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The selective immobilization of *mp*AFP RII octamere on gold surfaces for SMFS

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Abstract

A protein construct is made from antifreeze protein of the bacteria *Marinomonas Primoryensis;* the goal of this research is to immobilize this *mp*AFP construct on a gold surface using thiol gold bridges. *Marinomonas Primoryensis* uses the protein to attach itself to ice. As such the strength of the antifreeze protein is an interesting quality. The protein construct consist of eight RII monomere domains, which make up more than 90% of the antifreeze protein, and one green fluorescent protein. The strength of an unfolding monomere domain will be measured by single molecule force spectroscopy (SMFS) with an atomic force microscope (AFM). The protein construct has also two cysteines at the C-terminus. Not only can the *mp*AFP bind non-specific to the gold surface, like other proteins, but also via the sulfur group of the cysteine which can be used to form a thiol bridge with the gold. When the protein construct are adsorbed or attached to the gold surface with the thiol bridges, the AFM can pick up the protein construct on the other end. This will result in more domains unfolding compared to a non-specifically bound protein and thus a more reliable AFM measurement of the strength of the domain.

Two types of protein construct were used. Constructs which were able to form thiol bridges with the gold and the control group that could not. The adsorption curves of both constructs were measured with the help of surface plasmon resonance (SPR). It was found that the 10-20% of the constructs binds via thiol-gold bridges when a 100 mM Tris-HCL, 200 NaCl and 10 mM $CaCl_2$ buffer was used. This could be improved to 30-40% when a buffer without salt and with a neutral pH was used.

After these experiments were performed some AFM experiments were done to see if SMFS was already possible. This yielded some very good results. It was possible to see a typical saw tooth curve in about 1% of all the force curves. From these curves the force and length of the RII domains determined. The strength and length were 220±30 pN and 34±4 nm respectively.

Contents

Introduction

The bacteria *Marinomonas Primoryensis* lives in the deep ice-covered lakes of Antarctica. The bacterium wants to stay in the oxygen rich environment just below the ice. To ensure this the bacteria employs a large anti-freeze protein (ca. 1.5 MDa). This protein is attached with one end to the bacteria and with the other to the ice. The protein itself exists of five different domains of which the so called region II (RII) is the most interesting. This domain makes up the bulk of the antifreeze protein with more than 90% of the proteins mass consisting of 120 RII monomere repeats. The link between the ice and the bacteria consist primarily of the RII domain. For this reason the strength of this domain is interesting. [10] The strength of a protein can be measured with Single Molecule Force Spectroscopy (SMFS) in an Atomic Force Microscope (AFM).

To test the strength of this region 2 a protein construct has been made. These constructs have been made to ensure that when an unfolding can be seen in the force curve it is certainly a region 2 unfolding. This construct consist of 2 times 4 RII domains and a green fluorescent protein (GFP) which has been placed in the middle. The GFP will act as a reference point for the force measurements. Another construct consist of only 4 RII monomere domains linked together. The construct has 2 cysteines attached to the C'-terminus. These cysteines are included to covalently couple the protein construct to a gold layer. A cysteine can link to the gold with its –SH group to form a thiol-gold bond. The goal is to connect the protein construct to the gold using the cysteine.

The adsorption of the protein construct to bare gold will be measured with Surface Plasmon Resonance (SPR). This method measures the refractive index of different substances close to the gold layer over time. With these differences the surface concentration of the substance can determined. In this case when reduced protein constructs are compared to constructs with unblocked proteins something can be said about the effectiveness of the cysteine binding.

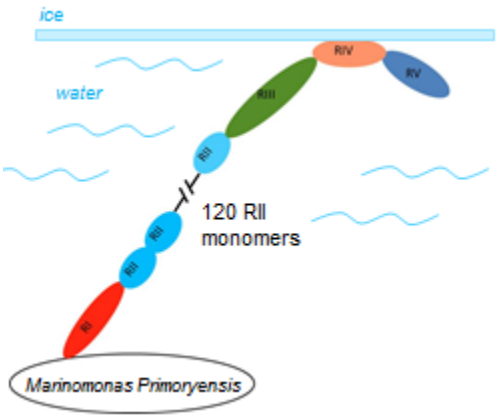

Figure 1 Schematic representation of MpAFP protein on the cell membrane of *Marinomanas Primoryensis*. [10]

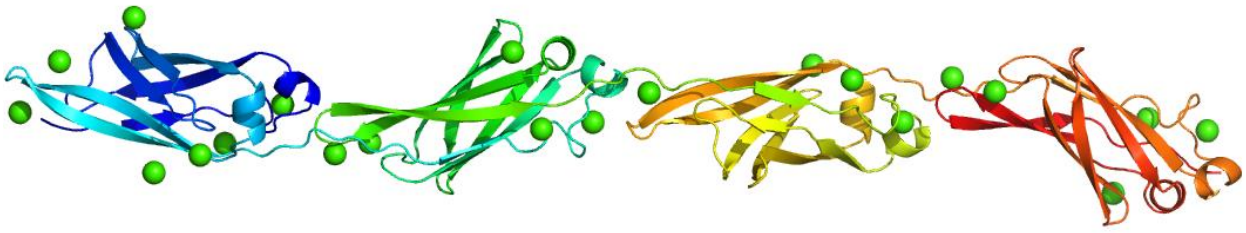

Figure 2 Cartoon representation the crystal structure of *Mp*AFP_RII tetramer. Ca^{2+} ions are indicated by green spheres. [10]

Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is a method to measure the refractive index of some substance near a surface, usually gold. This warrants the question: how does it work? First the plasmon needs to be defined. A plasmon is an oscillation of the electric charge, more precise the oscillation of the conduction electrons in a material. The eigenfrequencies of surface plasmons depend on the material it is excited in and the material properties of the material at the other side of the surface. The distance from which influences reach the surface plasmon is given by the so called evascent wave; this distance is 300 nm [11] for this experiment.

What happens in a SPR measurement is that basically a ray of light is shone on a gold surface and completely internally reflected. Depending on the wavelength and the angle of incident the intensity of the reflected light drops due to energy transfer to the plasmons. According to the following formula

$$\frac{\omega}{c}\sqrt{\epsilon_0}\sin\theta_0 = \frac{\omega_{sp}}{c}\sqrt{\left(\frac{\epsilon_1\epsilon_2}{\epsilon_1+\epsilon_2}\right)}$$

Were c is the speed of light in vacuum, ϑ_0 the angle of incidence, ω the frequency of the incoming wave, ω_{sp} the frequency of the plasmon wave and ε_0, ε_1, and ε_2 the relative permittivity's of the different materials as can be seen in Figure 3. The frequency of the plasmon wave is given by $\sqrt{\frac{ne^2}{\epsilon_3 m^*(1+\epsilon_1)}}$ with ε_3 the permittivity in vacuum, e the charge of an electron, n the electron density and m^* the effective mass of an electron. This formula is obtained by equalizing the wave vector for surface plasmons to the wave vector for light parallel to the surface. These are respectively $k_{sp} = \frac{\omega_{sp}}{c}\sqrt{\left(\frac{\epsilon_1\epsilon_2}{\epsilon_1+\epsilon_2}\right)}$ and $k = \frac{\omega}{c}\sqrt{\epsilon_0}\sin\theta_0$. In this case ε_0 is not the permittivity of vacuum but of glass. The wavelength, and thus the frequency of the light, is kept constant. This means the electric permittivity of the sample is only dependent on the angle of reflection. This angle is the angle were the intensity of the light drops due to energy transfer to plasmons. The angle can be measured using a CCD array and the electric permittivity can be derived with the formulas previously mentioned. [13, 21, 22, 23]

From this the refractive index can be derived using $n = \sqrt{\epsilon_1\mu_{water}}$ were μ_{water} is the relative magnetic permeability of water, which is 0.999992[1].

