Effect of temperature, pH and salt concentration on β-lactoglobulin deposition kinetics studied by OWLS

Marek Kroslak[#], Jan Sefcik^{\$}*, Massimo Morbidelli[#]*

[#]Institut für Chemie- und Bioinginieurwissenschaften, ETH Zürich, 8093 Zürich, Switzerland

^sDepartment of Chemical and Process Engineering, University of Strathclyde, James Weir Building, 75 Montrose Street, Glasgow, G1 1XJ, Scotland, UK

J. Sefcik: Phone: +44 141 5482410, Fax: +44 141 5522302, E-mail: jan.sefcik@strath.ac.uk

M. Morbidelli: Phone: +41 1 6323033. Fax: +41 1 6321082, E-mail: morbidelli@chem.ethz.ch

Deposition kinetics of β -lactoglobulin at a solid-liquid interface was studied with optical waveguide lightmode spectroscopy (OWLS) over a range of temperatures between 61°C and 83°C. A new temperature-controlled cell for OWLS measurements allows fast, on-line monitoring of the deposit formation at elevated temperatures. Primary protein layers were deposited at 25°C in order to precondition and stabilize the waveguide surface. Sustained deposition lasting from few minutes (around 80°C) to hours (below 70°C) resulted in multilayer deposits up to several tens of nanometers thick. The measured deposition rates were strongly influenced by temperature, pH and NaCl concentration. Deposition rates were decreasing with increasing pH from 5.5. to 7.4, in a trend similar to that for non-covalent aggregation of β -lactoglobulin in solution. Activation energies for deposition rates were decreasing with increasing pH, from 340kJ/mol at pH=5.5 to 230kJ/mol at pH=7.4, and were similar to the activation energies for denaturation of β -lactoglobulin in solution.

1 Introduction

β-lactoglobulin (β-LG) plays a crucial role during heat treatment of milk. It is the main protein in whey, comprising about 50% of the total whey proteins in bovine milk. At room temperature and at pH between 5.2 (the isoelectric point) and 7 it is well soluble in water, where it exists mainly as a dimer stabilized by hydrogen bonds ¹. At these conditions β-LG spontaneously adsorbs on most surfaces from aqueous solutions, resulting in an irreversibly bound single layer film. Further deposition on primary β-LG layers is very slow unless heat treatment is applied. At temperatures typical for milk processing (such as pasteurization) between 60°C and 90°C, voluminous deposits are rapidly formed at solid surfaces, leading to serious fouling problems. It is well known that fouling phenomena at this range of temperatures is related to thermal denaturation of β-LG ².

During thermal exposure at pH above the isoelectric point, the unfolding of native globular structure exposes the hydrophobic region of β -LG, containing disulphide bonds as well as one free thiol group ³. Moreover, at pH between 7 and 9 there is a gradual conformational change so that at pH=9 the thiol groups are exposed even at the ambient temperature ^{4, 5}. As a consequence of this reversible unfolding, the chemical reaction of the thiol group with disulphides bonds results in protein aggregation and thus irreversible denaturation. In addition to the covalent bridging via disulphide bonds, there is also physical (non-covalent) aggregation due to exposure of the hydrophobic residues, resulting in a complex aggregation mechanism influenced by temperature, pH, salt concentration, surfactant presence and protein concentration ^{1, 4, 6-11}.

Denaturation and aggregation of β -LG can thus be described in two successive, yet interlinked steps. The first step is a reversible transformation between the protein native state and the unfolded state, where unfolding follows a simple first order kinetics. Upon unfolding the inert hydrophobic part and the thiol group are exposed and become reactive, which allows for irreversible denaturation of the unfolded protein to proceed. The second step is then aggregation of unfolded proteins through covalent (irreversible reaction of thiol groups) and non-covalent mechanisms resulting in protein denaturation. Kinetics of denaturation is usually experimentally determined by monitoring time evolution of the fraction of protein remaining in its native state. Depletion of the native protein concentration in time is customarily described in literature ^{9, 10, 12, 13} using the n-th order kinetic expression

$$\frac{dN}{dt} = -k_{den}N^n \tag{1}$$

where *N* is the concentration of native protein and k_{den} is the corresponding denaturation rate constant. Depending on the experimental conditions, the apparent reaction order *n* obtained by fitting experimental data was found to vary between 1 and 2. Since the overall process involves the first order unfolding followed by multiple reaction and aggregation steps, it is not surprising that a complex dependence of denaturation kinetics on temperature, pH and protein concentration is observed.

