# Adsorption and Displacement of Beta-2-Microglobulin at Solid/Liquid Interfaces

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SUMMARY: Adsorption of beta-2-microglobulin from aqueous solution onto unmodified and methylated silicon wafers and subsequent displacement of the small globular protein by fibrinogen were studied by spectroscopic ellipsometry, immunosorbent assays and atomic force microscopy.
The results provide evidence that hydrophobicity of the substrate increases the maximum adsorbed amount of beta-2-microglobulin and the resistance of the adsorbed protein to displacement from the interface by competing species, respectively. Further, the dynamics of beta-2-microglobulin adsorption was found to induce significant differences in the degree of displacement achieved at given conditions. The observed variations in displacement behavior of adsorbed beta-2-microglobulin were interpreted based on information on the layer structure gained by atomic force microscopy. More compact and relatively smooth protein layers were formed on the hydrophobic surface corresponding to lower displacement by fibrinogen.

## Introduction

Adsorption, displacement and variations of the intramolecular structure of human proteins at polymer interfaces are at issue when considering biocompatibility and performance of medical devices, e.g. of medical membranes for artifical organs, cardiovascular and soft tissue implants. Due to the presence of many different dissolved species in biofluids the competition of the biomolecules for surface sites at the polymer interface is one key aspect with that concern. A pioneering hypothesis on protein-protein exchange at solid/liquid interfaces was developed by LEO VROMAN based on immunoassay studies with human plasma [1]. He found that fibrinogen, a key protein in blood coagulation, shows a transient maximum in the adsorbed amount on glass surfaces when adsorbed from plasma. Displacement patterns as described by the VROMAN effect were found with numerous different proteins [2].

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This article reports data on the competition of proteins at interfaces based on studies with beta-2-microglobulin, a small globular protein associated with cellular immune functions which is critically accumulated in uraemic patients because of its insufficient removal by the currently available hemodialysis membranes [3]. The removal of beta-2-microglobulin by permeation through hemodialysis membranes is difficult since the difference in size to other proteins like albumin (which must not be removed) is not great enough. It seems therefore attractive to develop membranes capable to remove beta-2-microglobulin by adsorption. This approach is, however, only realistic if the adsorbed beta-2-microglobulin can resist the displacement by other plasma proteins. Therefore, the sequential adsorption of beta-2-microglobulin and fibrinogen was investigated on model substrates of different characteristics. Since beta-2-microglobulin shows a relatively hydrophobic exterior surface differences in hydrophobicity of the substrates were preferentially examined.

## Materials and Methods

Beta-2-microglobulin from human plasma was kindly provided by ACORDIS Research GmbH, Obernburg, Germany. Fibrinogen was purchased from SIGMA, Deisenhofen, Germany. Properties of the proteins are given in table 1. The proteins were dissolved in phosphate-buffered saline (pH 7.4).

Table 1. Properties of beta-2-microglobulin and fibrinogen according to [4].

| | **Beta-2-microglobulin** | **Fibrinogen** |
|---|---|---|
| **Molecular weight [g mol$^{-1}$]** | 11.730 | 340.000 |
| **Structure** | Single Ig-like domain, high content of beta-sheet | Dimeric, each half molecule: three polypeptide chains |
| **Isoelectric point** | 5.8 | 5.1-6.3 |
| **Function** | Immune system | Blood coagulation |
| **Concentration in human blood** | 1-3 $\mu$g cm$^{-3}$ (healthy donors) 30-50 $\mu$g cm$^{-3}$ (uraemics) | 35 $\mu$g cm$^{-3}$ |

Silicon wafers were oxidized by thermooxidation. The thickness of the oxide layer was determined to 53 nm ± 1.5 nm by ellipsometry (for instrumentation see below, optical two-layer model for Si-$SiO_2$ [5]). The silicon wafers were used as substrates in the adsorption experiments either as obtained (unmodified Si-wafer/$SiO_2$) or after methylation by vapour-phase reaction with hexamethyldisilazane described in [6] (methylated Si-wafer/meth Si). Contact angle measurements (G 40, Krüss, Hamburg, Germany) prove moderately hydrophilic behavior of the unmodified Si-wafer ($\theta_{advancing}$ = 38°) and very hydrophobic characteristics of methylated Si-wafer ($\theta_{advancing}$ = 101°). The roughness of the substrates was controlled by AFM and found to be as low as expected (Ra 0.2 nm).