How much protein is attached to the gold surface can be found out this way. This can be done by computing the thickness of the protein layer with the formula [5]

$$d_a = (l_d/2)\,(n_{eff} - n_b)/(n_a - n_b)$$

Were d_a is the thickness of the layer, l_d the penetration depth of the evescent wave, and n_{eff}, n_b and n_a are respectively the measured refractive index, the refractive index of the reference and the refractive index of the protein. The used refractive index of the protein is 1.45[5]. When the density of the proteins is known the surface concentration in ng/mm^2 can be calculated. This density is taken to be 1.42[6] g/mm^3.

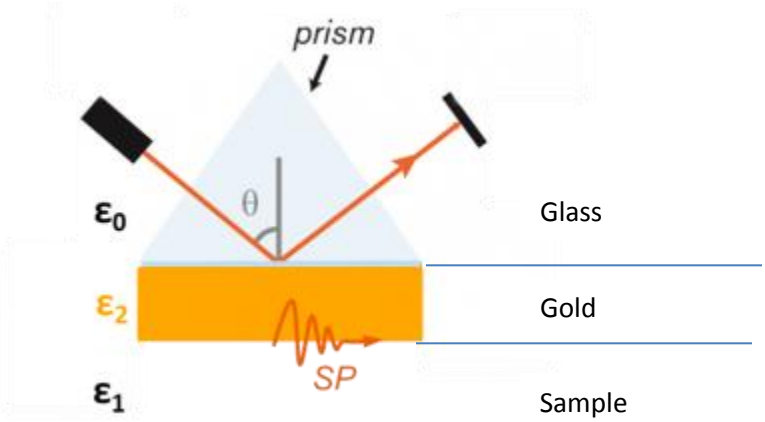

Figure 3 the setup for an SPR experiment. The side labeled with ε_1 is where the sample is applied. ε_1 is also the relative permittivity of the sample. [13]

A typical curve form an SPR machine looks like Figure 4. On the y-axis the refractive index is put in arbitrary units. In these units one response unit correspondents with a 10^{-6} change in the refractive index. This measures the change of the refractive index over time near the gold surface. In this case near the surface means a distance of 300 nm. The most important feature of the graph is that the signal is corresponding with the total amount of bound protein is the difference after the injection compared to the baseline. This is done to prevent the bulk refractive index of the buffer to interfere with the measurement. [11]

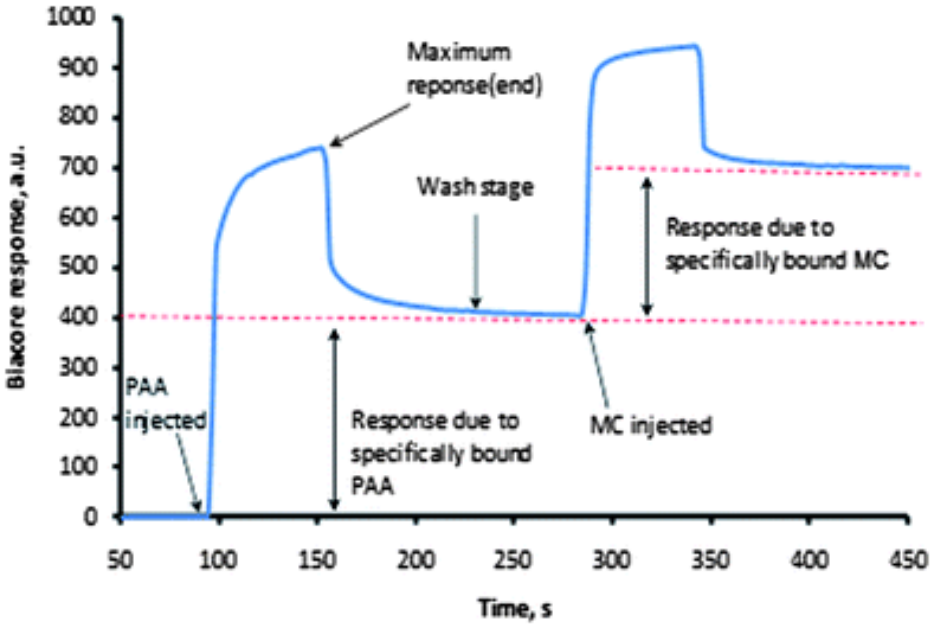

Figure 4 a typical SPR curve. This picture has been modified so that the zero is the baseline. The only thing to add is that the difference between the maximum response and the response in the wash stage is the bulk refraction. This bulk refraction is caused by the change in refraction brought on by the buffer. [14]

The SPR machine

The machine used for SPR is the Biacore T100. This machine is like all others of its kind very sensitive to temperature changes. The temperature sensitivity is 150 RU increase per K drop in temperature [7]. With an operable range of between 4-45 degrees Celsius, were there cannot be a difference bigger than 20 K between room and machine temperature. The measure range of this machine is a refractive index between 1.33 and 1.39 with a precision of 10^{-6} [8], which is 1 RU, and an accuracy of 10^{-5}. This accuracy is estimated from the drift of the machine over the course of the experiment, which is <0.3 RU per minute. The experiment itself is around 2 hours long. This machine has four flow channels in which it can perform experiments. [11]

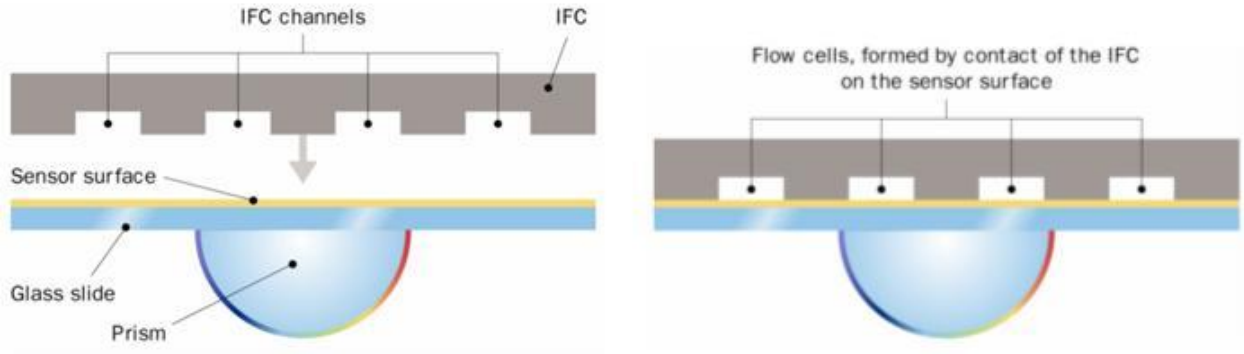

Figure 5 a schematic overview of the flow cells. [17]

Surface coverage

The surface concentration is in itself a rather uninteresting quantity. Far more interesting is the surface coverage, which is the percentage of the surface that is covered with protein. The surface coverage is important for AFM. You want an as high as possible surface coverage, because that gives the best chance of picking up a molecule. However a too high surface coverage causes the tip to pick up more than one molecule at a time. Obviously a balance between the two has to be reached.

This raises the question how the surface coverage can be calculated. This can be done when the area of one protein is known. The area of one protein can be calculated with the assumption that one protein construct covers an elliptic area [5]. The formula to calculate surface coverage is measured surface concentration divided by the theoretical (maximum) surface concentration. The theoretical area per protein assumes a monolayer and is calculated with

$$theoretical\ area\ per\ protein = \frac{M}{a * b * \pi * N_a}$$

Were *M* is the molecular weight of the protein construct in ng, N_a Avogadro's number and *a* and *b* are the short and the long axis of the projected ellipse. Which are the height and radius of a cylinder formed by the molecule.

When the surface coverage is calculated it has to be compared with ideal surface coverage for AFM, which lies around 50% [9] when the tip has a radius of 15 nm.

Atomic Force Microscopy (AFM)

The working principle of AFM is very simple. There is a tip transfixed on a cantilever. This lever bows under force according to hook's law *F=kx.* The displacement of the lever is measured by reflecting a laser on the cantilever, the reflection is then measured with a photodiode. This procedure can be seen in Figure 6.