Denaturation temperature of β -LG (i.e., the temperature where unfolding and subsequent depletion of the native form becomes observable) decreases from about 75°C at pH~5 to 60°C at pH~5, 50°C at pH=8 and 25°C at pH=9⁻¹. Measured denaturation rates increase with increasing pH between 5 and 8, while the activation energy corresponding to the denaturation rate constant in Eq. (1) decreases with increasing pH ¹⁴. It was found that denaturation rates of β -LG are closely related to the availability and reactivity of the free thiol groups, which increases with pH ¹⁵, so that denaturation kinetics is primarily driven by covalent bonding of unfolded proteins. On the other hand, formation of large β -LG aggregates as monitored by light scattering was found to be primarily driven by non-covalent physical aggregation.

Kinetics of physical aggregation of β -LG becomes slower with increasing pH between 5 and 8, due to increasing negative charge of the protein as pH is moving away from its isoelectric point ^{15, 16}.

Although there have been numerous studies on thermal stability, aggregation and fouling in β -LG solutions, there has been only few experimental studies addressing kinetics of early stages of B-LG deposition at solid surfaces at elevated temperatures. It was noted early on that the macroscopic fouling by β -LG becomes faster as pH is decreased from 7 to 5, in opposite to the trend observed for β -LG denaturation, and it was suggested that the deposition kinetics of β -LG essentially follows its aggregation kinetics in the bulk ¹⁷. Ellipsometry and reflectometry was used to study early stages of deposition kinetics of β -LG on chromium oxide, stainless steel and modified stainless steel surfaces. Elofsson et al. ¹⁸ studied deposition of rather concentrated solution of β -LG (48g/L) in phosphate buffer with 0.1 M NaCl at pH=6.9 and temperatures 65-68°C at chromium oxide. Jeurnink et al. ² used reflectometry to study effects of preheating on deposition of whey protein isolates and β -LG at various concentrations in water at pH=7.1 at temperatures 70-95°C on chromium oxide surfaces. Santos et al.¹⁹ used ellipsometry to study deposition of whey protein isolates at various concentrations in water at pH=6.7 and 7.8 at temperatures 85°C. Variously modified stainless steel surfaces were used, including one with a sol-gel based silica surface. They found that while most surface modifications did not change the deposition rate very much compared to the original stainless steel, some modifications resulted in about twofold reduction in the long term deposition rate, while the silica surface showed an about twofold increase. This also confirms that there is only a limited effect of the original surface properties on long term fouling rates after a multilayer deposit is formed on the solid surface.

In this work we present measurements of heat induced deposition rates of β -LG using optical waveguide lightmode spectroscopy (OWLS). OWLS belongs to a class of optical reflection techniques including also ellipsometry and reflectometry. It is an established technique to follow in-situ adsorption

and deposition at solid-liquid interfaces, especially for proteins at ambient temperature. We use OWLS to determine the kinetics of β -LG deposition at the silica-titania waveguide surface over a broad range of temperatures and pH values.

2 Methods

Optical waveguide lightmode spectroscopy is based on the change of the effective refractive index of an optical waveguide when a thin film forms at the waveguide surface. The presence of the thin film changes the evanescent electromagnetic field in a close vicinity of the waveguide surface and it affects the light reflection from the waveguide surface. This results in a shift of the incidence angle of a light beam leading to a lightmode guided along the waveguide. By measuring the incidence angle α corresponding to the resonance maximum of the light incoupled into the waveguide it is possible to determine an effective refractive index *N* of the light propagating along the waveguide as follows²⁰:

$$V = n_{air} \sin \alpha + l\lambda_0 / \Lambda \tag{2}$$

Here Λ is the grating period, l is the diffraction order (l=1), λ_0 is the laser beam wavelength in vacuum, α is the incoupling incidence angle for the resonance maximum and n_{air} is the refractive index of air. Under resonance conditions, the phase change that the electromagnetic wave undergoes during a round trip across the waveguide is equal to an integer multiple of 2π . By round trip we mean the reflection at the interface (F,C), between the liquid bulk (C) and the waveguide film (F), the crossing of the waveguide and than reflecting back at the opposite interface (F,S), i.e. the interface between the waveguide film (F) and its glass support (S). The zeroth order mode equation for such a standing electromagnetic wave propagating indefinitely along an asymmetric planar waveguide is given by:

$$0 = 2k_0 t_F \sqrt{n_F^2 - N^2} + \Phi_{F,S} + \Phi_{F,C}$$
(3)

