layer on pyrolytic carbon. When the thickness was calculated from the RIA experiments (a density of dry protein of 1.37 g/cm\(^3\) gives a thickness of \( \approx 97 \) Å at pH 4.8 and 56 Å at pH 7.4) and the McCrackin algorithm was used, two solutions \( N = 1.38 + 0.01i \) and \( N = 1.58 + 0.63i \) were found. This can be compared with \( N = 1.65 + 0.7i \) for pyrolytic carbon. When assuming that \( k_i = 0 \), then \( d = 107 \) Å, \( n_i = 1.50 \) at pH 4.8 and \( d = 50 \) Å and \( n_i = 1.44 \) at pH 7.4 respectively. For \( n_i = 1.465 \) the calculations yield a thickness of 34 Å at pH 4.8 and 19 Å at pH 7.4, respectively.

Others have discussed the validity of ellipsometry at low surface coverage [6,10,15,22]. In the present study there were many instances with a sub-monolayer coverage. The actual measured ellipsometric thickness’ of the HSA layers (with \( n_i = 1.465 \)) were close to 5 Å on hydrophilic silicon and 20 Å on hydrophobic. Our simple ellipsometric model apparently agreed well with RIA, when freshly labelled proteins were adsorbed onto smooth surfaces.

4.2. RIA

Radio labelled proteins certainly are a valuable tool for studies on adsorption processes. Under the assumptions that no iodine desorbs from the \( ^{125}\text{I} \) labelled proteins, that free iodine has a low binding affinity to surfaces and proteins and that the labelled protein preserves its nativity, then the measured activity in this study are supposed to be well correlated with the ‘true’ amount of adsorbed proteins.
Furthermore, a very low desorption of $^{125}$I-labelled HSA was noted during 20 h of incubation of protein in buffer by van der Scheer [4]. Others have found protein dimers after $^{125}$I-labelling [4], but neither IEF nor SDS-PAGE control experiments made in the present study indicated the presence of protein aggregates (not shown).

However, incubations of surfaces in low protein concentration solutions may give misleading results as adsorption to the chamber walls and diffusion limitations may occur and restrict the number of protein collisions with the surface. This may then lead to an overestimation of the amounts of adsorbed proteins by RIA. In the present study, we used 0.1 mg/ml solutions, which is a fairly high concentration. No decrease in the activity in the solutions before and after the incubations were observed. Also, a preferential adsorption of labelled proteins may be a problem in mixed protein systems; and probably, also in complex solutions like plasma [23]. In the present study the apparent adsorbed amounts increased at high dilutions of $^{125}$I-HSA in native HSA, see Fig. 7(a). This was, however, most likely a methodological bug because at these dilutions the activity was close to that of the background. This again underlines that the experimental set-up and manual handling of reagents and samples are extremely important for the final outcome (see also the different RIA values for hydrophilic silicon in Figs. 3 and 7(a)). The iodination process includes one oxidation step of the protein in question and exposure of this to high doses of ionising radiation. The $\gamma$-energy of $^{125}$I is 35.5 keV, with a relatively high cross section for the uptake by biological molecules. The probability then increases for the creation of Auger electrons which leads to an increased risk for protein ionisation and destruction. The above mechanism is important because this may add to the altered biological behaviour and adsorption properties, such as protein charge, hydrophobicity and flexibility. The $\gamma$-radiation may also split water, i.e. radicals could hypothetically disturb the integrity of biological molecules in the solution.

Another important issue is the freeze storage of proteins [24,25]. During freezing, the pH is lowered and the process leads to conformational changes in the protein. Chang suggests that denaturation of freeze-dried proteins is a result of an increased area of the water-ice interface [25]. This source of erroneous surface concentration calculation might become highly significant at low $^{125}$I-HSA/native HSA ratios (see Fig. 7), and during short equilibration times for the thawed protein mixtures. The reason for this seems to be a higher surface preference of the partly unfolded and denatured $^{125}$I-HSA, an error that may later be multiplied with the dilution factor. In the present study the observation was that during short equilibration times of the thawed labelled protein, the calculated adsorbed amount to hydrophilic silicon was up to 16 (data not shown) times too high at high dilutions. This could be corrected for, after an equilibration time of 4 h or more of the mixed protein solution at RT.

4.3. Comparison of the quantification methods

The main goal of the present study was to compare single wave length null ellipsometry with a pre-fixed protein refractive index ($n_f = 1.465$, $k_f = 0$), and RIA by use of $^{125}$I labelled and non-labelled HSA. The adsorbed amounts were measured with both methods. This made it easier to compare the methods, although the size of the probed area differs in respective case.

The statistical comparison of the methods relies on asking the proper questions, such as what is the repeatability of the methods and their relative bias [18]. The latter question may be answered from the results in Fig. 8, indicating a very small bias on flat silicon surfaces. The choice not to make a correlation analysis was due to the fact that correlation gives a measure of the association [18] and not of the desired agreement between the methods.

There exist many pitfalls for each method, although the understanding of them makes it easier to interpret some of the noted deviations. Overall, the methods agreed well on (Ångström scale) flat surfaces when freshly labelled proteins were used. The pyrolytic carbon surface was no longer smooth as is assumed in the McCrackin algorithm, and as expected, the simple model failed on this surface.
The dilution of $^{125}$I-HSA in HSA, and a constant total concentration requires careful handling and equilibration or refolding time for the labelled protein. This can be conveniently arranged, e.g. with overnight dialysis of the free iodine at 4°C.

As there inherently exist many difficulties in both methods, we do not feel confident with the statistical differences that occasionally show up between them on flat surfaces. On the contrary, in the case of pyrolytic carbon and aged labelled proteins there existed in this study a ‘safe’ significant difference between the quantification methods.

4.4. Summary

Simple ellipsometric quantification of adsorbed amounts of HSA was compared with $^{125}$I-labelled HSA which was quantified in a scintillation counter. It turned out that the methods agreed well on smooth hydrophilic and hydrophobic silicon, when freshly labelled HSA was used. Under certain circumstances, when frozen and long time stored labelled HSA was used, discrepancies appeared. The simple ellipsometric method with fixed protein refractive index, $n_f = 1.465$, grossly underestimated the adsorbed amount on rough pyrolytic carbon as compared with RIA. Thus, caution should be exercised about ellipsometric determinations of adsorbed amounts of proteins in such cases.

Overall, RIA and single wavelength null ellipsometry show a good agreement at smooth hydrophilic and hydrophobic silicon surfaces and at different pH (4.8 and 7.4).

Labelled proteins that were freeze-stored for 2 months indicated higher RIA values than freshly labelled. The discrepancy could be eliminated when the protein mixtures were allowed to settle (refold) for at least 4 h prior to the adsorption experiments.

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