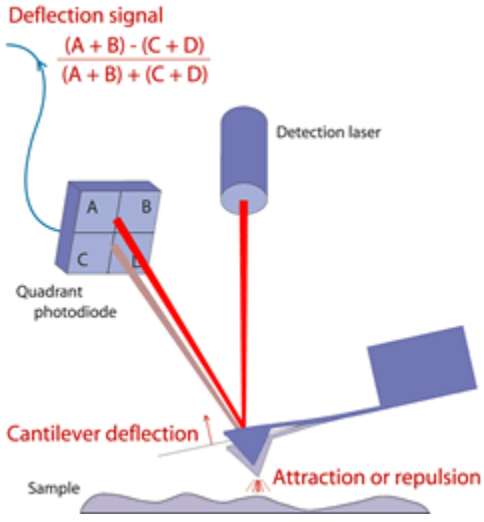

Figure 6 the principle of AFM imaging. [15]

This method can be used to make a map of the surface by measuring the height of the cantilever when it encounters the surface or a force curve, which is the force against the z position of the cantilever. The focus of this research is the creation of force curves. Specifically force curves of the unfolding of the protein constructs. These force curves have a very specific form an example can be seen in Figure 7. A few regions are of particular interest. The first region is when the cantilever encounters the surface. This is the region labeled A which is governed by Hooke's law. Second comes the liftoff from the surface, the tip has a protein fixed to it with non-specific interactions. This is the region just after the first dotted line. The irregular peak is a result of this. Third are the regular peaks which correspond with the unfolding of the domains, this is the main of the region labeled B. The force on the lever increases until the domain unfold, which results in a drop in the force. The fourth region is when all domains are unfolded and the protein is pulled free from the surface.

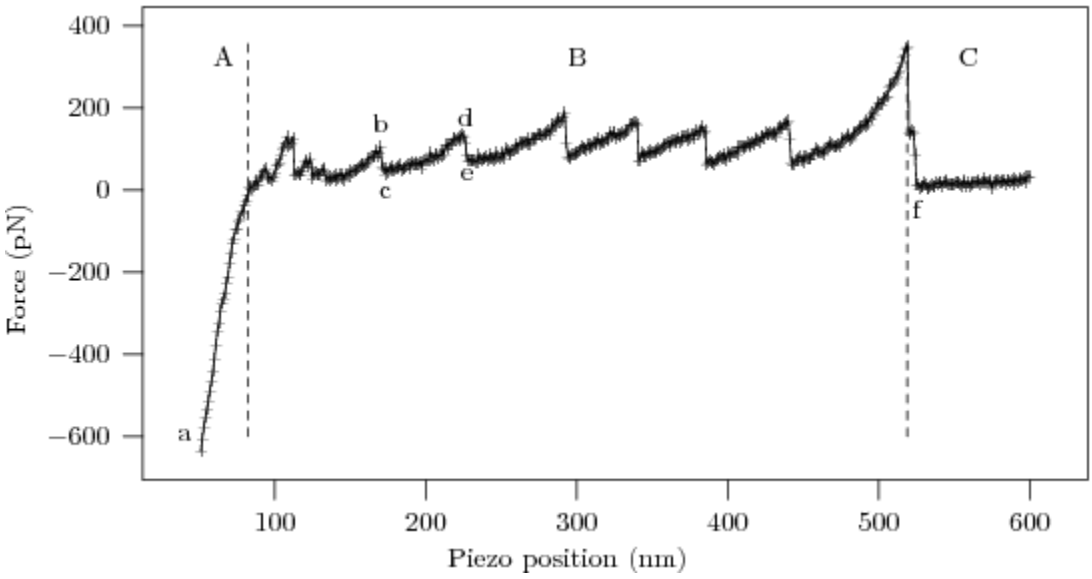

Figure 7 A typical force curve of the unfolding of a protein. [16]

The amount of peaks in region B is variable depending on where the tip picks up the protein. This is also the reason why protein construct with multiple repeats of the same domain are favored over regular proteins for single molecule force spectroscopy (SMFS). This makes sure the peaks correspond with the unfolding of the right domain. The ideal force curve would have as much peaks as possible in region B and an as small as possible.

Goal and outline

The goal of this project is the selective immobilization of *mp*AFP tetramers on a gold surface with the help of thiol-gold bridges. This to later perform SFMS experiment with an AFM on them. Also the oriented immobilization will be discussed of the protein construct. Some discussion of the methods and theoretical background has been done in the introduction. The results will be discussed next, from these result the conclusion will be drawn.

Results and discussion

The protein construct

The construct consist of two times four RII pieces and a green fluorescent protein (GFP) which has been placed in the middle. This GFP will be used as a reference in the single molecule force spectroscopy because the properties of this molecule are well known. The unfolding force of the GFP is 450 pN [18, 19]. Another construct consist of only 4 region 2 linked together, but will not be the focus of this project. The construct have 2 cysteines attached to the C'-terminus. A schematic overview of the protein construct can be seen in Figure 8. The protein is approximately a cylinder with radius 0.55 nm and length 36 nm for the octamere and length 16 nm for the tetramere, these dimensions have been determined with the help of the program Pymol. The weight of this molecule is 110 kDa for the octamere and 42 kDa for the tetramere. One important property of this construct is the fact that it needs Ca^{2+} ions to keep it from denaturing [12,20]; the stock solution contains 10 mM $CaCl_2$ for this purpose. Since this is construct is created from an anti-freeze protein, it is reasonable to assume this protein works optimal around the freezing point of water. Another property of the protein construct is their isoelectric point (pI); this determines at which pH the protein is negative or positive. The pI for the octamere is estimated on pH 4.03 and for the tetramere on pH 2.97[2]. This means that at neutral pH it is negative just like the gold, which has an isoelectric point at pH 4.95±0.15 [3].

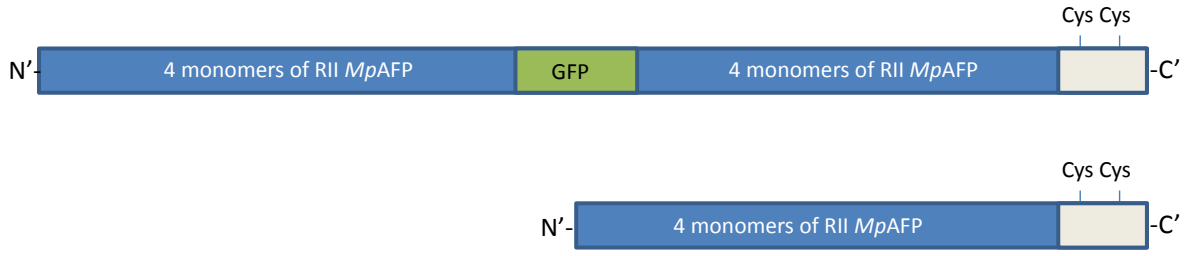

Figure 8 the used protein constructs, the upper is the octamere and the lower the tetramere. [10]

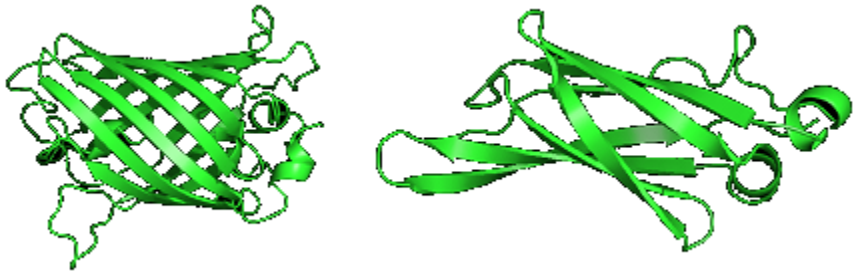

Figure 9 Cartoon representation of a GFP and a RII monomere, on the left the GFP and on the right the RII monomere. These images were created with the help of Pymol. The dimensions of the tetramere and the octamere were derived from the length and height of these figures. It is assumed that both are cylinders.

The protein construct can be immobilized in two different ways: attaching the molecule with aspecific binding to the surface or binding the construct with the –SH group of the cysteine to the gold. The thiol binding with the –SH group is stronger than the aspecific binding, 47 KJ/mol compared to 6 KJ/mol [4].

