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Label-free detection of amyloid growth with microcantilever sensors

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Abstract
We present an approach for sensing protein aggregation using microcantilever systems. Results from both single cantilever experiments with internal reference and multicantilever array measurements with dedicated reference cantilevers are discussed. We show that in both cases protein aggregation on the sensor can be detected through associated changes in surface stress.

1. Introduction

Protein aggregation and the resulting formation and growth of insoluble proteinaceous structures are processes associated with important normal and aberrant biological pathways [1–7]. Such processes in particular underlie a range of clinical disorders, including Alzheimer's and Parkinson's diseases, where the transition of proteins from their normal biologically active soluble form into supra-molecular aggregates leads to accumulation of non-native proteinaceous nanostructures such as amyloid fibrils. Biology has, however, also found ways to exploit amyloid structures for functional purposes under controlled conditions in applications which include catalytic scaffolds [8], functional bacterial coatings [9] and non-genetic information transfer [5–7].

Detecting and measuring amyloid growth has proved to be a challenging task. The currently used fluorescent label based assays for determining the concentration of fibrillar material in solution rely on the knowledge of the label-fibril binding stoichiometries, which can be variable for different types of aggregates or as a function of extrinsic conditions. Recently, alternative label-free approaches for monitoring protein aggregation have emerged, and it has been shown that techniques based on biosensors, in particular quartz crystal microbalances (QCM) [10–13] or surface plasmon resonance sensors (SPR) [14], could represent a valuable addition to the palette of methodology available for studying the supra-molecular self-assembly of proteins. Both QCM and SPR techniques, however, are currently limited with respect to possibilities for miniaturization and scalability.

Microcantilever sensors have recently been developed as highly sensitive miniaturized transducers for label-free detection of biomolecules [15–18]. The molecular interactions on one side of a cantilever surface are directly transduced into mechanical bending, which in turn can be precisely detected either optically or electronically. The bending of the cantilever is driven by the surface stress changes arising as a result of specific interactions between biomolecules on the cantilever surface. A wide range of interactions have been detected using such an approach, including pairwise interactions between biomolecular partners such as DNA–DNA [15] hybridization, protein–protein interactions [19] and protein–DNA interactions [18, 20].

In this paper, we demonstrate the detection of protein aggregation and resulting amyloid growth through surface stress measurements by the use of microfabricated cantilevers and cantilever array systems.