Variable angle spectroscopic ellipsometry was applied to determine the adsorbed amount of protein *in situ*. A commercial instrument (VASE M 44, WOOLAM Inc., Lincoln, NE, USA) was used for that aim as described elsewhere [5]. Briefly, the thickness of an approximated homogeneous protein layer was determined based on the evaluation of the ellipsometric data $\Delta$ and $\Psi$ using an optical four layer model. Referring to the approach of DE FEIJTER [7] the surface concentration of protein was derived from the thickness and the refractive index of the analyzed layer. (The refractive index increments of the proteins required by this approach were determined to 0.188 $cm^3\ g^{-1}$ for both proteins.) The experiments were performed at an angle of incidence of 68° in a cuvette made by HELLMA, Müllheim, Germany.

An enzyme-linked immunosorbent assay (beta-2-microglobulin-ELISA ID59041, Gesellschaft für Immunchemie und Immunbiologie mbH, Hamburg, Germany) was used for the determination of the displaced beta-2-microglobulin [8]. Photometric measurements were performed by means of an Anthos 2010 reader (Anthos Mikrosysteme, Krefeld, Germany). The immunoassay was calibrated for the performed study by reference samples related to the displacement experiments.

Selected protein layers were investigated by atomic force microscopy (Bioscope, DI, Santa Barbara, CA, USA) using the tapping mode in buffer solution to prevent artefacts [9, 10]. (SiN-cantilevers were used k = 0,3 N/m, scan rate was adjusted to ≤ 0,8Hz, scan size and height were set to 1$\mu$m and 10 nm, respectively.)

Adsorption-displacement experiments were performed at conditions of stagnant solution. The time-dependent variation of the adsorbed amount of protein was monitored by ellipsometry at different solution concentrations of beta-2-microglobulin between 0.2 and 100 $\mu g\ cm^{-3}$. After the adsorption experiment and rinsing with pure buffer the cuvette was changed. The substrate was brought into contact with fibrinogen solution (100 $\mu g\ cm^{-3}$) in a second cuvette where again the time-dependent variation of the adsorbed amount of protein was determined by

ellipsometry. Afterwards, the solution was collected and analyzed for beta-2-microglobulin by means of the enzyme-linked immunosorbent assay. Protein layered substrates for analysis by atomic force microscopy were prepared in a similar procedure.

## Results and Discussion

Time dependent variations of protein surface concentrations on the unmodified Si-wafer and on the methylated Si-wafer in contact with protein solutions are given in figure 1 and 2. On the left side of each figure the beta-2-microglobulin adsorption is shown, on the right side the subsequent fibrinogen adsorption. For clarity only the adsorption experiments using 2 $\mu$g cm$^{-3}$ and 50 $\mu$g cm$^{-3}$ solution concentration of beta-2-microglobulin are indicated. Maximum adsorbed amounts of beta-2-microglobulin (first part of sequential experiment) at several solution protein concentrations and of the total protein at the interface after fibrinogen adsorption (second part of sequential experiment, always 100 $\mu$g cm$^{-3}$ solution concentration) are shown in figure 3.

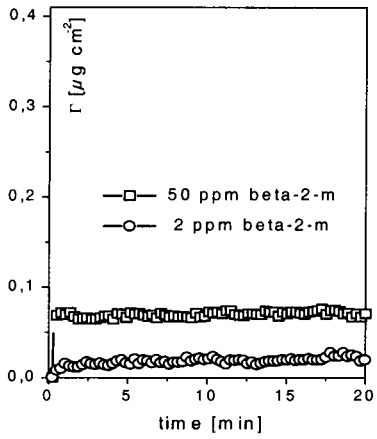


Fig. 1a: Beta-2-microglobulin adsorption onto unmodified Si-wafer at protein solution concentrations of 2 $\mu$g cm$^{-3}$ and 50 $\mu$g cm$^{-3}$ (ppm).