The creation of the sample and substrate

The protein constructs are stored in a 50 mM Tris-HCl (pH 9), 200 mM NaCl, 10 mM CaCl2 and 1 mM DTT storage solution. The samples were created by first removing the DTT form the storage solution. This was done to prevent the DTT from forming thiol-bridges with the gold. DTT can do this because it has a –SH group like the cysteines. The next step was to alkylate the cysteines on half the protein constructs with the help of iodoacetamide. To do this first 100 µg of the sample was added to 5 µl 2% SDS and 45 µl 200 mM ammonium bicarbonate. Water was added to get a volume of 100 µl. Some Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was added, 5 µl 200mM, and it was incubated for 20 min at 20 degrees Celsius. Finally the iodoacetamide was added, 9.3 mg, with 132 µl 200 mM ammonium bicarbonate. This was incubated for 30 min at room temperature.

After this the iodoacetamide was removed and the protein constructs put into the buffer used for the experiment, also 3 mM TCEP was added to prevent the construct form forming disulfide bridges. After the creation of the right concentrations, the sample was ready to use.

To perform the experiment the gold substrates were first immersed in piranha solution (5:1, sulfuric acid (65%): hydrogen peroxide (35%), v/v) for 10 seconds after letting the solution cool for 90 seconds. After this the substrate was inserted in the SPR machine and running buffer, 100 mM Tris-HCl, 200 mM NaCl and 10mM $CaCl_2$ with a pH of 9 or 10 mM $CaCl_2$ buffer with the pH of 7.3, was flown over it to create a stable baseline. Following this the flow program was run. This program consisted of 4200 s running buffer to ensure a stable baseline. The next step was the injection of the sample for 20 min followed by 15 min running buffer, this was repeated for three different protein concentrations, 50 µg/ml, 100 µg/ml and 200 µg/ml. This program was repeated for all 4 of the flow channels. With the flow channels 1 and 4 used as reference. The reference used was buffer solution. The used buffer for this experiment was mainly 100 mM Tris-HCl, 200 mM NaCl and 10mM $CaCl_2$ with a pH of 9. The protein immobilization experiments were additionally performed with a different buffer. This was a 10 mM $CaCl_2$ buffer with the pH of 7.3. This buffer was used with the tetramere to investigate whether the salt concentration had any influence on the adsorption of the protein construct on the gold surface. The absence of any electrolytes would allow the gold and the protein to repel each other with the help of electrostatic forces. This would help orient the gold construct straight up since that is the most favorable position. All of the experiments were performed at a temperature of 6 degrees Celsius.

Control measurements

To analyst the mass and purity of the protein constructs a SDS page gel was made. The result of this can be seen in Figure 10. As can be seen the protein constructs have the expected weight. Lane ten is the octamere and lane eleven the tetramere, they have their respective weights of 110 kDa and 42 kDa.

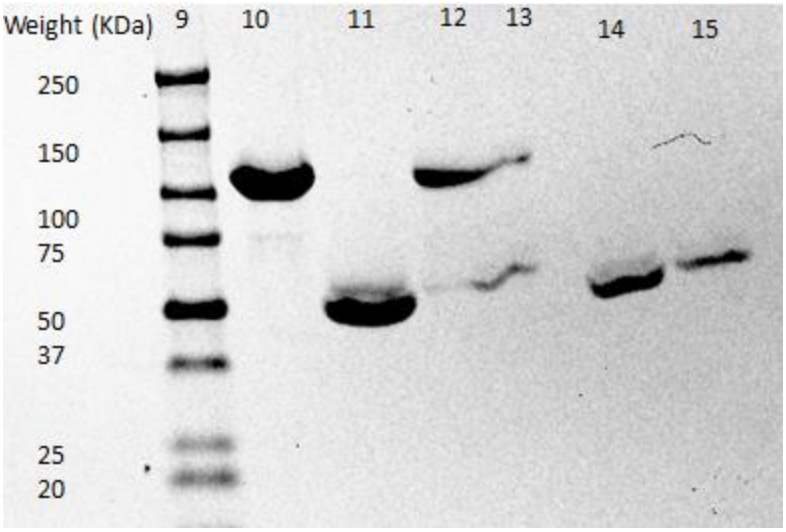

Figure 10 the SDS page weight measurement of the tetramere and the octamere. Lane 9 = dual color protein marker (Precision Plus), lane 10 = RII octamere (3.11 mg/ml), lane 11 = RII tetramer (2.77 mg/ml), lane 12 and 13 failed, lane 14 = RII tetramer (1 mg/ml) and lane 15 = RII tetramer (0.5 mg/ml). The tetramere should weight 42 kDa and the octamere 110 kDa.

SPR measurements

The first SPR measurement of the protein construct can be seen in Figure 11. There are a few things of interest here. One of them is the instable baseline. This makes the results unreliable since a shift in the response can either be caused by the injected protein or by the shifting baseline. However when the buffer is flown for some time over substrate the buffer and the surface equilibrate which results in a stable baseline.

Another thing is the temperature of the measurements which was 25° C. This results in the denaturation of the protein as can be seen after the injections of the protein. The response decreases while only buffer is flown over the surface. This means that either the protein is desorbing from the surface or the protein is changing. The denaturation of the protein constructs is likely at this temperature as this is part of an antifreeze protein that operates at temperatures near zero. This is also backed up by later experiments at a lower temperature were this does not happen. This is shown by the period were only buffer is flown over the substrate. The response is there a horizontal straight line, indicating that the amount and form of protein on the substrate is constant.

The third thing to note is the equilibrium of the protein adsorbing and desorbing. This can be seen best by the first injection were the curve becomes flat during the injection. This means that there is no more protein accumulation on the surface and the adsorption of the protein at the surface is in equilibrium with the desorption. This in turn means that the surface is getting in equilibrium with the protein. Another thing that points towards equilibrium are the higher protein concentrations. The amount of protein on the surface still increases when the concentration increases. This means the surface is not completed saturated

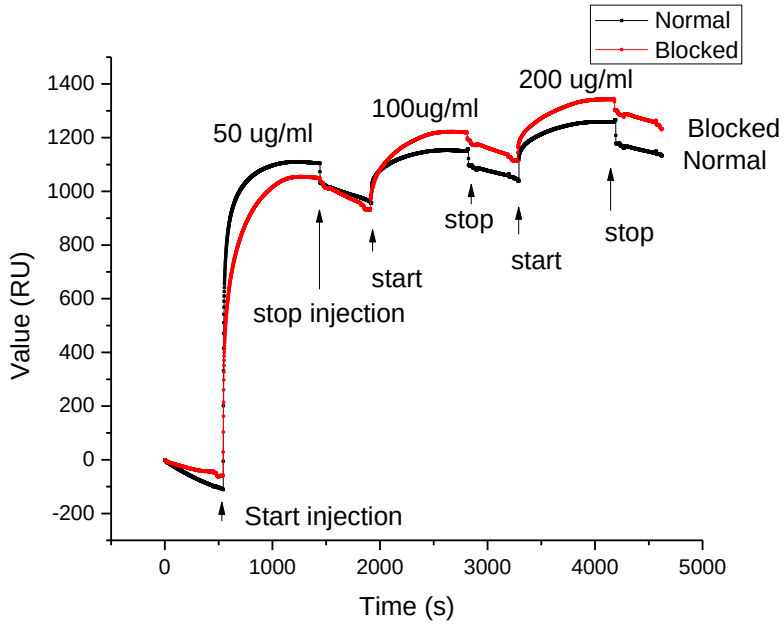

Figure 11 the first SPR picture made of the octamere. There are three different concentrations injected in the machine and flown over the substrate. The flow time of the protein is 15 min. There is also no time taken to ensure a stable baseline. The third thing is that these measurements are made at a temperature of 25 degrees Celsius. The results have a reference of only buffer subtracted from them and the start of the first injection is set to zero.