The above equation gives a relation between the measured effective refractive index and the optogeometric parameters of the composite waveguide. Here $\Phi_{F,S}$ and $\Phi_{F,C}$ denote the phase shifts

upon reflection from the waveguide surfaces, $k_0 = 2\pi/\lambda_0$ and n_F , t_F represents the refractive index of the waveguide film and its thickness, respectively. Assuming that all optical interfaces are abrupt and the layers are homogeneous and isotropic, Eq. (3) can be written for both the transverse electric (TE) and the transverse magnetic (TM) modes as follows:

$$0 = k_0 t_F \sqrt{n_F^2 - N^2} - arc \tan\left[\left(\frac{n_F}{n_S}\right)^{2\rho} \left(\frac{N^2 - n_S^2}{n_F^2 - N^2}\right)^{1/2}\right] - arc \tan\left[\left(\frac{n_F}{n_C}\right)^{2\rho} \left(\frac{N^2 - n_C^2}{n_F^2 - N^2}\right)^{1/2}\right]$$
(4)

where $\rho = 0$ for the transverse electric (TE) mode and $\rho = 1$ for the transverse magnetic (TM) mode, n_s is refractive index of the substrate (below the waveguide), and n_c is the bulk medium refractive index (above the waveguide). If we measure the effective refractive indices N(TE) and N(TM) in the absence of any additional layer at the waveguide surface, and the refractive indices of both the substrate n_s and the cover medium n_c are know, then we can solve the two equations (Eq. (4) for TE and TM) to calculate the unknown waveguide refractive index and thickness, n_E and t_E .

When a thin film is present at the waveguide surface, its optical properties with respect to the reflected light can be represented by the excess polarization density γ^{21} . The phase shift $\Phi_{F,C}$ at the waveguide surface can be also expressed in terms of γ , so that a generalized form of Eq. (4) is obtained ²², accounting for the presence of the thin film. This equation is then solved for γ , resulting in the following:

$$\gamma = \left(\frac{(n_F^2 - n_C^2)}{(n_F^2 - N(TE)^2)}\right)^{0.5} \left(\frac{-k_0 t_F (n_F^2 - N(TE)^2)^{0.5} + arc \tan\left(\frac{(N(TE)^2 - n_C^2)}{(n_F^2 - N(TE)^2)}\right)^{0.5} + arc \tan\left(\frac{(N(TE)^2 - n_S^2)}{(n_F^2 - N(TE)^2)}\right)^{0.5} \right)$$
(5)

The excess polarization density is related to the distribution of optical density at the interface ²²:

$$\gamma = k_0 \int_0^{\infty} (\mathcal{E}_{\Box}(z) - \mathcal{E}_0(z)) dz = k_0 \int_0^{\infty} (n_{A\Box}^2(z) - n_C^2(z)) dz$$
(6)

where the subscript || refers to the direction parallel to the waveguide surface and n_A is the refractive index of adsorbed thin film. We note that the dielectric constant of the non-adsorbing medium is equal to the second power of its refractive index ($\varepsilon = n^2$). In the case of optically homogeneous isotropic film the integral in the above equations simplifies to $Md\varepsilon/dc$, i.e. the total mass deposited per unit area of the film multiplied by the derivative of the dielectric constant with respect to the mass concentration of deposits. The corresponding value of $d\varepsilon/dc$ applicable for various protein solutions and thin films is 0.527 cm³/g²³. We note that the value of $d\varepsilon/dc$ varies only very little (less than 1%) with temperature between 25 and 80°C. Therefore the deposited mass can be expressed for all temperatures considered here in terms of the excess polarization density γ as follows:

$$M = \frac{\gamma}{k_0} \frac{dc}{d\varepsilon} \tag{7}$$

Using Eq. (5) and (7) we can calculate the deposited mass per surface area M, from the experimentally measured value of N(TE).

3 Experimental Section

 β -LG from bovine milk used in this study was from Fluka (β-Lactoglobulin A and B, purity ~80%, Lot # 1087564). Protein solutions were prepared by proper addition of β-LG into 10mM HEPES buffer from Acros Organics (Lot # A019289801). Buffers were adjusted to target pH and salt concentration by NaOH and NaCl addition, respectively. Protein solutions were left to equilibrate for at least two hours before being used or stored at 4°C for a maximum of three days. Ultra pure water (Millipore, 18mΩ) was used for solution preparation. All solutions were degassed under vacuum before use in order to reduce creation of bubbles in the OWLS cell.

Deposition experiments were performed using the instrument OWLS 110 made by Microvacuum Ltd, Budapest, Hungary, with the integrated temperature control unit OWLS TC. The OWLS

waveguides (75% SiO₂, 25% TiO₂) were supplied by Microvacuum. The waveguide layer of thickness $t_f \approx 180 nm$ and refractive index $n_f \approx 1.76$ (at 25°C) is supported by glass substrate of refractive index $n_s = 1.52578$ with dimensions $12 \times 8 \times 0.5 mm$. The waveguides were stored in a NaOH solution at pH=11 for 3 hours to uniformly activate hydroxyl groups on the surface prior to protein deposition. They were subsequently rinsed with water and dried with nitrogen. The waveguide was placed adjacent to the flow-through cell in the shape of a rectangular channel (8mm long, 0.8mm high, 2mm wide) with the total volume of 12.8µl, with entrance and exit ports. All delivery tubes and the measuring cell were made of Teflon[®].