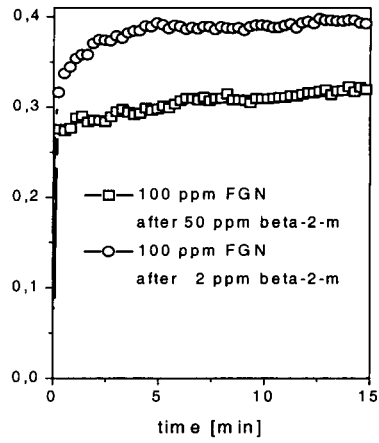


Fig. 1b: Fibrinogen adsorption onto unmodified Si-wafer after preadsorption of beta-2-microglobulin (beta-2-m displacement experiment).

The adsorbed amounts of beta-2-microglobulin for a given solution protein concentration determined were considerably higher at the hydrophobic surface as compared to that at the hydrophilic surface. Further, overshoot-effects were observed in the dynamics of beta-2-microglobulin adsorption onto the hydrophobic substrate at solution concentrations higher then 10 $\mu$g cm$^{-3}$ (fig. 2a), but not at the unmodified, hydrophilic Si-wafer. Overshoot effects can be caused by the time-dependent reorientation of adsorbed proteins leading to an increase in the protein substrate contact area for the individual adsorbed molecules and, thus, inducing desorption of a part of the initially adsorbed amount. In the discussed case the occurence of overshoot effects might be interpreted as an additional indication of higher affinity between protein and substrate.

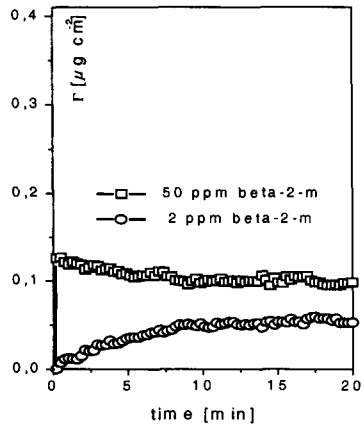


Fig. 2a: Beta-2-microglobulin adsorption onto methylated Si-wafer at protein solution concentrations of 2 $\mu$g cm$^{-3}$ and 50 $\mu$g cm$^{-3}$ (ppm).

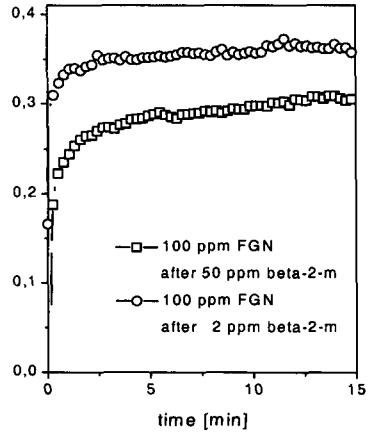


Fig. 2b: Fibrinogen adsorption onto methylated Si-wafer after preadsorption of beta-2-microglobulin (beta-2-m displacement experiment).

After beta-2-microglobulin adsorption the samples were rinsed with pure buffer solution at least three times. No variation of the adsorbed amount of protein was observed at this stage of the experiment indicating the absence of reversibility of the adsorption upon dilution. Subsequently, the samples were incubated with fibrinogen solution in a second cuvette (figs. 1b and 2b). The time-dependent variation of the adsorbed amount of protein included both the potential displacement of beta-2-microglobulin and the adsorption of fibrinogen. (There is no access to the composition of the protein layer on the level of the ellipsometric data.) In

general, the maximum adsorbed amount of protein after contact with fibrinogen solution was significantly reduced if any beta-2-microglobulin was adsorbed in advance as compared to the adsorption of fibrinogen from similar solutions onto the native substrates ($\Gamma_{max}$ of FGN onto native unmodified Si-wafer 0.42 $\mu$g cm$^{-2}$, $\Gamma_{max}$ of FGN onto native methylated Si-wafer 0.51 $\mu$g cm$^{-2}$). Furthermore, we obtained lower totally adsorbed amounts of protein in this second part of the sequential adsorption experiment at higher amounts of preadsorbed beta-2-microglobulin.

