A catalytic surface for amyloid fibril formation

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Abstract. A hydrophobic surface incubated in a solution of protein molecules (insulin monomers) was made into a catalytic surface for amyloid fibril formation by repeatedly incubate, rinse and dry the surface. The present contribution describes how this unexpected transformation occurred and its relation to rapid fibrillation of insulin solutions in contact with the surface. A tentative model of the properties of the catalytic surface is given, corroborated by ellipsometric measurements of the thickness of the organic layer on the surface and by atomic force microscopy. The surfaces used were spontaneously oxidized silicon made hydrophobic through treatment in dichlorodimethylsilane.

1. Introduction

We have observed an unexpected phenomenon in model studies of amyloid fibril formation on a hydrophobic surface. Insulin monomers under denaturing conditions, an often used model system for fibril formation [1,2], were used also in our studies.

We only briefly describe the preparation of the surface; details of the procedure will be given in future publications. Since the surfaces were prepared by standard methods this should not make the main message of this contribution difficult to understand. We use ellipsometry to obtain the amount of organic material adsorbed on the surface after different incubation cycles and atomic force microscopy (AFM) to elucidate the structural appearance of the layers. Ellipsometry is a well known technique for the study of protein adsorption and protein – surface interactions [3]. There have been numerous AFM studies of amyloid fibrils picked up or deposited from a solution of the fibrils [4, 5]. We make no attempt in the present communication to compare our AFM – results with previous results with respect to the appearance and density of the protein material deposited on our hydrophobic surfaces. The AFM studies were made only to further understand the unusual kinetic behaviour observed with ellipsometry and to corroborate our model for the observed phenomenon. We should say that the term catalytic is used in a broad sense. The surface we have discovered may also serve as a reservoir for seeds for fibril formation, seeds which are released by insulin monomers in solution.

2. Surface preparation

The surfaces were thus prepared on pieces of silicon wafers cleaned by standard methods and incubated in dichlorodimethylsilane (DDS) 1 % in xylene at room temperature. In short the silicon pieces (5 x 5 mm²) were boiled in a 5:1:1 mixture of water, ammonia and hydrogen peroxide for 10
minutes and then in a mixture 6:1:1 mixture of water, hydrochloric acid and hydrogen peroxide for further ten minutes. This is a standard way of making silicon surfaces clean and hydrophilic. The silicon pieces were rinsed and dried in nitrogen and put into the DDS /xylene mixture for 5 minutes, treated by ultrasound in xylene for 10 minutes and dried. The silicon surface is now hydrophobic.

3. Results
To investigate amyloid growth on a solid surface we used insulin which is notorious for its amyloid fibrillation tendencies especially under denaturing conditions. We used ellipsometry to estimate the amount of organic material adsorbed on the surface of differently treated pieces of silicon. Silicon substrates (hydrophobic and hydrophilic) were incubated for different lengths of time in solutions of unfolded bovine insulin (BI) monomers (200 μM BI, 65°C and pH 1.6, 25 mM HCl). After the incubation the substrates were rinsed with distilled water and dried in a stream of nitrogen, and the amount of organic material estimated through ellipsometric measurements in air after rinsing and drying. The following observations were made:

During continuous incubation of the surfaces for several hours, only small amounts of adsorbed protein (1-2 nm) were observed on either the hydrophilic or hydrophobic silica surface. It should be stated that from the dimensions of an insulin molecule a monomolecular layer of molecules would correspond to less than ~2 nm, hence continuous incubation merely induces a monolayer deposit on these surfaces. However, an interesting observation was made when the hydrophobic substrate was incubated a second time and third time in the same insulin solution. The observations are summarized in Fig. 1. Over the first 2 h the adsorbed layer increased in thickness to ~7.0 nm. After the third incubation step the thickness increased during the following incubation steps to an optical thickness of 40 nm (at 3h), followed by a decrease of the deposited layer decrease down to a thickness of 15-20 nm. The number of incubation – air cycles appeared to be the driving factor and not the total incubation time. When the surface with the 15-20 nm layer was immersed into a fresh solution of unfolded insulin monomers (200 μM BI, 65°C and pH 1.6, 25 mM HCl) the subsequently deposited layer was immediate and very large, leading to equivalent thicknesses in the range 100 – 200 nm, corresponding to hundreds of insulin molecules thick. After this large increase the a following incubation in the same monomer solution resulted in a rapid decrease in the measured amount of adsorbed material, leaving a layer with a thickness around 15-20 nm thick. This type of behavior could not be observed for the hydrophilic surface where an increase in the adsorbed layer thickness could not be obtained in similar series of incubation – air exposures. The details of the phenomenon observed in Fig.1 depended somewhat on the length of the different incubation periods, but the general appearance was always that shown in the figure. After a number of incubation-air cycles the total amount of insulin on the surface in a fresh solution initially increased rapidly but finally decreased upon subsequent incubations in the same solution to a constant level. Immersion in a fresh solution of insulin induced a rapid formation of a 100-200 nm layer of protein deposits followed by an equally rapid decrease in the layer thickness upon further incubations in the same solution.