Prior to the β -LG deposition, a buffer solution was flowed through the deposition cell and the waveguide parameters were determined. The flow rate was held constant at 2ml.hr⁻¹ (corresponding to a residence time of about 30s in the deposition cell) by the programmable syringe pump Vit-Fit (Lambda, Czech Republic). The syringe pump provided steady injection of the sample without flow rate pulses, which is particularly important for temperature measurements, because fluctuating flow rates induce oscillations of the fluid temperature, resulting in substantial noise in the experimental data. The response of the bare waveguide at elevated temperature was recorded prior to the deposition measurements.

4 Results and Discussion

4.1 Primary layer creation at ambient temperature

Primary exposure of oxide surfaces to protein solutions typically leads to spontaneous adsorption of thin protein layers. In this work, bare silica-titania waveguides were first equilibrated at 25°C with the blank buffer solution at the flow rate of $2ml.hr^{-1}$. Then the waveguide was exposed to a β -LG solution in 10mM HEPES, pH 5.5, at the standard flow rate for 60 minutes to create a primary protein layer. In

Figure 1 we show the time evolution of the deposited mass of β -LG on the bare waveguide surface for two subsequent exposures to 0.5mg.ml⁻¹ solution of β -LG followed by rinsing with the blank buffer.

The first exposure to the β -LG solution for 60 minutes resulted in the total primary layer coverage of 1.4mg.m⁻². After reaching this coverage a further growth of deposited mass becomes very slow. Subsequent desorption in the blank buffer for 60 minutes resulted in a decrease of the deposited mass to 0.7mg.m⁻². The subsequent exposure to the same β -LG solution for another 60 minutes resaturated the surface to the same total coverage as observed in the first exposure. Also, the second desorption resulted in the same deposited mass as in the first desorption. This mass of 0.7mg.m⁻² corresponds to the irreversible primary layer, where the protein is irreversibly bound to the waveguide surface so that it cannot be removed from the surface by desorption in the blank buffer at 25°C.

The density of the primary layer depends on various factors like pH, salt concentration, protein concentration, and surface properties. We examined the effect of solution pH on the primary layer deposition from 10mg.ml^{-1} β -LG in 10mM HEPES for the range of pH 5.5-7.4. Experiments showed the same deposition pattern as discussed in Figure 1, i.e., alternating between the total primary layer coverage and the irreversible primary layer coverage, as for 0.5mg.ml⁻¹ β -LG solution discussed above. The values of mass corresponding to the total primary layer coverage after one hour deposition at ambient temperature as well as to the irreversible primary layer coverage after one hour rinsing with the buffer are shown in Figure 2 as a function of pH.

The observed adsorption behavior of β -LG is consistent with the classical protein deposition mechanism ^{24, 25}. The adsorbed protein is present in two states: one irreversibly and the other reversibly adsorbed. Proteins slowly undergo conformational changes after being adsorbed on oxide surfaces and these changes result in a stronger interaction between protein and surface. Such strongly bounded

proteins loose their native structure, spread in time over the surface thus hindering further protein irreversible deposition. Reversibly bound proteins are weakly attracted to the surface, which results in their accumulation at the interface, but they can be relatively easily removed by washing.

4.2 Deposition at elevated temperatures

4.2.1 The temperature response of the bare waveguide in pure buffer

Exposure of the bare waveguide to aqueous solutions at various temperatures leads to changes of the measured incoupling angle α and the corresponding effective refractive index *N*. This is due to the temperature dependence of the system optogeometric parameters: refractive indices of the substrate n_s and cover medium n_c , as well as the thickness t_F and refractive index n_F of the waveguide itself. We can calculate the waveguide parameters t_F and n_F as a function of temperature from the equation (4) for TE and TM modes, using the measured values of N(TE) and N(TM) at the corresponding temperatures. The expressions used to describe the temperature dependence of n_s (glass substrate) and n_c (10mM HEPES solution at pH=7.4 and water) are reported in Table 1. It is worth noting that calculations of the deposited mass using the cover medium refractive index either for HEPES ($n_{C,HEPES}$), or for water ($n_{C,water}$), shown in Table 1, resulted in virtually identical results. This means that for the diluted aqueous solutions used here, the OWLS response, eq. (2.4), is insensitive to changes of n_c due to the cover medium composition.

