Change in Rigidity in the Activated Form of the Glucose/Galactose Receptor from *E.coli*: A Phenomenon That Will Be Key to the Development of Biosensors

Running title: Change in Rigidity in the Glucose/Galactose Receptor.

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Abstract

Recently a periplasmic glucose/galactose binding protein, GGRQ26C, immobilized on gold surface has been used as an active part of a glucose biosensor based on quartz microbalance technique (QCM). However the nature of the glucose detection was not clear. Here we have found that the receptor protein film immobilized on the gold surface increases its rigidity when glucose is added, which explains the unexpected detection signal. To study the rigidity change, we developed a new fast and simple method based on using atomic force microscopy (AFM) in tapping mode. The method was verified by explicit measurements of the Young’s modulus of the protein film by conventional AFM methods. Since there are a host of receptors that undergo structural change when activated by ligand, AFM can play a key role in the development and/or optimization of biosensors based on rigidity changes in biomolecules.

Key words: glucose biosensors; atomic force microscopy; quartz crystal microbalance; periplasmic receptor proteins

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INTRODUCTION

The potential use of periplasmic binding proteins as biosensors has arisen due to their solubility, stability, and ability to reversibly bind a large variety of small ligands including sugars, amino acids and inorganic ions. These proteins comprise a large family of functionally similar receptors with a two-domain structure and a hinge cleft mechanism for binding substrate. (Blair, 1995; Felder et al., 1999; Mowbray and Sandgren, 1998; Stock and Mowbray, 1995) Whereas the proteins are open and flexible when ligand is absent, they are more structurally compact and closed when ligand is bound. (Salopek-Sondi and Luck, 2002; Salopek-Sondi et al., 2003) This large conformational change is the key to subsequent events in the chemotaxis pathways and transport of the bound substrates into the cytoplasm of the bacteria and can be used as a bioplatform for sensing small ligands. (Dwyer and Hellinga, 2004; Hsieh et al., 2004; Marvin and Hellinga, 1998; Salins et al., 2001) The most widely exploited receptor is the glucose/galactose binding protein (GGR) which binds D-glucose (K_d = 0.2 \mu M) and D-galactose (K_d of 0.4 \mu M). (Vyas et al., 1988) This receptor is an ideal biomaterial since it is structurally and functionally well characterized. There are no disulfide bonds or free cysteine residues present in the native protein. However, a mutant of GGR (GGRQ26C) with an engineered single cysteine at amino acid 26 replacing a glutamine residue was the utilized as a means to attach the protein to the gold surface via a covalent gold-sulfur bond for use in the biosensor experiments. (Careaga and Falke, 1992) In our quest for a glucose sensor, we have found that single cysteine mutants of the glucose/galactose binding protein can immobilized on the gold surface. This film is capable of binding glucose. This binding event has been demonstrated by a number of techniques including electrochemical impedance, (Wang et al., 2005) surface plasmon resonance (SPR) (Luck et al., 2003) and piezoelectric quartz crystal (QCM) (Carmon et al., 2004).
The principle of the QCM technology is based on detecting the frequency decrease of the piezoelectric crystal resulting from mass changes on the surface when biomolecules are attached. (Janshoff et al., 2000; Marx, 2003) Recently it has been shown that GGRQ26C directly attached to the gold surface of the QCM can be used as an effective glucose sensor even though the target sugars are predicted to be too low in mass to be detected (Carmon et al., 2004). Applying the Voight model of a viscoelastic films to interpret the QCM data in the study indicated that the protein film should be considerably more viscous and/or possibly more rigid when glucose was bound. (Hook and Kasemo, 2001). Direct rigidity measurements shown in this present study corroborate that hypothesis.

The atomic force microscopy (AFM) technique (Binnig et al., 1986; Sokolov, 2003c; Sokolov and Henderson, 2000) is a natural choice to study mechanical properties of molecular films at the nanoscale. Several studies have been done on essentially atomically smooth surfaces (Kovalev et al., 2004; Sokolov et al., 1997; Sokolov, 2003a; Tsukruk et al., 2000). In the case of more rough surfaces, i.e., gold surface of the piezoelectric crystal of the QCM, the inhomogeneity of the films can be considerable. Furthermore, the surface geometry should be measured to derive the Young’s modulus, a geometry-independent characteristic of rigidity. Consequently, a large amount of statistical data is required to make conclusion about the mechanical properties of the film. Although these data can potentially be collected automatically (force-volume mode (A-Hassan et al., 1998 Mar; Berdyyeva et al., 2005a; Hoh and Schoenenberger, 1994 May)), it still takes a considerable amount of time.