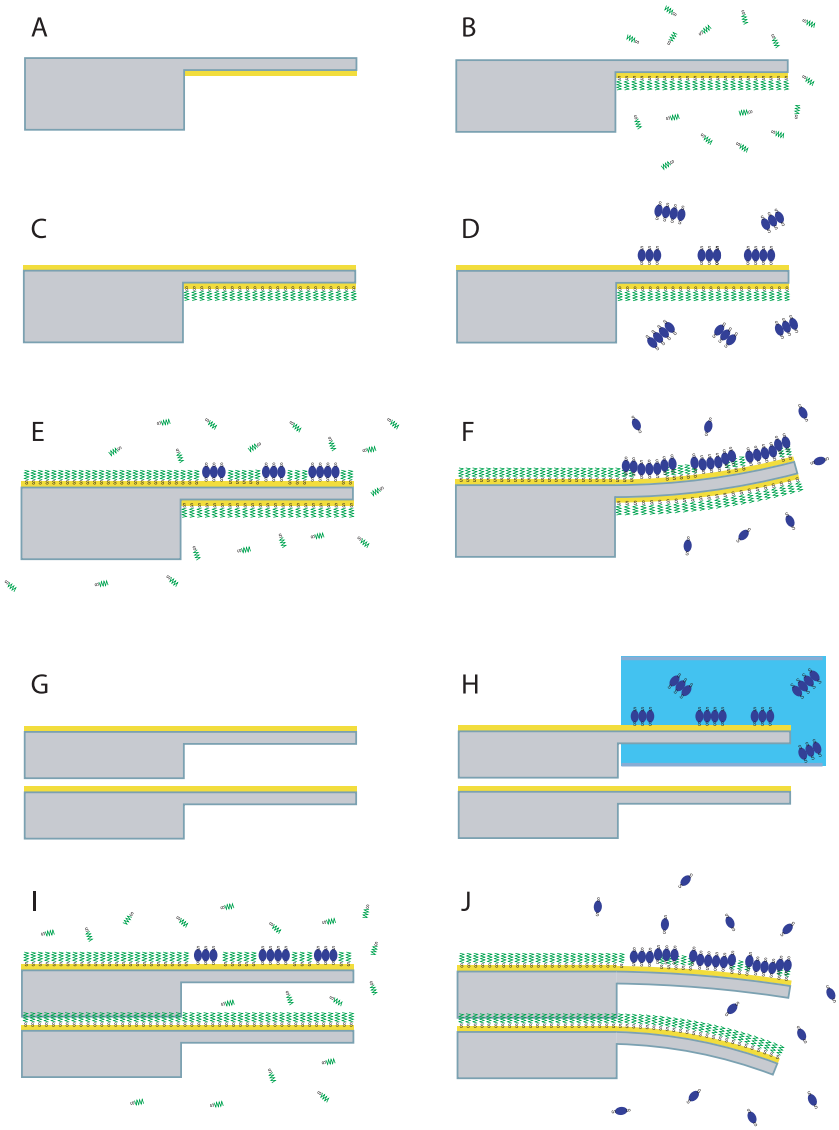

Figure 1. Functionalization of a single internal reference microcantilever ((A)–(E)) as described in section 2 of the main text. The cantilever is shown in gray, the gold layer in yellow, the PEG layer in green, and protein molecules in blue. In (F) protein is injected into the flow cell, and protein molecules can add onto the seed fibrils present on the cantilever resulting in tensile stress. In ((G)–(I)) functionalization of a multi-cantilever array is illustrated; in (J) soluble monomer is injected into the flow cell, resulting in a net differential tensile stress between the sensor cantilever and the control cantilever lacking the seed functionalization.

2. Experimental details

We use here the protein insulin to illustrate the detection of protein aggregation with cantilever sensors. The general approach is similar to earlier experiments on quartz crystal microbalances [10] although the micron scale size of the cantilevers allows for significantly smaller sample quantities to be used. Seed fibrils were prepared by incubating bovine insulin (Sigma Aldrich) at a concentration of 10 mg ml^{-1} in water adjusted to pH 2.0 using hydrochloric acid. The solution was kept at 60 °C for 24 h and then for 7 days at room temperature. The fibril suspension was then diluted 1:5 into 10 mM HCl and sonicated in a bath sonicator until the length of the fibrils as assessed by atomic force microscopy was reduced from the initial values in excess of a micron to a few hundreds of nanometers.

For the single cantilever measurements, v-shaped silicon nitride cantilevers (Veeco Instruments Inc., USA, length 220 μm, thickness 0.6 μm and a typical spring constant of 0.03 N m^{-1}) were coated on the frontside with 2 nm of chromium followed by 20 nm of gold (figure 1(A)) using a thermal evaporator (BOC Edwards) with a base pressure below 10^{-6} mbar. The cantilever was then incubated for 20 min in a 0.02% solution of mercapto-PEG in HCl (figure 1(B)) in order to form a protective inert monolayer which prevents non-specific protein adsorption. The cantilever then received on the backside a chromium and gold coating (figure 1(C)) identical to the one previously deposited on the frontside. We verified that the passivating PEG layer was intact after the second evaporation step by testing the wetting properties of the film using an optical meter before and after the second gold evaporation; no measurable change in contact angles

(≈65.9° ± 2°) was observed [21]. The cantilever was then inserted into a pipette tip which was filled beforehand with 10 μl of the sonicated seed suspension prepared as described above, and left for 60 min for the seed fibrils to attach onto the backside of the cantilever (figure 1(D)). The cantilever was subsequently immersed for 30 min in a 0.02% mercapto-PEG solution to passivate unexposed areas on the backside of the cantilever (figure 1(E)). We have previously characterized such functionalized surfaces by atomic force microscopy [10], and have shown that the PEG layer does not displace the fibrils under the conditions used. Finally, the cantilever was inserted into the flow cell and left to equilibrate for 24–36 h in 10 mM HCl.