The temperature response of the bare waveguide was in the presence of the buffer solution (10mM HEPES, pH 5.5). Flow rate through the heated deposition cell was kept constant at 2ml.h⁻¹. The set of two nonlinear equations (4) for the TE and TM modes was solved to obtain the waveguide parameters t_F and n_F , using the values of n_C and n_S in Table 1. The values of t_F and n_F measured at steady temperatures were fitted as a function of temperature T (in °C) using the following expressions and the corresponding results are shown in Figure 3:

$$t_F(T) = 184.31 + 0.0096872T - 9.5054 \cdot 10^{-6}T^2$$

$$n_F(T) = 1.7881 - 2.7434 \cdot 10^{-5}T - 2.2582 \cdot 10^{-7}T^2$$
(8)

A similar magnitude of the change in the waveguide thickness and refractive index with temperature was also measured in the presence of pure water, in agreement with previous results ²⁶. When the waveguide was cooled back to the ambient temperature, a change of about $\Delta n_f=0.001$ in the waveguide refractive index was observed. This observation was made for several waveguides exposed to the HEPES solution at pH between 5.5 and 7.4. In all cases the temperature response of the bare waveguide to the buffer solution revealed gradual modification of the waveguide optical properties at elevated temperatures. The same effect was also observed for silica and niobia coated waveguides. Coating of waveguide surfaces (i.e., by polymer or protein) can be used to effectively suppress this effect, by providing a barrier between the waveguide and the liquid solution. In particular, a primary protein layer adsorbed at ambient temperature can stabilize the waveguide at elevated temperatures, as will be discussed shortly.

4.2.2 The temperature stability of primary protein layers in pure buffer

Before discussing thermally driven deposition of β -LG, we address the issue of durability of irreversibly bound primary protein layers deposited at ambient temperature, when exposed to the buffer solution at elevated temperatures. A typical experiment is shown in Figure 4, starting with the protein layer deposited at 25°C from 10mg.ml⁻¹ β -LG solution. The detailed experimental conditions are summarized in Table 1. At ambient temperature the irreversibly deposited protein amount was 1.6mg.m⁻² (period A). After 30 minutes of heat treatment at 72°C in 10mM HEPES solution at pH 5.5 (period C) and cooling back to the ambient temperature (period D), the measurement becomes steady at the end of period E. Here, the layer exhibits a partial loss of deposited mass being the protein coverage decreased to 1.0mg.m⁻². Subsequent resaturation of the protein layer by native β -LG solution for 60 minutes (period F) at ambient temperature resulted in the complete recovery of the total coverage (4.2mg.m⁻²) reached in the saturation step at ambient temperature (not shown in Figure 4) preceding the

heat treatment. Subsequent rinsing with the blank buffer (period G) resulted in the recovery of the same irreversible coverage (1.6mg.m^{-2}) as observed before the thermal treatment.

The same observation was made for repeated thermal exposures of the same deposited protein layer at six different temperatures between 60°C and 80°C. Irreversibly deposited amounts measured before temperature exposure (period A) and at the end of buffer washing (period G) were always very similar (difference $\pm 20\%$). These experiments showed that the protein layer, which partially degrades during the thermal treatment, can be fully recovered by subsequent resaturation at ambient temperature. Moreover, it has been observed that the optical properties of the waveguide covered by the primary protein layer are stable in the buffer solution at the temperatures considered in this work. Since this was not the case for bare surfaces as mentioned above, it is apparent that the protein layer effectively protects the waveguide beneath it from heated buffer solutions.

4.2.3 Secondary deposition of β-LG on thin protein layers at elevated temperature

As discussed above, the first step in the β -LG deposition process is the formation of a primary layer, which occurs spontaneously even at ambient temperature. This primary layer is irreversibly bound to the original surface of the sensor, resulting in a significant modification of its surface properties. Further deposition at elevated temperature is then essentially driven by interactions between the deposited proteins and the proteins in the bulk. In order to decouple the interaction between surface-protein (initial deposition) and protein-protein (secondary deposition) we measured the secondary deposition rates always starting with protein layer several nanometers thick. In Figure 5 we show results from a typical secondary deposition experiment from 10mg.ml⁻¹ β -LG solution at 75°C. The detailed experimental conditions are reported in Table 3. A previously deposited layer (13mg.m⁻²) was present on the top of the waveguide (period A), so that the influence of the original waveguide surface on the deposition rate is expected to be negligible. After the system was heated to the desired temperature

(periods B and C), the β -LG solution was injected. After a very short transition time (in this case about one minute) the rate of change of the deposited mass became constant and the deposition rate at the given temperature could be determined from the slope of the linear growth observed in period D.