In this paper we suggest a simple and fast AFM method to detect the rigidity change in protein film before and after addition of ligand. In this study we explicitly show that GGRQ26C protein film on a gold surface of the piezoelectric crystal indeed increases its rigidity when activated with
glucose. To show consistency of this method with the more “traditional” direct measurements of rigidity (detecting not just the rigidity change), we explicitly measure the Young’s modulus at a few points on the surface. The latter study shows both changes in rigidity and effective thickness of the surface layer that arises from ligand induced conformational change of the protein.

This AFM method for detecting rigidity changes in proteins can be effective in the study and optimization of any sensors where the ligand induced structural change occurs.

MATERIALS AND METHODS

Expression and Purification of GGRQ26C

The GGRQ26C plasmid was expressed in *E. coli* BL21 cells and purified as described previously in (15,19). Protein was then dialyzed against two changes of 250 ml 3M guanidinium chloride (GnHCl), 100mM KCl, 20mM EDTA, 10mM Tris pH 7.1, and four changes of 500 ml buffer containing 100mM KCl, 10mM Tris pH 7.1, and 0.5 mM CaCl₂. Quantitation of the protein was determined by extinction coefficient ($\varepsilon_{280}$) of 0.93 mL mg⁻¹ cm⁻¹ and a $M_f$ of 33400.(Luck, 1995)

Each molecule of GGRQ26C has a cysteine residue at position 26, which can be attached to a gold surface by a sulfur-gold covalent bond as illustrated in Fig. 1. The size of each protein molecule of is ~ 3.5 x 6.5 nm.

Protein Immobilization on the Gold Surface of the Piezoelectric Crystal and Subsequent Activation by Glucose

A piezoelectric quartz crystal used in the previously described QCM experiments (Carmon et al., 2004) was used for the AFM experiments. This crystal was attached to a Petri dish by double-sided
tape to prevent movement of the crystal during the measurements. The gold surface of the piezoelectric quartz crystal to which the protein was to be immobilized was first cleaned with UV short-wave light for 5 minutes. An AFM image of bare gold surface is shown in Fig.2. One can see that the surface has granular structure. Despite that the surface is rather flat. With the area of 3x3 µm², the height difference in the image is only 19nm. It also can be quantified by roughness (RMS) parameter, which is equal here to ~2nm.

A droplet (~ 500 µL) of 27µM GGRQ26C protein in a buffer (100mM KCl, 10mM Tris pH 7.1, 0.5 mM CaCl₂) was then introduced to the gold surface. This solution incubated for 1 hour in a closed Petri dish to insure the formation of the gold-sulfur bond immobilizing the protein to the surface. Water was added around the glass slide to prevent possible evaporation of the buffer solution and drying of the protein surface. The immobilized protein surface was then washed with the above described buffer to reduce any non specific binding of the protein to the already immobilized protein or the gold surface. Specifically, the droplet was removed by tilting the slide, and ~ 2mL of the buffer was added, and then also removed by tilting.

The first AFM scanning experiments were performed in the buffer after this wash. The second AFM scans were done after adding glucose as follows. Without disassembling the AFM liquid cell, 100 µL of 1mM of glucose in the buffer was added to 2mL buffer in the fluid cell, and left quiescent for 15 minutes before the starting scanning. So, the protein immobilized on the gold surface was exposed to ~ 50 µM glucose solution for 15 minutes. The surface was then rescanned by AFM.

_Atom Force Microscope_
Dimension 3100 Nanoscope™ IIIa by Digital Instruments/Veeco (Santa Barbara, California) with an extender box was used in this study. The imaging was done in liquid using a standard fluid holder. There were two types of the AFM cantilevers used for the imaging in tapping mode. The first (tip 1), FESP AFM cantilevers (Digital Instruments/Veeco, CA) with silicon tip was used for tapping mode of scanning in liquid. The radius of the probe was tested on a 3-Dimensional tip characterization gratings (TGT1 by Micromash, Inc.). A typical AFM tip used had the radius of the apex of ~10 nm. The driven oscillating amplitude was taken 20mV, the oscillating frequency in liquids was ~30KHz. The second type (tip 2) of cantilever used was a regular V-shaped silicon nitride cantilever with integrated pyramidal tip (Digital Instruments/Veeco, CA). The driving amplitude was set to 3V, while 6Khz. Both tips were cleaned before each series of measurements by a UV short-wave lamp for 2 min. The scan rate was set 0.5-1 Hz to optimize the image quality. Each image was collected in resolutions of 512x512 pixels. It is worth noting that there is no need in knowledge of the force constant of the used in the suggested here method.