In Figure 12 a far better result can be seen. There is a far bigger difference between the normal protein constructs and the constructs with a blocked cysteine. Not only that there is also less saturation of the surface and degeneration of the protein on the surface. Also the baseline is stable.

This experiment has been repeated three times. The results of which are shown in Table 1. The uncertainty is calculated from spread of the values. The uncertainty in the measurement is constant. However in the case of the blocked cysteines the uncertainty is very high. This might be remedied when more measurements are done.

Table 1 in this table the average of three measurements is shown. The amount of protein construct that is attached via cysteine is calculated form the difference between blocked and normal. It is assumed that the difference is completely caused by proteins that are adsorbed with thiol-gold bridges.

Injection (µg/ml)	Average Normal(KRU)	Average Blocked(KRU)	Average difference(KRU)	% attached via cysteine
50	1.7±0.3	1.4±0.5	0.3±0.6	18±36
100	2.0±0.3	1.7±0.5	0.3±0.6	15±30
200	2.1±0.3	1.9±0.5	0.2±0.6	10±30

The difference between the normal and blocked cysteines is around 0.3 KRU which is also the size of the error. This means that it is difficult to produce a reliable difference between the blocked and the unblocked cysteines. However when we take a look at Figure 12 there is a clear difference between the normal and the unblocked cysteines.

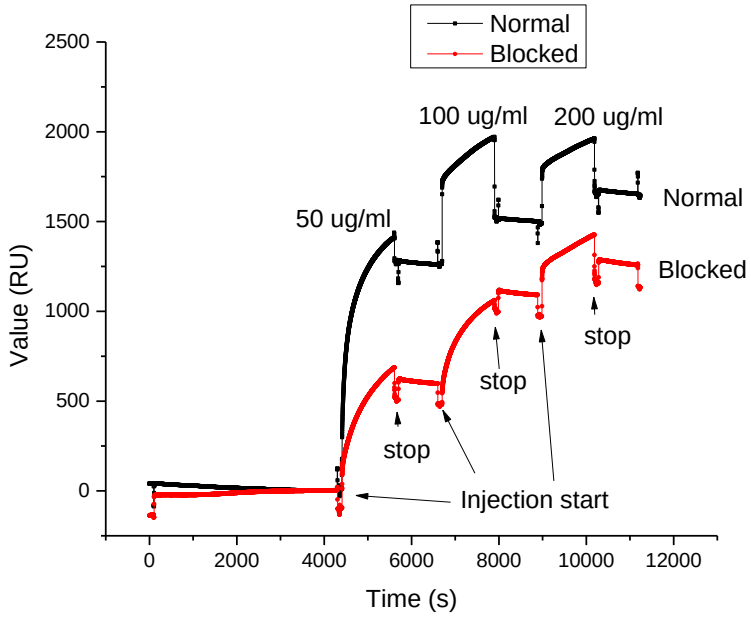

Figure 12 Another SPR image made with the octamere construct. A definite improvement over the last picture can be noticed. These results have the reference subtracted from them. This is also the first of the three pictures used to calculate the average.

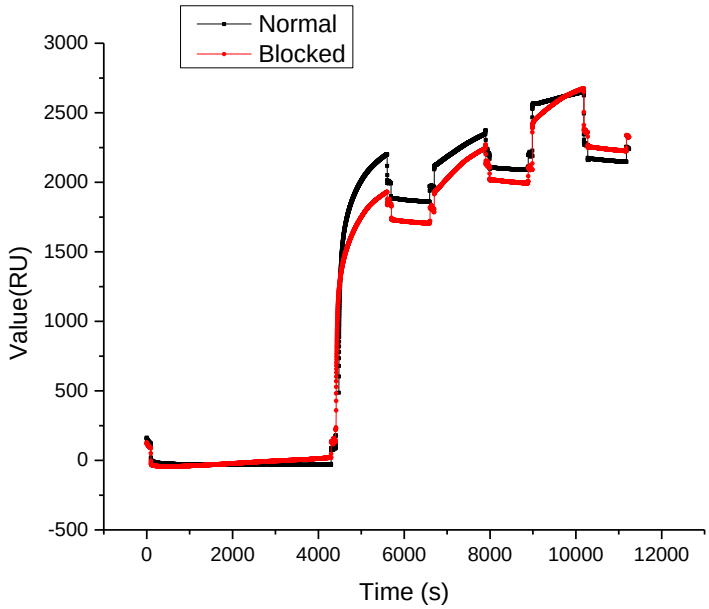

Figure 13 the second of the three SPR images used to average. The black or upper one is the normal the other is the blocked protein construct.

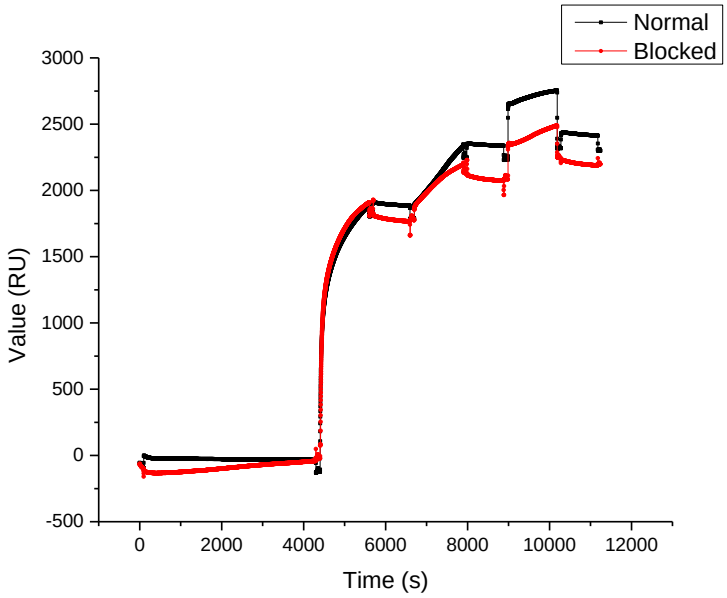

Figure 14 the third of the three SPR images used to average. The black or upper one is the normal the other is the blocked protein construct.

The averages in Table 1 can be converted to a surface coverage as can be seen in Table 2. These values are well in the range needed for a good AFM measurement.

Table 2 the average surface coverage from three measurements.

Injection (µg/ml)	Average Normal coverage (%)	Average Blocked coverage (%)
50	48±8	39±15
100	57±10	49±13
200	59±9	53±12

In Figure 15 an SPR image can be seen of the tetramere in a buffer without salt. The most important thing here is the difference between the normal and the blocked protein this difference here is bigger than in the regular with values more than double of the previous buffer. This means that in this buffer the cysteine bound protein is way higher than in the other buffer. This in turn means that it is far more likely that the protein stands straight up. When comparing surface coverage, the surface covered by the normal protein in this buffer is bigger than 100%. If only a monolayer has formed this means the calculation of the surface coverage is wrong. This is good as the calculation assumes the construct lies down. When the construct stands straight up it takes up less space and thus more protein can be on the surface, which results in a higher surface coverage than 100%. The amount of protein construct that stand straight up can be derived from this and can be seen in Table 4.

Table 3 the stable values of the tetramere measurement with little salt.

Injection (µg/ml)	Normal (KRU)	Blocked (KRU)	Difference (KRU)
50	3.3±0.3	2.7±0.5	0.6±0.6
100	4.4±0.3	3.2±0.5	1.2±0.6
200	4.9±0.3	3.6±0.5	1.3±0.6

Table 4 the surface coverage of the surface by the protein constructs when a buffer without salt is used. The percentage of construct that stand straight up is given too.