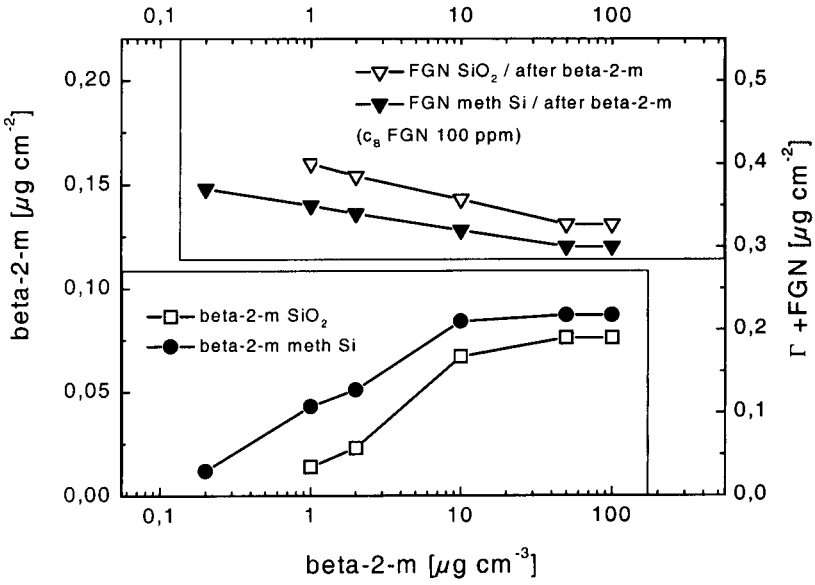


Fig. 3: Maximum adsorbed amounts of protein at unmodified and methylated Si-wafer after beta-2-microglobulin adsorption at different solution protein concentrations (left axis, lower plots) and after subsequent contact of the samples with fibrinogen solution (right axis, upper plots).

In order to analyze the composition of the adsorbed protein layers after the displacement experiment the applied fibrinogen solution was checked "after use" for desorbed beta-2-microglobulin by means of an beta-2-microglobulin immunoassay. Due to the experimental procedure the detected protein could be attributed to displacement from the sample exclusively. The displaced amounts and displaced fractions of initially adsorbed beta-2-microglobulin, respectively, are shown in fig. 4. Considerably higher amounts of beta-2-

microglobulin were released from the hydrophilic substrates although those samples had initially lower amounts of beta-2-microglobulin adsorbed. Therefore, the fractions of displaced beta-2-microglobulin are much higher at unmodified Si-wafer than at the methylated hydrophobic Si-wafer. For a given substrate, the displaced amount and fraction apparently increase with increasing solution concentration of beta-2-microglobulin in the adsorption step. The latter trend roughly correlates with the maximum adsorbed amounts observed for lower solution concentrations. However, the comparison of the displaced amount for the highest solution concentrations in the adsorption experiments considered in this study clearly indicates that the displaced amount was not solely affected by the surface concentration but by the adsorption dynamics as well: Although the adsorbed amounts at 50 $\mu g\ cm^{-3}$ and 100 $\mu g\ cm^{-3}$ beta-2-microglobulin solution concentration were almost constant at both substrates the displaced amount was higher in the latter case. This may lead to the conclusion that the structure of the adsorption layers could differ at similar surface concentrations due to differences in the dynamics of the layer formation.

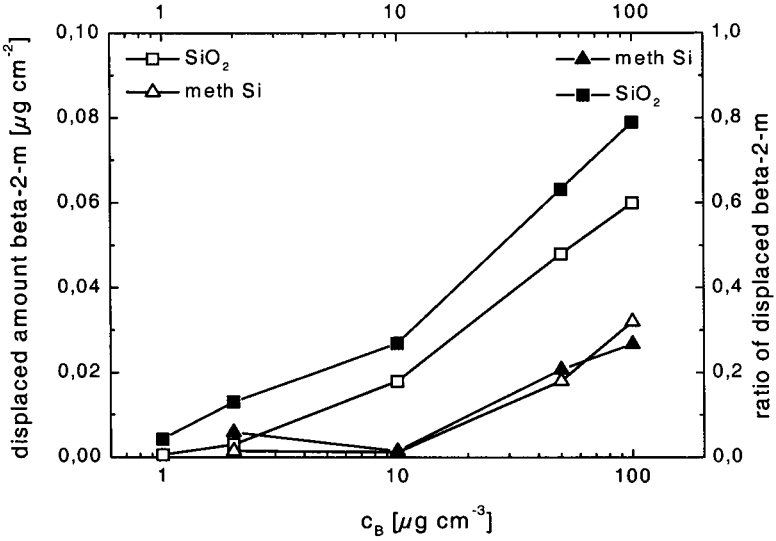


Fig. 4: Displaced amounts (left axis) and displaced fractions (right axis) of beta-2-microglobulin from unmodified and methylated Si-wafers after adsorption at different solution protein concentrations and subsequent contact of the samples with fibrinogen solution.