For the force-volume mode, a V-shaped silicon nitride cantilever with integrated pyramidal tip (similar to tip 2 above) was used. The radius of curvature of the tip was found by using the same method as above, and was ~20nm. The force constant was found to be 0.04N/m by using the resonance shift method (built-in option of Nanoscope 5.12r4 software).

RESULTS AND DISCUSSION

Detection of the Rigidity Change with AFM: Rationale
A more traditional approach to measure rigidity of thin film would be to record the force-distance curves and derive the Young’s modulus directly from the curves. To do that, however, one has to (a) collect enough statistics to take into account a possible inhomogeneity of the film, and (b) know the topography of the film. In the case of the gold film on the QCM sensor, the surface can be approximately described as a flat covered with spherical protrusions (illustrated later in Fig. 4). The formulas describing the interaction between the AFM tip and surface are quite different if the AFM tip scans above the top of the spherical protrusion, or the valley in-between the protrusions. To measure simultaneously the tip position and the geometry of the surface, one needs to use the AFM force-volume mode (A-Hassan et al., 1998 Mar; Berdyyeva et al., 2005a; Hoh and Schoenenberger, 1994 May). However, this particular mode requires a much longer time than just regular mode of scanning. It also is quite limited in the size of area it can examine because we are limited up to 64x64 pixels to record the image. In addition, the film on the surface of QCM sensor is not ideal. One area can differ from another area on the surface quite dramatically. Therefore, one needs to repeat the force-volume measurements many times at different areas to collect enough statistical data. And finally, to get reliable contact in the force-volume mode, one needs to use force that might damage a soft molecular layer.

Here we show a novel technique of AFM scanning that is considerably faster and gentler to the sample to obtain qualitative observation of the change of rigidity of the protein film. Such an observation is needed, for example, when developing a new biosensor, when it is not known if the presence of ligand influences the rigidity or not. The suggested technique of scanning is based on the AFM tapping in liquids with small amplitudes. We have previously used a technique to observe multilayer growth of liquid crystals with no destruction (Sokolov et al., 1997). Here we show that the suggested new technique requires only one tapping scan before and one scan after the addition of
ligand. Both scans have to be taken on the same surface area. So, one obtains reliable statistics of the rigidity change.

The time required by our new method takes about 10 min to collect both required scans (512x512 pixels each). To collect comparable statistics in the traditional force-volume mode it would take more than 40 hours (~ 40 min per 64x64 pixel scan). It should be noted, however, that the amount of calculation time can be larger for the suggested method due to the lack of customized software.

Method of Detection of the Rigidity Change with AFM: Theory

Here we show that the increase of rigidity, the Young’s modules, of the surface layer can be estimated using a relatively simple experimental method, which requires just two regular AFM scans without special calibrations and measuring the forces. In this method we scanned the immobilized protein on the surface and then the same area with ligand (glucose) added to obtain two topographical images of the surface. The change of rigidity of the surface layer can be found by using various indentation models. It is intuitively clear that the conclusion about either increase or decrease of the Young’s modulus is independent of a specific model. To demonstrate the method, we will use the classical Hertzian model, see, e.g. (Landau and Lifshitz, 1986). The same conclusions about the rigidity can be obtained by using more sophisticated semi empirical multi-layer models overviewed in (Kovalev et al., 2004). However, to show it here is beyond the scope of the present work.

We model the AFM tip – sample contact by two deformed spheres, Fig. 3. Deformation distance \( d \) (penetration) of such two spheres of radii \( R \) and \( R' \), which have different modulae \( E \) and \( E' \), is shown in Fig.2.
The relation between the applied load force $F$, which induces the deformation, with deformation $d$ is given by the following formula (Landau and Lifshitz, 1986)

$$d = F^{2/3} \left\{ D^2 \left[ \frac{1}{R} + \frac{1}{R'} \right] \right\}^{1/3}, \quad (1)$$

where

$$D = \frac{3}{4} \left\{ 1 - \frac{\nu'^2}{E'} + \frac{1-\nu^2}{E} \right\} \quad (2)$$

is a combination of the Young's modulae and the Poison ratio $\nu$ and $\nu'$.