Injection (µg/ml)	Surface covered by alkylated protein constructs (%)	Surface covered by normal protein constructs (%)	% normal constructs standing up
50	78	95	0
100	94	128	29
200	104	141	43

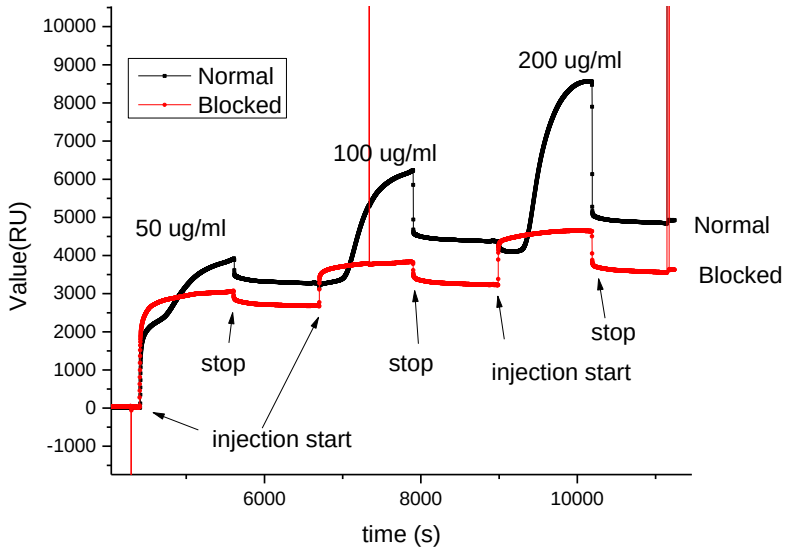

Figure 15 AN SPR image of the tetramere in a buffer without salt 3 mM TCEP was added to prevent the construct from forming disulfide bridges with each other. The used buffer is a 10 mM $CaCl_2$ buffer with pH 7.3.

Calibration of the AFM

The deflection sensitivity of the AFM was 19.4 nm/V. This was measured from the linear part of an engagement of a hard surface, which corresponds to the uttermost right part of Figure 16.

The thermal tune method was used to calibrate the spring constant of the cantilever of the AFM. The movement of the cantilever while withdrawn was measured and a fit was made with this data. The fit was a Lorentzian for air and one of a simple harmonic oscillator for fluid. From this the spring constant

could be calculated. This was done three times. The average spring constant used for the measurements was 0.1657 N/m, compared with the 0.1 N/m that was given by the manufacturer.

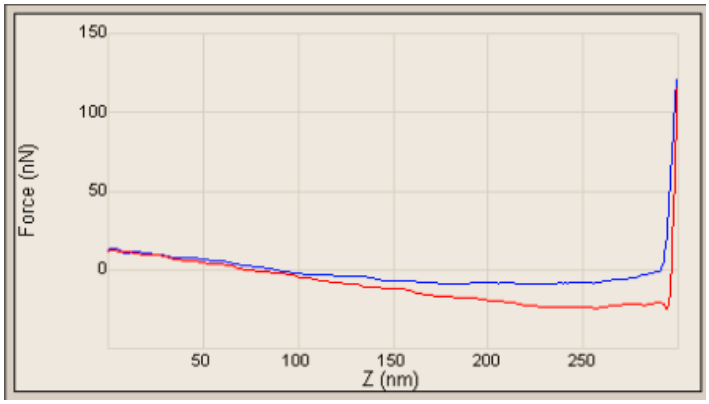

Figure 16 A force curve of an empty gold surface.

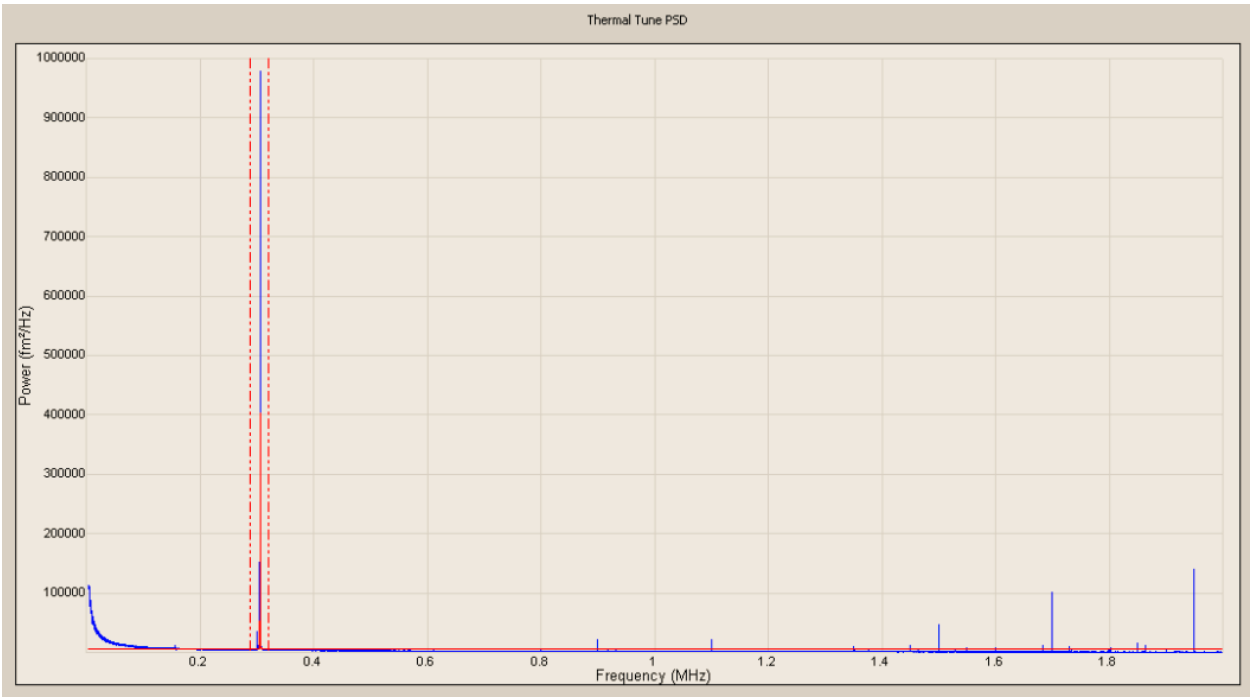

Figure 17 the graph used for the thermal tune method.

The AFM experiment

For the AFM experiments the gold substrate was also cleaned in piranha solution as described before. After this a 50 µg/ml drop of protein construct in 100 mM Tris-HCl, 200 mM NaCl and 10mM $CaCl_2$ buffer with 3mM TCEP and pH 9 was put upon the substrate. The drop was left for 15 min on the gold. When the 15 minutes were over the drop was washed off with buffer. This buffer consisted of

The used cantilever had an expected spring constant of around 0.1 N/m. The used tip had a radius of 20.0 nm. This tip was chosen because this was the tip with the smallest spring constant that gave

reliable results. The cantilever was first calibrated on a hard surface were the deflection sensitivity was determined. After that the actual spring constant was found by using the thermal tune method. The force curves were produced by continuously ramping a 500 nm ramp with a speed of 1µm/s. When the surface was reached the tip stopped for 1 second before retracting. The total number of samples made was 1024 in an area of 1280x1280 nm^2.

AFM measurements

In Figure 18 the unfolding of different domains of the octamere can be seen. The most important feature of this graph is showing that single molecule force spectroscopy is possible.