When considering potential reasons for the observed higher resistance of adsorbed beta-2-microglobulin to displacement by fibrinogen on the hydrophobic substrate information on the adsorption layer structure is very valuable. Insights on the layer structures can be provided by atomic force microscopy. However, attention has to be paid to the potential disturbance of the layer structure by the scanning force inspection itself. Therefore, the tapping mode of the AFM was applied. Figures 5 and 6 give selected examples for the obtained morphology of beta-2-microglobulin layers at unmodified (fig. 5) and methylated (fig. 6) Si-wafers where two solution concentrations of the protein had been applied in the adsorption step.

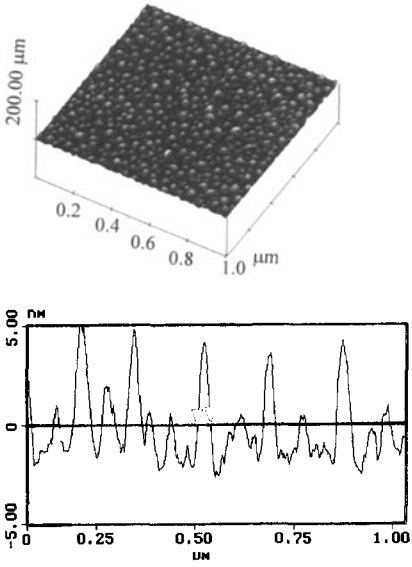


Fig. 5a: Beta-2-microglobulin layer on unmodified Si-wafer adsorbed from 2 $\mu g\ cm^{-3}$ beta-2-microglobulin solution

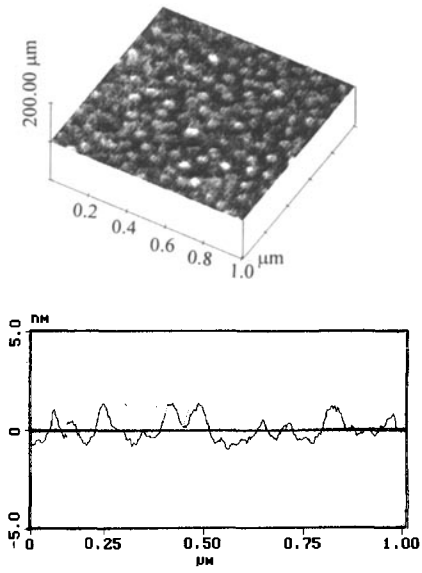


Fig. 5b: Beta-2-microglobulin layer on unmodified Si-wafer adsorbed from 50 $\mu g\ cm^{-3}$ beta-2-microglobulin solution

The characterized beta-2-microglobulin layer structures could be distinguished according to the different substrates and different solution protein concentrations applied. In order to consider varying features in detail two values for the description of the morphology were derived: Rms and Ra (tab. 2). Rms (Rq) is the standard deviation of the Z value within the given area and is calculated as:

$$R_q = \sqrt{\frac{\sum (Z_i - Z_{ave})^2}{N}}$$

where $Z_{ave}$ is is the average of the Z value within the given area, $Z_i$ is the current Z value, and N is the number of points within this area.

The mean roughness (Ra) is the mean value of the surface relative to the center plane and is calculated using:

$$R_a = \frac{1}{L_x L_y} \int_0^{L_y} \int_0^{L_x} |f(x,y)| dxdy$$

where f(x,y) is the surface relative to the center plane $L_x$ and $L_y$ are the dimensions of the surface.