It is a good approximation to consider the AFM tip to be considerably more rigid than the sample surface. Hereafter we put $E' \to \infty$ (let the AFM tip be the upper sphere). Furthermore, we will use $\nu = 0.5$, which is the case for incompressible materials. It should be noted that our conclusions do not depend on the latter assumption. These two assumptions reduce eqs.(1), (2) to

$$d = F^{2/3} \left( \frac{3}{4} \right)^{4/3} \left\{ \left( \frac{1}{E} \right)^2 \left[ \frac{1}{R} + \frac{1}{R'} \right] \right\}^{1/3} \quad (3)$$

If the protrusion is not spherical but elliptical, there is a simple modification of the above formula (Johnson 1985). In such a case, the radius factor $1/R + 1/R'$ is changed by an effective one, geometrical average of the multiplication of two radius factors for both major axis of the ellipsoid $R_{\text{min}}, R_{\text{max}}$:

$$\frac{1}{R_{\text{eff}}} = \sqrt{\left( \frac{1}{R_{\text{min}}} + \frac{1}{R'} \right) \left( \frac{1}{R_{\text{max}}} + \frac{1}{R'} \right)} \quad (3a)$$
Let us now consider a case when the AFM tip scans over two protrusions of radii $R_1$ and $R_2$, which are covered by a layer that has rigidity $E$, Fig.3. If scanning with the load force $F$, the AFM tip causes deformations $d_1$ and $d_2$ over the protrusions $R_1$ and $R_2$, $(R_{eff1}$ and $R_{eff2})$ respectively. Here we consider $R_{1,2} \gg d_{1,2}$, which corresponds to the our experiment. Therefore we will not consider the change of radius of the protrusions due to the film deformation.

The height difference $\Delta H$, see Fig.3, as measured in the AFM scan is given by

$$\Delta H = (h_1 - d_1) - (h_2 - d_2)$$

$$= h_1 - h_2 + F^{2/3} \left( \frac{3}{4} \right)^{4/3} \left( \frac{1}{E} \right)^{2/3} \left\{ \frac{1}{R_{eff1}} \right\}^{1/3} \left( \frac{1}{R_{eff2}} \right)^{1/3}$$

(4)

where $h_1$ and $h_2$ are the heights of the non-deformed protrusions.

If the material (film) rigidity changes, the height $\Delta H$ will have different value. For example, as we demonstrate in this paper, the protein film changes its rigidity if we add glucose. Scanning the same area with the AFM before and after adding glucose, we can measure the changes of heights $\Delta H_{no \ glucose}$ and $\Delta H_{with \ glucose}$ between the same two protrusions. Subtracting these two values, and using eq. (4), one gets

$$\Delta = \Delta H_{no \ glucose} - \Delta H_{with \ glucose}$$

$$= F^{2/3} \left( \frac{3}{4} \right)^{4/3} \left\{ \frac{1}{E_{no \ glucose}} \right\}^{2/3} \left( \frac{1}{R_{eff1}} \right)^{1/3} \left( \frac{1}{R_{eff2}} \right)^{1/3}$$

(5)

where $E_{no \ glucose}$ and $E_{with \ glucose}$ are the Young’s modules of the film in the absence and presence of glucose.

One can see that the difference $\Delta$ is an indicator of the film rigidity change after adding glucose. Because $R_1$ and $R_2$ can be directly measured from the AFM scans, the difference $\Delta$ gives
unambiguous answer based on the sign of the rigidity change. For example, as one can see from eq.5, if $E_{\text{no glucose}} < E_{\text{with glucose}}$ than the difference $\Delta$ is positive, provided $R_{\text{eff1}} > R_{\text{eff2}}$.

It should be noted that applying the above derivation to a film on a rigid surface, we assumed that the deformation of the film is small, and as a result, the influence of more rigid surface is negligible. Indeed, a more exact model (Kovalev et al., 2004) is needed if more quantitative results are required. However, using that more complex model here would not change the qualitative result.