Silicon multi-cantilever arrays (length 500 μm, width 100 μm and thickness 1 μm, fabricated by the IBM Zurich Research Laboratory [16]) were gently cleaned in piranha solution (2/3 H_2O_2 30% and 1/3 H_2SO_4 98%) and washed in copious amounts of water followed by washing with absolute ethanol 98%. The microcantilevers were then blow dried using a gentle flow of nitrogen gas. Then the microcantilevers were coated on the backside with 2 nm of titanium and 20 nm of gold using an electron beam evaporator (BOC Edwards). The cantilevers were subsequently functionalized using an array of capillary tubes with diameters chosen to match the pitch of the array. The tubes connected to the sample cantilevers were immersed at the other end into small reservoirs containing a suspension of seed fibrils prepared similarly as for the single cantilever setup. After 60 min of functionalization, the array was immersed in 0.02% mercapto-PEG in 10 mM HCl for 30 min, passivating the unexposed areas of the sample cantilever as well as the entire surface of the control cantilever. Both for the single cantilever and for the cantilever array, the deflection detection was optical through reflection of a diode laser beam off the backside of the cantilever and onto a single position sensitive photodiode, whereby time multiplexing was employed in the case of the multi-cantilever array.

The measured cantilever deflections Δz were converted to changes in surface stress σ using Stoney's equation [22]:

$$\sigma = \frac{Yh^2}{3(1-\nu)l^2}\Delta z$$

where Y is the elastic modulus and ν Poisson's ratio, and h and l are the height and length of the cantilever.

3. Results and discussion

We demonstrate the detection of protein aggregation through measurements of changes in surface stress by directing the deposition of misfolded proteins to the surface of a microcantilever sensor. This localization was achieved through the use of seed fibrils, fixed to the sensor, which have previously been shown to sequester proteins from solution by functioning as a template to propagate the fibril polymerization reaction resulting in amyloid growth. As shown in figure 2, when a cantilever passivated on one side with an inert self-assembling monolayer and containing seed fibrils on the other is brought into the presence of soluble protein, an increasing amount of tensile surface stress is generated over the time

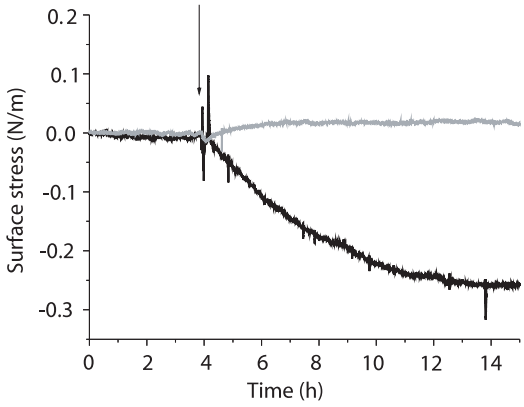

Figure 2. Single cantilever detection of amyloid growth. Starting from a flat baseline (0–4 h) a cantilever with seed fibrils on the backside and an inert PEG layer on the frontside is brought into the presence of soluble insulin (arrow), and the subsequent growth of amyloid on the cantilever results in tensile stress (2–10 h, black curve). The reaction comes to completion after 10 h due to the depletion of soluble precursor protein and the absence of free space on the sensor required for the fibrils to continue growing. When both sides of the cantilever are covered with an inert monolayer only a small deflection is observed (gray curve), demonstrating that the signal of the functionalized cantilevers originates from the growth of the seed fibrils. The small deflection observed for the control could originate from small physical or chemical differences between the front and backsides of the cantilever.

period of 10 h as the fibrils grow. The maximum surface stress observed was 261 mN m^{-1} or 441 nm of bending. On the other hand, for a cantilever coated only with an inert monolayer and no seed fibrils, no significant surface stress was observed even in the presence of protein molecules in solution (figure 2), showing that the observed surface stress originates from the growth of the seed fibrils.