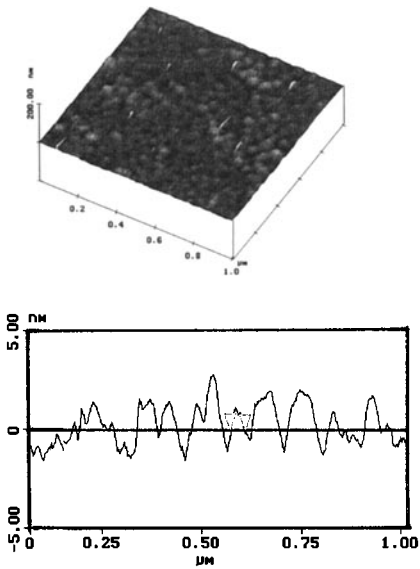


Fig. 6a: Beta-2-microglobulin layer on methylated Si-wafer adsorbed from 2 $\mu$g cm$^{-3}$ beta-2-microglobulin solution

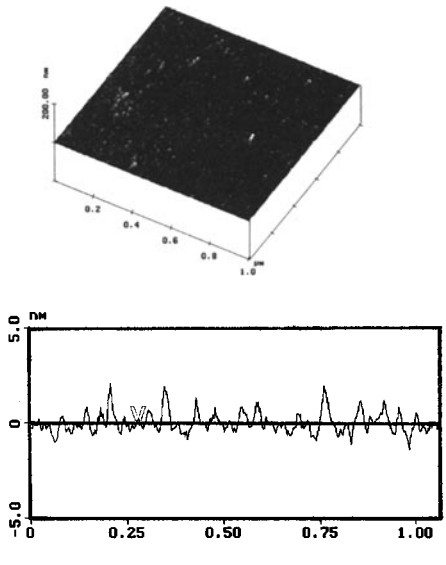


Fig. 6b: Beta-2-microglobulin layer on methylated Si-wafer adsorbed from 50 $\mu$g cm$^{-3}$ beta-2-microglobulin solution

As evident from table 2 significantly higher values of Rms and Ra were obtained for the beta-2-microglobulin layers on the hydrophilic substrate than for the protein layers on the hydrophobic surface at given conditions. Referring to the results of the immunoassays discussed above less morphology of the beta-2-microglobulin layer is obviously correlated

with stronger resistance to displacement. This might be attributed to the facilitated interaction between the competing protein and the substrate and to the easier fragmentation of the adsorption layer upon approach of fibrinogen. Also, decreasing values of Rms and Ra were found for beta-2-microglobulin layers on the hydrophilic substrate at increased solution protein concentration whereas the corresponding values for the layers on the hydrophobic surface remained almost constant. Considering the AFM results given above it is also important to note that the resolved structures do not coincide with the dimensions of individual molecules but should rather be attributed to aggregates. Since beta-2-microglobulin is known to form aggregates [11] the latter observation does not surprise.

Table 2. Rms and Ra values of the analyzed beta-2-microglobulin adsorption layers.

| | native $SiO_2$ and meth Si | **$SiO_2$, 2 $\mu g\ cm^{-3}$ beta-2-m soln.** | **$SiO_2$, 50 $\mu g\ cm^{-3}$ beta-2-m soln.** | **meth Si, 2 $\mu g\ cm^{-3}$ beta-2-m soln.** | **meth Si, 50 $\mu g\ cm^{-3}$ beta-2-m soln.** |
|---|---|---|---|---|---|
| **Rms** [nm] | 0.3 | 1.64 | 1.12 | 0.54 | 0.58 |
| **Ra** [nm] | 0.2 | 1.28 | 0.85 | 0.43 | 0.41 |

## Concluding remark

The reported study was motivated by recently suggested strategies for adsorptive removal of beta-2-microglobulin from the blood of uraemic patients [12, 13]. The results support the conclusion that the degree of hydrophobicity of the adsorbent surface is a key parameter for the effiency of this principle. Based on the described methodology the authors currently perform screening experiments for the selection of polymers to be used as membrane materials with promising beta-2-microglobulin adsorption capacity. However, although the general idea of strong adsorption of beta-2-microglobulin onto hydrophobic surfaces is confirmed by the collected data the determined adsorbed amounts do not indicate that purely adsorptive beta-2-microglobulin removal from blood could fullfill the requirements of blood purification.

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