There is one natural limitation in usability of our new method, which occurs due to a possible change in long-range forces acting between the tip and surface. Because both scans should be collected while using the same force of interaction between the tip and surface, the load force is the same, if and only if, the tip-surface interaction is the same. If the addition of ligand alters the long range force, it makes our method much more complicated. In our case, the use of buffer with 50 $\mu$M glucose as ligand in buffer of 0.1M ionic strength should not change possible long-range forces. In any case the strongest component of the long-range forces, the electrostatic interaction is shielded by the high ionic strength of the buffer (Debye length is $\sim$ 1nm).

Another method of estimation the rigidity might be the changes in surface roughness. Roughness depends on the variation of the surface heights. Looking at formula (4) that gives such variations, one can see, however, that any change of rigidity can lead to either decrease or increase of roughness depending on the surface geometry. To make even a qualitative statement, one would need to calculate deformation of the surface at each point, which is impractical.

**Method of Detection of the Rigidity Change with AFM: Experiment**

To study the change of rigidity with the AFM, two scans were taken as described above. A representative scan without glucose done with tip 1 is shown in Fig. 5 a. To exclude a possible
simple removal of the protein film during scanning three scans were executed. The last scan was recorded and used for the further analysis. Glucose was added and the same region was scanned, Fig.5b. Despite some thermal drift, all features in the images can be easily identified. Fig.5 (c) and (d) show the same type of images obtained with tip 2 before and after adding glucose, respectively.

One can see in Fig.5 that relatively high noise in images (a) and (c) is gone in images (b) and (d). This is a typical behavior when the film increases its toughness, and it is more durable. However, to exclude ambiguity of radii calculations in the noisy area, we did not use those areas in further calculations. Fig. 6 shows bearing analysis of depth distributions highlighting the changes in the film morphology before and after adding glucose. Each point on the curve shows the fraction of the film in the imaginary plane drawn at corresponding depth below the topmost point of the surface. One can clearly see smaller number of highs (slower increase of the shown histogram portion with increase of depth near zero) before adding glucose. Comparing this result with Fig.5 (a) and (c), one can conclude that this is due the higher amount of spiky noise, which almost disappears after adding glucose. We can observe similar behavior with calculation of roughness. After adding glucose roughness drops from 1.63nm (Fig.5a) to 1.47nm (Fig.5b) and from 1.81nm (Fig.5c) to 1.68nm (Fig.5d).

To analyze the change of the Young’s modules, we measure the radii of the protrusions in Fig.5 and the change of height $\Delta$of formula (5). Fig.7 shows an example of cross-section of two protrusions before and after adding glucose. Because we need to find the radii of the protrusions and their relative height, it is worth of processing the image though the low pass filter. Random noise can be removed in this way. This fairly simple procedure should be watched, however, not to overdue to possibly change the data (heights and radii). The radii of curvature were found using SPIP software (Imagemet, Inc.). Then we need to find the effective radii (eq.3a). For example, for one protrusion
we found $R_{\text{min}}=(144\pm5)\text{nm}$ and $R_{\text{max}}=(232\pm8)\text{nm}$. Taking the tip radius of 20nm, one gets $R_{\text{eff}}=(18.0\pm0.1)\text{nm}$. It should be noted that it is not an easy task to estimate the load force during the tapping scanning. Fortunately, it is not necessary to use the load force to find the rigidity change, see the theory Section. For our estimate, we use $F = 0.1nN$. This number comes from the fact that we were able to image liquid crystals (Sokolov et al., 1997) using similar tapping mode, whereas the crystal destruction starts from forces $\sim 1nN$ (Sokolov, 2003b). Table 1 shows the effective radii and measured $\Delta$, and the numerical results for

$$\left(\frac{1}{E_{\text{no glucose}}}\right)^{2/3} - \left(\frac{1}{E_{\text{with glucose}}}\right)^{2/3} = \frac{\Delta}{F^{2/3}(3/4)^{1/3}} \left(\frac{1}{R_{\text{eff}2}}\right)^{1/3} - \left(\frac{1}{R_{\text{eff}1}}\right)^{1/3}, \quad (6)$$

which we called the “rigidity factor change” (RFC). Histograms of these results are presented in Fig.8. In these calculations we choose to keep the definition of radii so that $R_1 > R_2$. Therefore, the positive factor, eq. 6, corresponds to the increase of the Young’s modulus of the film.