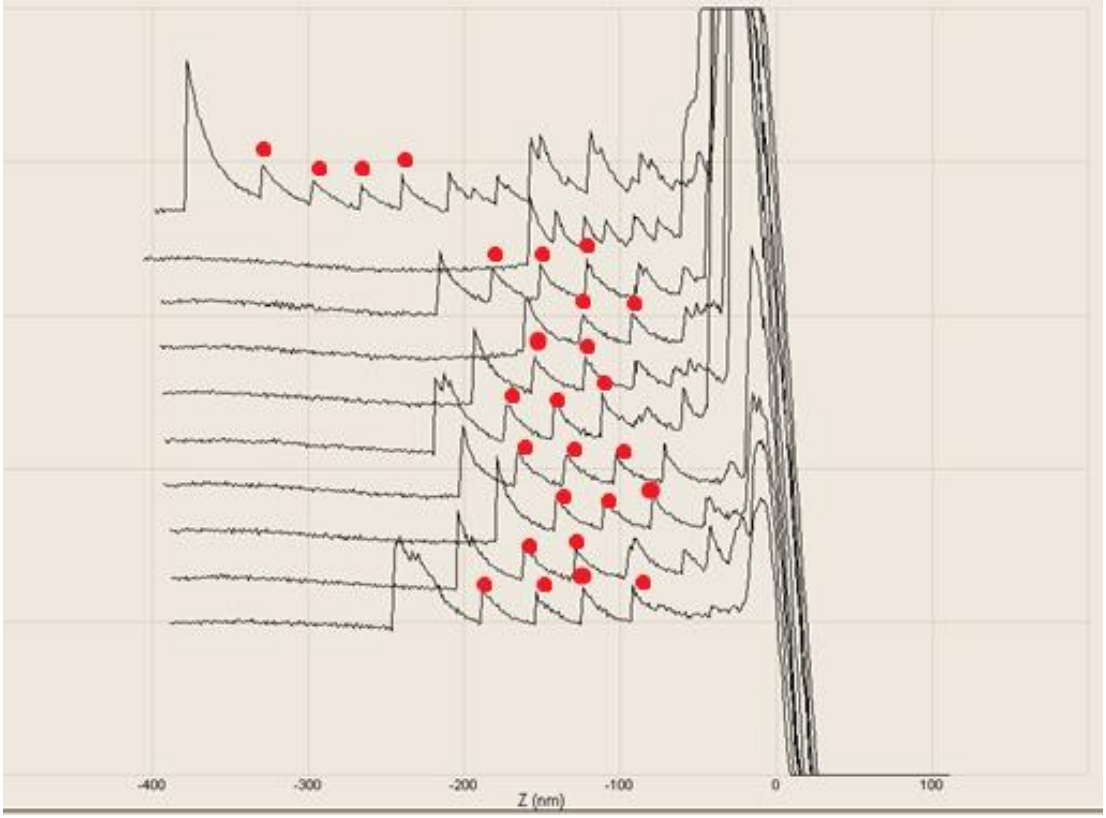

Figure 18 all force curves that show unfolding of their domain. This is the octamere on gold. The peaks with red dots above them are used to measure the force and distance of the domains.

Not only is protein on the gold, it can also be picked up by the AFM tip. It also shows that the AFM picks up only one molecule. The problem with this is the percentage of picked up proteins. This is only 12 form the 1024 curves or around 1%. This is not a big problem as it is very simple to create a lot of force curves. One of the things that can be done to improve the amount of force curves is using a buffer with little salt. Another possibility is using a bigger tip. This leads to more protein pickups.

The curves that are not giving any useful information are the same as the curve that can be seen in Figure 16. This means that for the AFM the surface looks like a clean gold surface. These empty curves

have been filtered out of the available curves by demanding that a force curve has at least a peak bigger than 150 pN more than 100 nm from the surface. These demands might have filtered out some curves that were shorter or smaller and might give a reason why none of these curves are without the big GFP peak, since this would require the tip to pick up less than half the protein construct and have fewer peaks that are also closer to the surface.

When looking over this data it seems that every curve has several smaller peaks followed by a larger peak. This pattern can be explained by the fact that the protein construct consists of several of the same RII monomere domain and one green fluorescent protein. The big peak at the end of the curve is than the GFP unfolding. Since this requires a bigger force the domains unfold first and the GFP last. The GFP requires 104±40 pN to unfold [18] when the pulling speed is 300 nm/s and the spring constant 30 pN/nm. This translates to a force of 450 pN [19] when a pulling speed of 1000 nm/s and spring constant of 0.1 nN/nm.

With this the total length of the region can be determined and also the strength of these regions. This can be seen in Figure 19 and Figure 20. The strength is 220±30 pN and the length 34±4 nm.

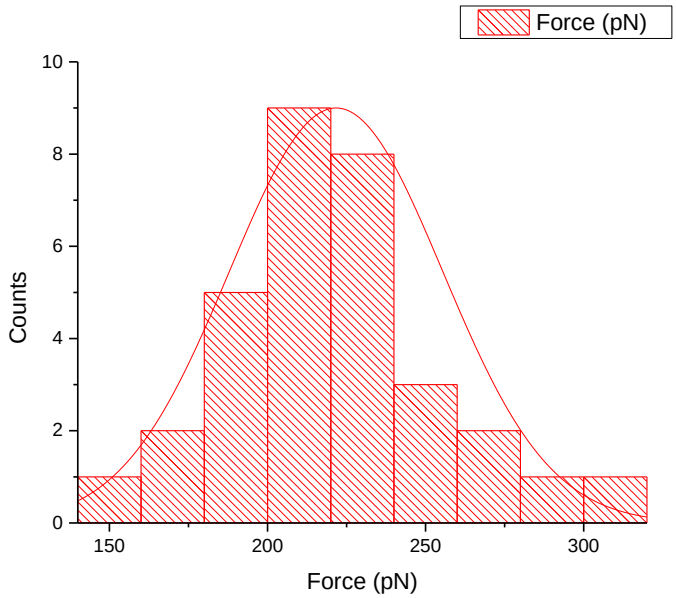

Figure 19 the strength of the domain put into a histogram with a normal distribution fit through it.

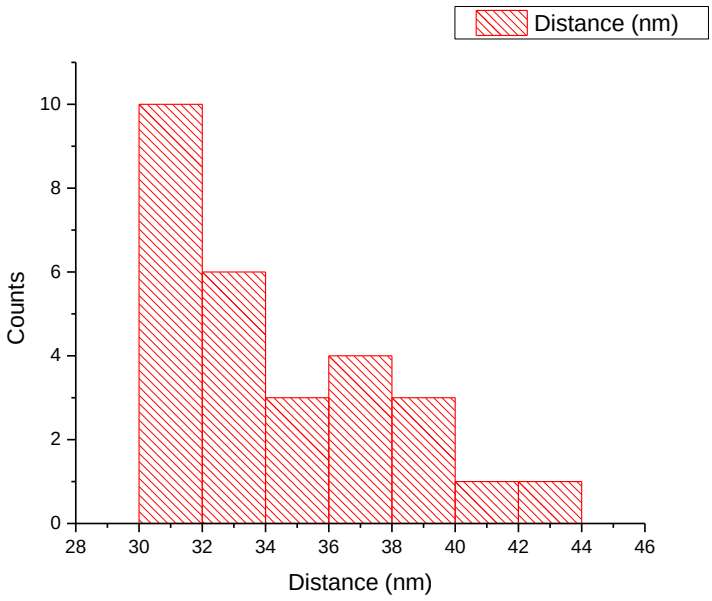

Figure 20 the length of the domain put into a histogram.

One possible source of this spread is that there might be geometry in play. This means that not every molecule is pulled under an angle of 90 degrees with the surface, but that some molecules have lower angles. This means that the distance measured is shorter than the real length of the molecule. A similar argument can be said for the force measurement.

Conclusion

This project goal of researching the feasibility of immobilizing protein construct made of RII monomere domains on a gold surface with thiol-gold bridges to investigate the possibility of SMFS with AFM has been reached. When using this protein construct some very good force curves can be made, around 1% of the amount of force curves. The force and length of the domains could already be calculated from the data and are respectively 220±30 pN and 34±4 nm.

The experiments show that a significant amount of octamere is attached to the gold, at least 2.5±0.4 ng/mm^2. The difference between the cysteines that are alkylated and those that are not is as big as the error, with a difference of 0.3±0.3 KRU, this translates in around 10-20±30% of the construct that are attached via cysteines. However when a buffer without almost all salt is used, this difference goes up to 1.3±0.3 KRU. This result means more thiol bridges are formed with the gold surface also it can be derived from these results that the protein constructs are standing straight up, around 30-40%. This is exactly what is needed for the AFM. Unfortunately there is also a higher surface coverage, around 100% for the alkylated construct, involved when using this buffer. The surface coverage might go down when the octamere is used instead of the tetramere.