**Figure 1** Summary of determined thicknesses of organic material on the silicon surface at different stages of the incubation experiment. The thickness was estimated using a a refractive index of 1.46 (SiO₂) which fitted the built in evaluation program of the ellipsometer used. The samples were taken out of solution, rinsed and dried before each measurement.
Apparently the remaining layer had a catalytic effect on the process in the solution controlling the adsorption behavior. If, however, a catalytic surface was incubated too long in a fresh solution no extra layer could be observed. In all cases upon incubation in a solution containing insulin monomers, the return to the remaining catalytic layer was accompanied by a light scattering from the solution as seen by eye. As it turns out and as described further below the transient behavior in Fig. 1 was due to a rapid formation of amyloid fibrils in solution.

Fig. 2 shows a collection of AFM-pictures and line scans obtained at the three different stages in Fig. 1. During the development of the catalytic surface, i.e. before the first large increase of the thickness of the adsorbed organic layer there appears to be a loose network of rather narrow fibrils as seen in Fig. 2 (stage 1), which corresponds to a layer with an average optical thickness of about 7 nm as determined by ellipsometry (stage 1 in Fig. 1). A surface taken out of the monomer solution at the peak of the adsorbed layer (represented by stage 2 in Fig. 2) shows a very thick and uneven organic layer, probably consisting of small as well as large fibrils organized in a three dimensional network. The layer imaged in Fig. 2, stage 2, had an optical thickness of 100-200 nm. Fig. 2, (stage 3) shows a collage of images of different parts of a surface after the large peak has disappeared with a remaining optical thickness of about 15-20 nm. This is the surface which shows the catalytic activity. The collage suggests that it is a surface with a number of dynamic processes occurring since there are areas of very different appearances in terms of the fibrils and other structures remaining on the surface.

![AFM images and line scans](image)

**Figure 2** Collection of AFM images of the organic layer at different stages during incubation studies. The different stages refer to the notations in Fig. 1

### 4. Tentative model for the surface induced fibril formation

The mechanisms suggested below are based on the following observations and suggestions:

i) It takes at least three (in the present experiments) exposures to air and drying to obtain a layer of insulin with an estimated thickness of ~7 nm on the hydrophobic surface. This layer induces formation of protein deposition on the surface and fibril formation.

ii) When the monomers in solution are consumed the thick deposited protein layer is desorbed and leaves a layer 15–20 nm thick.

iii) The 15-20 nm layer catalyzes insulin deposition upon incubation in a fresh insulin monomer solution and also fibril formation in the solution.

iv) During the fibril formation phase a layer with a thickness of 100–200 nm can be detected which rapidly desorbs when the monomers in solution have been consumed. The AFM
picture of the this layer shows a structures with hills much higher than the average thickness and valleys down to the initial substrate

vi) The AFM micrograph of the catalytic surface shows a large variation in structure and packing of the remaining protein layer (with an average thickness of 15 – 20 nm)

vi) Since fibrillation occurs in the whole solution it is suggested that the catalytic surface produces/ provides seeds for further fibrillation in solution

We conclude that through subsequent air exposure and drying a catalytic surface is created consisting of a layer of insulin with an average thickness of about 15 – 20 nm. This layer appears to be very heterogeneous with a number of different structures and distributions of the deposited protein layer. The surface thus seems to provide several interaction possibilities between monomers in solution and the surface. The fact that the thick protein layer is not observed when the monomers in solution have been consumed indicates that fibrils in general do not stick permanently to the surface. A suggestion is therefore that the thick layer detected consists of fibrils or fragments formed or under formation, which are not removed by the rinsing in distilled water. When the monomers in solution are consumed, there is no further production of fibrils/fragments on the surface and most of the protein material on the surface desorbs and we are left with the catalytic layer again. The tentative model is illustrated in Fig. 3.

Figure 3 Tentative model for the catalytic surface. The drawing illustrates that seeds of different sizes as well as fibrils may be formed on the surface. The seeds are helping to fibrillate the bulk of the solution. When the monomers are consumed most of the organic material leaves the surface. The remaining layer acts as a catalyst upon addition of monomers

5. Discussion

The molecular reason for the catalytic activity of the surface can only be speculated upon. One possible explanation can be that in the presence of new monomers some of the already formed seeds are desorbed and replaced by new monomers to keep the number of occupied adsorption sites constant, which would be thermodynamically favourable. The newly adsorbed monomers aggregate to form new seeds etc. The observations described in this contribution need many more studies to reveal the nature of the catalytic surface, e.g. to decide if it is truly catalytic or only acting as a self filling reservoir of seeds for fibrillation in solution. Kinetic studies with surface plasmon resonance on hydrophobic gold at different stages are one possibility. We should say that preliminary experiments indicate that the hydrophobicity of the surface is the key parameter. It will of course be of the highest interest to correlate our findings with the standard procedures used to create and detect fibrillation in solution (without catalytic surfaces) [6].

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