One can see from Fig. 8 that the film statistically increases its rigidity. The average increase is $+2\times10^{-4}\text{ Pa}^{-2/3}$ when using tip 1 (Fig.5a), and $+1\times10^{-4}\text{ Pa}^{-2/3}$ for tip 2 (Fig.5b). The observed decrease in same cases could probably be explained by irregularity of the film properties, adsorption of additional layers after adding glucose. In some cases ($\sim 20\%$) we were not able to detect the height change because it was too small, below the sensitivity of the instrument. Those data are not plotted in Fig.8.

One note should be done about the resolution and optimal scan size of the collected images. Because we need to access relatively small features, protrusions, it is worth taking as much pixel resolution as possible. For the lateral size of the scan, it needs to be large enough to provide enough statistical data. We found that 1.5-2 microns is close to optimum with this type of surface features.
Quantitative Measurement of the Young’s Modulus with the Force-Volume Mode

To validate our new method, we compared the above results with direct measurements of the Young’s modulus by collecting the force curves in the force-volume mode. An integrated pyramidal tip was used in these measurements (similar to tip 2). Radius of curvature of the tip and the cantilever spring constant were measured as described in Materials and Methods. To analyze our data from the force-volume mode, we used the Hertzian model as described by eqs. (1), (2). Analysis of the force-volume data was done as follows. 20 to 30 force curves measured on the tops of the protrusions were averaged. Fig. 9 shows an example of three averaged force curves collected before and three after adding glucose. The procedure of finding the Young’s modulus from this type of curves is described in detail elsewhere (Berdyyeva et al., 2005b). Each average force curve was processed to calculate the Young’s modulus vs. penetration $d$, by using eq.(1). The results of the analysis of six measurements before and six after adding glucose are presented in Fig.10. One can see unambiguous change of the Young’s modulus after adding glucose.

The increase of the rigidity with the tip penetration is expected due to approaching the much more rigid gold substrate. One also can see the change of thickness of the protein film. While the thickness before adding glucose is ~ 6-7 nm, after adding glucose, the film becomes ~ 3-4 nm thick. Schematically, it is shown in Fig.11.

It should be noted that the values of the Young’s modulus and the film thickness obtained in this study are in good agreement with the values estimated by (Carmon et al., 2004) to explain the QCM data. It is also interesting to compare the results presented in Fig.6 with the calculations used for the change of the rigidity factor, eq. (5). Assuming $F = 0.1nN$, $R_1=140nm$, $R_2=50nm$, and taking the Young’s modules $E_{no\, glucose} = 0.25\times10^5 Pa$ and $E_{with\, glucose} = 0.5\times10^6 Pa$ from Fig.6, one can get $\Delta=0.4nm$. The experimental data corresponded to those radii show $\Delta=0.6-0.8nm$. This small
discrepancy can be explained by using the Hertzian model, which is too simplified for quantitative analysis. Moreover, force $F$ is not really known for the tapping mode. The use of more sophisticated model for deformation of multi-layered materials (Kovalev et al., 2004) gives $\Delta=0.4-0.6\text{nm}$ for the same parameters as the used above. This shows consistency of both methods. To make the statement of consistency more convincing, let us note that there is some basic difference between these two methods. First, the rigidity change method is more statistically sound. The analysis in that method covers a considerably larger area, and a larger number of surface spherical protrusions. Secondly, in this method, the areas of study were the same before and after adding glucose, whereas those areas were different in the force-volume measurements. This will add more uncertainty to the direct comparison of the methods. In the force-volume method, the radii of gold spherical protrusions were found with less precision because of the limited spatial resolution (limited number of pixels). Furthermore, we did not have ability to exclude some “noisy” areas in the force-volume mode (it was not possible to detect with the limited number of pixels), which was done in the other method. And finally, the force-volume method requires attaining considerably higher AFM tip-surface forces to observe reliable tip-surface contact. This can result in the destruction of possibly multilayered film, which could be responsible for the decrease in the film rigidity presented in Fig.8. Therefore, it quite expected to have some quantitative discrepancy between these two methods.