It is worth noting that the deposited mass shown by the circles in Figure 5 was calculated using the temperature correction of the optical properties described above in section 4.2.1 using the temperature measured on-line by a thermocouple located close to the deposition cell. It can be seen that the apparent deposited mass with temperature correction exhibits a small peak soon after the temperature started to increase at the beginning of period B. By the time the system reached a steady temperature (period C), this transient effect disappeared. This is an artifact due to the fact that in the non-steady state regime the temperature measured by the thermocouple does not correspond to the real waveguide temperature. Therefore, the temperature correction used in period B of the experiment does not accurately represent the optical parameters of the system and incorrect values of the deposited mass are obtained.

For the sake of illustration, the squares in Figure 5 show the values of the deposited mass calculated without applying the temperature correction. In this case, the calculated apparent deposited mass is significantly lower than the actual one represented by the circles in the same figure. However, the deposition rates (corresponding to the slope in region D) determined from the data calculated with and without temperature correction are very similar.

It is worth noting, that an alternative simplified approach to the evaluation of the deposition rates can be used, based on treating the previously deposited layer of protein as part of the waveguide. In this case, apparent optogeometric parameters of the so modified waveguide (consisting of waveguide and thin protein layer) n_F and t_F are determined using Eq. (4) at the relevant deposition temperature just before the injection of β -LG. We have seen that this approach yields very similar deposition rates as the more rigorous one based on the temperature correction of the waveguide parameters. The values of mass deposited during the secondary deposition from $10\text{mg.ml}^{-1}\beta$ -LG solution (10mM HEPES, pH 6.4) at various temperatures are shown in Figure 6 as a function of time. The deposition below 60°C was so slow that it was not detectable with our instrumentation. The linearity of the mass growth in time was confirmed by long experiments lasting up to two hours. Of course, such long experiments are only possible for conditions where the deposition rate is slow enough so as to avoid the saturation of the waveguide evanescence region, which occurs at mass coverage of about 35mg.m⁻².

The deposition rates were found to increase exponentially with the temperature over the range of temperatures considered here. They were highest at pH 5.5 (close to the isoelectric point of β -LG) and decreased as pH was increased from 5.5 to 7.4. This pattern of dependence on pH is consistent with the trend observed for the growth of large aggregates in β -LG solutions ^{5, 27}. When pH is above the isoelectric point of β -LG, increasing pH results in increasing negative charge on β -LG, which implies increasing electrostatic repulsion and thus slowing down of aggregation as well as deposition kinetics.

It is worth noting that the residence time of the protein inside the deposition cell at elevated temperature is around 30 seconds. Based on previous experimental studies on β -LG aggregation ¹⁰ it is expected that growth of protein aggregates due to non-covalent (physical) aggregation is not significant for such short times, especially at higher pH (i.e., higher charge and thus stronger electrostatic repulsion) and lower temperatures (i.e., slower denaturation). On the other hand, at higher pH values and higher temperatures (where denaturation is faster), formation of small covalently bound clusters observed in bulk solutions ¹⁰ can not be excluded before proteins are transported to the surface.

In Figure 7 we show the Arrhenius plots for secondary deposition rates at various pH values. From these the activation energy E_a for the secondary deposition process can be estimated, and the obtained

values are shown in Figure 8 as a function of pH. These activation energies for β -LG deposition are similar to those previously reported for β -LG denaturation at comparable pH values found in the literature ^{1, 5, 11, 14}. On the other hand, the denaturation kinetics itself becomes faster as pH increases ^{4, 5, 28, 29}, which is opposite to the trend observed here for the deposition kinetics.

A possible explanation for the observed dependence of deposition rates and activation energies on pH is as follows. Let us consider that the deposition process consists of two steps in series. Unfolding and denaturation of β -LG (facilitated by covalent binding of unfolded proteins) with the denaturation kinetics according to Eq. (1) with rate constant k_{den} , is followed by deposition of denatured β -LG, with the first order kinetics and the deposition rate constant k_{dep} . Assuming that the conversion of the native protein is quite small within a short residence time in the deposition cell, so that the extent of both denaturation reaction and the subsequent deposition step stays small (i.e., only small proportion of protein denatures and only small proportion of denatured protein is deposited), it is easy to see that the overall rate of β -LG deposition R_{dep} would be proportional to the product of the two rate constants k_{den} and k_{dep} . Let us now consider the two rate constants written as the usual Arrhenius-type expression:

$$k_{den} = k_{den}^{0} e^{-\frac{E_{a}^{den}}{kT}}$$

$$k_{den} = k_{den}^{0} e^{-\frac{E_{a}^{den}}{kT}}$$
(9)

where k^0 and E_a are corresponding prefactors and activation energies, respectively. The overall rate of deposition R_{dep} is then proportional to

$$k_{den}k_{dep} = k_{den}^{0}k_{dep}^{0}e^{-\frac{E_{a}^{den} + E_{a}^{dep}}{kT}}$$
(10)