We next performed analogous measurements using microcantilever arrays. Multi-cantilever arrays have the advantage that during the experiments dedicated reference cantilevers can simultaneously be monitored. The reference levers have an identical geometry and mechanical properties to the sensor levers and can be used to eliminate any non-specific drift effects of chemical or physical origin. For single cantilevers this type of reference is not possible; however as the observed signal results from the difference in surface stress between the front and the backsides, the inert PEG layer used on the front side as described above can conceptually be viewed as acting as an 'internal reference', in the sense that resulting surface stress can be identified as stemming from the backside functionalization. This conclusion is valid as long as in the absence of such functionalization no surface stress is observed as shown in figure 1. For multi-cantilever arrays, the differential bending between the sensor cantilever and the reference cantilever was monitored, yielding the net contribution from the surface reaction as any non-specific drift is canceled in the differential signal. In this case, as shown in figure 3, the detection of amyloid growth can also be achieved through the resulting tensile differential stress between sample and reference cantilevers, consistent with the observations for the single cantilever sensor. To give a measure of the quality

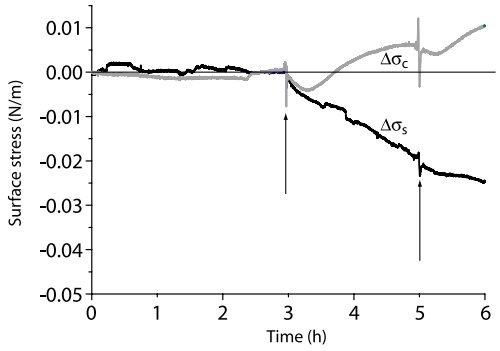

Figure 3. Multi-cantilever measurement of amyloid growth. The differential stress $\Delta\sigma_s = \sigma_{sample} - \sigma_{control1}$ of the sample cantilever versus the reference cantilever is shown in black. For comparison, the differential bending $\Delta\sigma_c = \sigma_{control2} - \sigma_{control1}$ from an additional reference cantilever is shown in grey to give a measure of the quality of the drift compensation as described in the text. Soluble protein was injected at the time corresponding to the first arrow, and the injection of 10 mM HCl solution without protein is indicated with the second arrow.

of the reference approach through cancelation of non-specific drift, we also show a differential signal between two identically prepared individual reference levers (figure 3, gray line). The absolute values of the rates of change in the surface stress between the single and multi-cantilever experiments differ here typically by up to a factor of 4, a fact which can be attributed to the difficulty in repeatedly functionalizing the cantilever systems with identical amyloid seed material and density.

The advantages of microcantilever systems as sensors are multiple [16–18, 23–27] and range from the scalability of the approach in the form of parallel arrays to the very small sample volumes required implying possibilities of integration with microfluidic systems in a lab on a chip type setup. Due to the relatively recent development of the technique, however, no unified theory exits yet as to the origins and magnitude of surface stresses generated by reactions on the sensors. Short range surface forces between adsorbed molecules and the surface in the form of van der Waals or hydrophobic forces, as well as steric repulsion or longer range electrostatic effects [21] have been proposed to contribute to varying degrees in different cases, but an *a priori* prediction of the magnitude or even sign of surface stresses generated for a broad class of chemical reactions is yet lacking. The situation for proteins is especially complex as at most pH values these exist as zwitterionic species, complicating an electrostatic analysis. In the present case, it is interesting to speculate that the mechanism of stress generation could be analogous to what has been proposed [28] previously for simple protein adsorption, where in plane attractive protein–protein forces, taking here the form of hydrogen-bonds characteristic of the amyloid structure, lead to a tendency to contract and therefore translate into the upwards bending of the cantilever sensor. For sensor systems such as quartz crystal microbalances, in contrast, the physical basis for the generation of the observed signal, namely frequency shift of shear oscillations, is less convoluted, thereby facilitating the interpretation of kinetic measurements of protein aggregation. On the other hand, the fact that cantilever sensors are sensitive to a wide range of different interactions implies the possibility of acquiring information on the different surface related forces which result from protein aggregation; in this context the deconvolution of the respective contributions can be seen as a major target and challenge for future developments of cantilever sensor technology.

4. Conclusions

In conclusion we have shown that detection of protein aggregation using microcantilever sensors, both on single cantilever sensors and multi-cantilever arrays is possible through measurement of the generated surface stress. These findings lay the foundations for and open up the possibility of using the palette of technologies available in connection with parallel cantilever biosensors to explore protein aggregation in more complex settings such as starting from *ex vivo* material or parallel screening for modulators or inhibitors of protein aggregation.

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