The measured increase of rigidity is plausible to expect from biochemical point of view. When glucose binds to the receptor, a large conformational change takes place and the glucose is buried deep in the interior of the protein. The overall surface of the protein does not change significantly and one would not expect a major change in chemical composition of the GGR-glucose complex from the unbound GGR. The glucose binding is through a large network of hydrogen bonds that do not change the ionic character of the protein in solution. Within the cavity when the protein is open
there are hydrogen bonds to the water solution that encompasses that baths the protein. When glucose binds to the cleft, the OH groups on the sugar molecule replace the hydrogen bonds to water. Several hydrogen bonds are formed between the two lobes of the protein as the hinge closes. This change in the protein upon glucose binding causes many secondary elements within the structure to change. These shifts are presumably responsible for the increase of rigidity and compactness to the protein which has been measured by AFM here. The glucose is held within the interior by a network of hydrogen bonds that secures the two domains together sequestering the ligand away from the solvent.

CONCLUSION

We studied mechanical behaviors of the protein film used for detection of glucose in a QCM-based biosensor. A receptor protein, GGRQ26C was immobilized on gold surface of the sensor. We found that the binding of glucose to the protein on the sensor surface resulted in the increase of rigidity of the film. A straightforward approach to measure rigidity of the thin protein film would be to record the force-distance curves and derive the Young’s modulus directly from the curves. However, for a number of applications such a method is impractical mostly due to its large time consumption. Here we developed a simple and substantially faster method based on taking scans of the surface with the atomic force microscope. The method allows one to detect a qualitative change of the rigidity of a molecular (protein) layer when activated by ligand.

The AFM data described supports the reason for the large increase in the QCM frequency when glucose is bound to the receptor film (Carmon et al., 2004) and can explain the biophysical mechanism of detection of glucose by piezoelectric biosensors. This is very important to the future development of such biosensors for small ligands. Since there are a host of receptors that undergo
structural change when activated by ligand, AFM can play a key role in the development and/or optimization of biosensors based on rigidity changes in biomolecules.

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Table 1.
FIGURE LEGEND

**Fig.1.** The RasMol depiction of GGRQ26C on the gold surface attached via a gold-sulfur bond to the cysteine residues at position 26. The protein is illustrated in the closed form with the ligand trapped in the cleft between the two domains.

**Fig.2.** AFM scan of an area of 3x3 µm², the height difference in the image is only 19nm. The insert in the upper right corner is a zoomed view of 350x350nm².

**Fig.3.** A scheme of the AFM tip-surface contact.

**Fig.4.** A configuration of an AFM tip and two spherical protrusions.

**Fig. 5.** (a) AFM scan with tip 1 of an area of gold with the receptor GGRQ26C proteins attached; (b) the scan with tip 1 of the same area but with glucose added; (c) scan of another area with tip 2 of gold with the receptor GGRQ26C proteins attached; (d) scan of the area shown in (c) with glucose added. The bar size is 200nm.

**Fig.6.** Bearing analysis of depth distributions highlighting the changes in the film morphology before and after adding glucose of the surfaces shown (a) in Fig.5 a,b, and (b) in Fig.5 c,d.
**Fig. 7.** An example of cross-section of two protrusions before and after adding glucose. A small vertical shift is artificial, and introduced for better presentation as well as to stress the fact that the absolute vertical shift is meaningless in the AFM imaging. The insert shows the corresponding part of the AFM scan.

**Fig. 8.** Histogram of the rigidity factor change calculated from eq.6. Positive values correspond to the increase, the negative values to the decrease of the Young’s modulus (rigidity) of the film. The average rigidity factor change is $+2 \times 10^{-4}$ Pa$^{-2/3}$ when using tip 1 (Fig.5a), and $+1 \times 10^{-4}$ Pa$^{-2/3}$ for tip 2 (Fig.5b), which corresponds to an overall increase of rigidity.

**Fig. 9.** An example of three averaged force curves collected before and three after adding glucose. Raw data of the AFM cantilever deflection (in nm) vs. z-position of the scanner are shown.

**Fig. 10.** Dependence of the Young’s modulus on penetration (deformation) the AFM tip into the surface. Six curves before and six after adding glucose are presented.

**Fig. 11.** A schematic of spatial organization of GGRQ26C proteins before and after adding glucose.

**Table 1.** Measured effective radii, corresponding change of heights $\Delta$, and the rigidity factor change (RFC).
Fig. 1
Fig. 3
Fig. 5
Fig. 6
Fig. 7
Fig. 8

(a)

(b)
Fig. 9

- Before adding glucose
- After adding glucose

Z-position of scanner [nm] vs. Cantilever deflection [nm]
Fig. 10

Young's modulus [MPa] vs. penetration [nm]

- △ after adding glucose
- ○ before adding glucose
Fig. 11