Now we can see that the activation energy corresponding to the deposition rates observed in our experiments is actually the sum of the two activation energies for the denaturation and the deposition step of the denatured proteins. On the other hand, the prefactor is given by the product of the two prefactors for the two steps. If the activation energy for denaturation is much larger than the activation energy for deposition of denatured protein, then the activation energy of the overall deposition step will

be close to the activation energy for denaturation, as observed in our experiments. This is a reasonable assumption, since typical energy barriers for reaction limited aggregation of charge stabilized colloids are not more than 10-20kT ³⁰, which translates into the activation energy of 25-50 kJ/mol, which is only about 10-20% of the activation energy values for the deposition rates in Figure 8. On the other hand, if the prefactor for the denaturation rate constant increases moderately with pH, while the prefactor for the denatured proteins decreases strongly with pH (as expected for aggregation in reaction limited aggregation regime ³⁰), then the prefactor for the deposition rate given by the product of the two decreases with pH, as observed in our experiments. It can then be concluded that the activation energy for the overall deposition rate follows the trend of the activation energy for denaturation, i.e. decreases with increasing pH, while the deposition rate follows the trend opposite to that of denaturation, but consistent with aggregation, i.e. decreasing with increasing pH.

Finally, we analyze the effect of salt concentration on the deposition kinetics of β -LG. In Figure 9 we show the measured deposition rates as a function of NaCl concentration for two temperature values. A maximum in the deposition rate was observed at a concentration of the NaCl in the 10mM HEPES buffer equal to about 30mM. After reaching the maximum, deposition rate decreases when further increasing the salt concentration. This qualitative behavior follows the well-known pattern of protein solubility in electrolyte solutions ¹. In general, the salt concentration influences both steps described in the deposition mechanism above. The observed maximum is caused by the combined effect of a reduced denaturation rate and an increased aggregation rate which follow an increase of salt concentration. NaCl belongs to the salting-out class of salts and thus stabilizes the native protein conformation thus decreasing the denaturation rate ³¹.

On the other hand, when increasing the salt concentration the charge of the protein is screened, and therefore the non-covalent aggregation is enhanced. Thus the maximum in the overall deposition rate with increasing NaCl concentration, observed in Figure 9, is related to the simultaneous decrease in the rate of the denaturation reaction and increase in the rate of aggregation.

5 Conclusions

Deposition of thin β -LG layers at the surface of silica-titania waveguides was monitored on-line by OWLS, an optical reflection technique related to ellipsometry and reflectometry. This is the first time that OWLS was used to quantitatively monitor kinetics of temperature driven deposition. The initial step in β -LG deposition is the primary coverage of the bare surface, which is partially reversible at ambient temperature. Primary coverage density at ambient temperature depends on β -LG concentration and pH. The irreversibly bound part of the primary layer is partially desorbed when exposed to pure buffer at elevated temperatures, but can be resaturated to its original density from the protein solution at ambient temperature. The temperature driven secondary deposition of β -LG is strongly dependent on temperature and pH. Deposition rates were decreasing with increasing pH from 5.5. to 7.4, in a trend similar to that for non-covalent aggregation of β -lactoglobulin in the bulk solution. Activation energies for deposition rates were decreasing with increasing pH, from 340kJ/mol at pH=5.5 to 230kJ/mol at pH=7.4, and were similar to the activation energies for denaturation of β -lactoglobulin in the bulk solution. This is consistent with the fact that protein deposition is the result of two processes occurring in series: protein denaturation followed by deposition of the denaturated protein. The effect of NaCl concentration on the deposition rate at elevated temperatures follows the inverse of a well-known pattern of protein colloidal stability, with a maximum deposition rate at moderate salt concentrations.

Figures

Figure 1. Primary exposure of bare waveguide to 0.5 mg.ml ⁻¹ β-LG in 10mM HEPES, pH 5.5, at 25°C. Arrows above the time axis indicate change in liquid phase
Figure 2. Effect of pH on total deposited mass (circles) and irreversibly deposited mass (diamonds). 10 mg.ml ⁻¹ β-LG in 10mM HEPES at 25°C
Figure 3. Waveguide parameters measured in 10mM HEPES, pH 5.5 as a function of temperature. Crosses represent the measured thickness t_F and squares the refractive index n_F . Lines represent the fit given by Eq. (8)
Figure 4. Temperature exposure of the thin protein layer in 10mM HEPES solution, pH 5.5 at 72°C. The protein layer was resaturated in 10mg.ml ⁻¹ β -LG solution
Figure 5. Secondary deposition from a 10mg.ml^{-1} β -LG solution at, pH 5.5 and 75°C on previously deposited protein layer. Values computed with temperature correction for the optical properties (circles) and without correction (squares)
Figure 6. Growth of protein mass during secondary deposition from a $10\text{mg.ml}^{-1} \beta$ -LG solution in 10mM HEPES at pH 6.4 and various temperatures
Figure 7. Arrhenius plot for the deposition rate from a 10mg.ml^{-1} β -LG solution in 10mM HEPES. Points are the experimental values, the solid lines represent the linear fit and the doted lines are the upper and lower estimates corresponding to a 95% confidence level
Figure 8. pH dependence of the activation energy for β -LG deposition
Figure 9. Deposition rates as a function of NaCl concentrations at two temperature values. 10mg.ml^{-1} β -LG solution in 10mM HEPES at pH 5.9



Figure 1. Primary exposure of bare waveguide to 0.5 mg.ml⁻¹ β -LG in 10mM HEPES, pH 5.5, at 25°C. Arrows above the time axis indicate changes in liquid phase.



Figure 2. Effect of pH on total deposited mass (circles) and irreversibly deposited mass (diamonds). 10 mg.ml⁻¹ β -LG in 10mM HEPES at 25°C.



Figure 3. Waveguide parameters measured in 10mM HEPES, pH 5.5 as a function of temperature. Circles: measured thickness t_F ; Squares: measured refractive index n_F ; Lines: fits given by Eq. (8).



Figure 4. Temperature exposure of the thin protein layer in 10mM HEPES solution, pH 5.5 at 72°C. a) Temperature in deposition cell; b) Deposited mass. The protein layer was resaturated in 10mg.ml⁻¹ β -LG solution.



Figure 5. Secondary deposition from a 10mg.ml^{-1} β -LG solution at, pH 5.5 and 75°C on previously deposited protein layer. a) Temperature in deposition cell; b) Deposited mass. Values computed with temperature correction for the optical properties (circles) and without correction (squares).



Figure 6. Growth of deposited protein mass during secondary deposition from mg.ml⁻¹ β -LG solution in 10mM HEPES at pH 6.4 and various temperatures.



Figure 7. Arrhenius plot for the deposition rate from 10mg.ml^{-1} β -LG solution in 10mM HEPES at pH values as indicated. Points are the experimental values, the solid lines represent the linear fit.



Figure 8. pH dependence of the activation energy for β -LG deposition.



Figure 9. Deposition rates as a function of NaCl concentration at two different temperatures. mg.ml⁻¹ β -LG solution in 10mM HEPES at pH 5.9.

Tables

Table 1. Refractive indices as a function of temperature. T denotes temperature in °C29
Table 2. Experimental procedure for temperature exposure of primary protein layers at 72°C shown in Figure 4
Table 3. Experimental procedure for secondary deposition experiment at 75°C shown in Figure 531

Parameter	25°C	T [°C]	Source			
n _{C,water}	1.33114 4	$n_{C,H_2O}^{25^{o}C} - \frac{75453.41\Delta T + 2340.431\Delta T^2 + 6.363191\Delta T^3}{T.10^7 + 65.7081.10^7};$ where $\Delta T = T - 25$	а			
n _{C,HEPES}	1.33369 9	$1.33156 - 5.89189 \cdot 10^{-5}T - 1.06617 \cdot 10^{-6}T^2$	Ь			
n _s	1.52571 8	$1.52542 - 1.23 \cdot 10^{-6}T$	a			
a - reference 26						
b - refere	nce ³²					

Table 1. Refractive indices as a function of temperature. T denotes temperature in °C.

Table 2.	Experimental	procedure for	temperature	exposure	of primary	protein	layers at
72°C sho	own in Figure 4	ŀ.					

Period	Time	Bulk Solution	Flow Rate	Temperature
	[min]		$[ml.hr^{-1}]$	[°C]
А	0-81	buffer	2	25
В	81-89	buffer	2	heating
С	89-112	buffer	2	72
D	112-129	buffer	2	cooling
Е	129-163	buffer	2	25
F	163-230	β-LG in buffer	2	25
G	230-400	buffer	2	25

Period	Time	Bulk Solution	Flow Rate	Temperature
	[min]		[ml.hr ⁻¹]	[°C]
А	0-15	buffer	2	25
В	15-22	buffer	2	heating
С	22-36	buffer	2	75
D	36-45	β-LG in buffer	2	75
Е	45-48	buffer	0	cooling
F	48-57	buffer	0	25
G	57-80	buffer	2	25

Table 3. Experimental procedure for secondary deposition experiment at 75°C shown in Figure 5.

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