Literature

1. R. Belmans & K. Hameyer, (1999), *Elektrische energie. Fundamenten en toepassingen,* Garant, p30 table 2.3.
2. L.Stryer*, (*1988), *Biochemistry, 3rd edition*W.H. Freeman & Company.
3. D. Barten (2003)*,Adsorption of Charged Macromolecules on a Gold Electrode,* Thesis, Wageningen Universiteit
4. R. Di Felice & A. Selloni,(2004), *Adsorption modes of cysteine on Au(111): Thiolate, amino-thiolate, disulfide*, J. Chem. Phys. 120, 4906
5. S. Balasubramanian, A. Revzin & A. Simoniana,(2006), *Electrochemical Desorption of Proteins from Gold Electrode Surface*, Electroanalysis 18
6. H. Fischer, I. Polikarpov & A. Craievich, (2004), *Average protein density is a molecular-weight-dependent function*, Protein Sci. 13(10): 2825–2828.
7. M. Taulés & J. Comas, *Handbook of instrumental techniques from CCiTUB Overview of molecular interactions using Biacore*
8. *Biacore T100 Software Handbook*
9. P. Klapetek, M. Valtr, [...], and P. Dzik,(2011)*, Atomic force microscopy analysis of nanoparticles in non-ideal conditions,* Nanoscale Res Lett. 2011; 6(1): 514.
10. *Hooking proteins onto ice,* an introduction to a bachelor project, Eindhoven university of technology
11. *Biacore T100 Instrument Handbook*
12. Garnham, C.P., R.L. Campbell, and P.L. Davies*, Anchored clathrate waters bind antifreeze proteins to ice. Proceedings of the National Academy of Sciences, 2011.* ***108****(18): p. 7363-7367.*
13. http://www.bionavis.com/technology/spr/
14. http://pubs.rsc.org/en/content/articlehtml/2012/sm/c2sm25652e
15. http://usa.jpk.com/what-is-atomic-force-microscopy.432.us.html
16. http://blog.tremily.us/posts/Force_spectroscopy/
17. http://web.bf.uni-lj.si/bi/sprcenter/BiacoreT100.pdf
18. Dietz, H. and M. Rief*, Exploring the energy landscape of GFP by single-molecule mechanical experiments. Proceedings of the National Academy of Sciences of the United States of America, 2004.* ***101****(46): p. 16192-16197*
19. Chih-Kung Lee, Yu-Ming Wang, Long-Sun Huang, Shiming Lin, A*tomic force microscopy: Determination of unbinding force,* Micron Volume 38, Issue 5, July 2007, Pages 446–461
20. Guo, S., et al*., Re-Evaluation of a Bacterial Antifreeze Protein as an Adhesin with Ice-Binding Activity*. PloS one, 2012. **7**(11): p. e48805.
21. Cottam, Michael G. (1989). Introduction to Surface and Superlattice Excitations. New York: Cambridge University Press. ISBN 0750305886.
22. Kittel, Charles (1996). Introduction to Solid State Physics (8th ed.). Hoboken, NJ: John Wiley & Sons. ISBN 0-471-41526-X.
23. Homola, Jirí (2006). Surface Plasmon Resonance Based Sensors. Springer Series on Chemical Sensors and Biosensors, 4. Berlin: Springer-Verlag. ISBN 3-540-33918-3

Appendix

Most errors were calculated by from the spread in the measurements. The error in the difference was calculated with the formula

$$S_y^2 = S_{x_1}^2 + S_{x_2}^2$$

With the $y=x_1-x_2$ used as formula to calculated the difference.

All the graphs used over which the value is averaged in Table 1 and Table 2. With the used values summarized in Table 5. The third graph is Figure 12.

Table 5 the used values for summarizing in Table 1 and 2.

Injection (µg/ml)	Normal (RU) 1	Normal (RU) 2	Normal (RU) 3	Blocked (RU) 1	Blocked (RU) 2	Blocked (RU) 3
50	1876	1889	1258	1711	1771	596
100	2094	2343	1473	1998	2084	1064
200	2155	2424	1603	2240	2201	1210

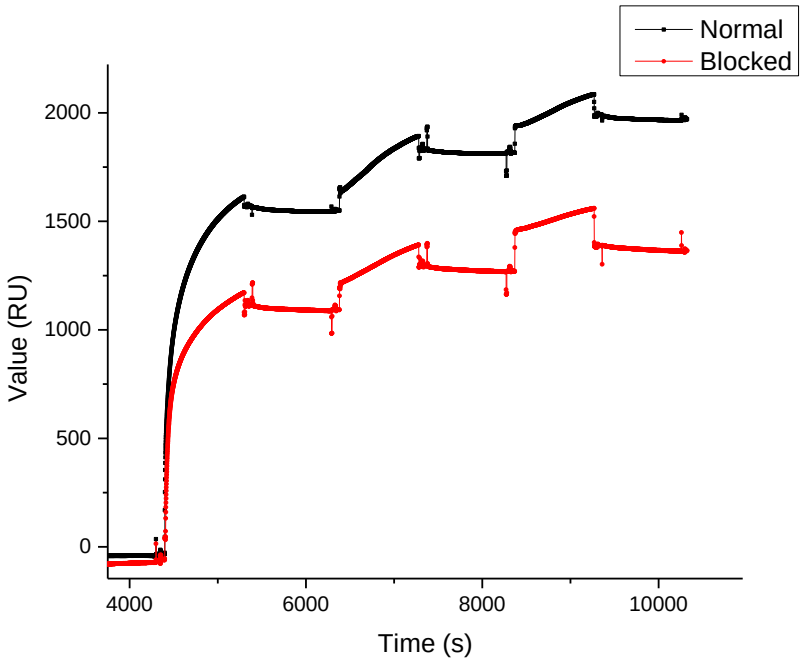

Figure 21 in this experiment there is DTT in the buffer. This leads to a more pronounced difference between blocked and normal, but this might be because the DTT is binding to the gold and not the protein. Also the protein is flowing 5 shorter over the gold than in other experiments.

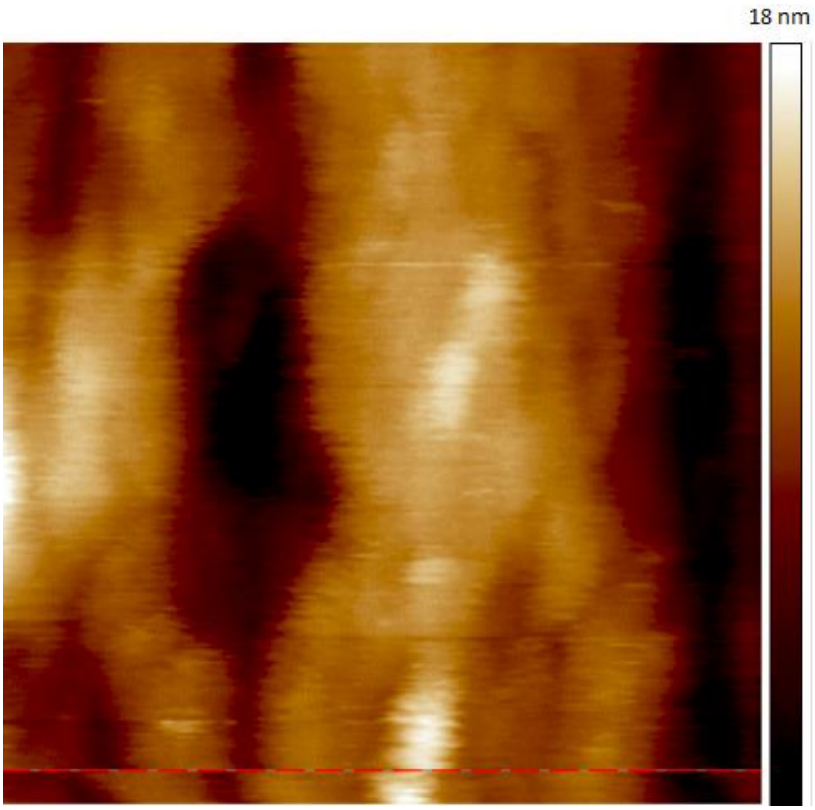

Figure 22 Scan of the gold surface with protein. This is a 500×500 nm surface.

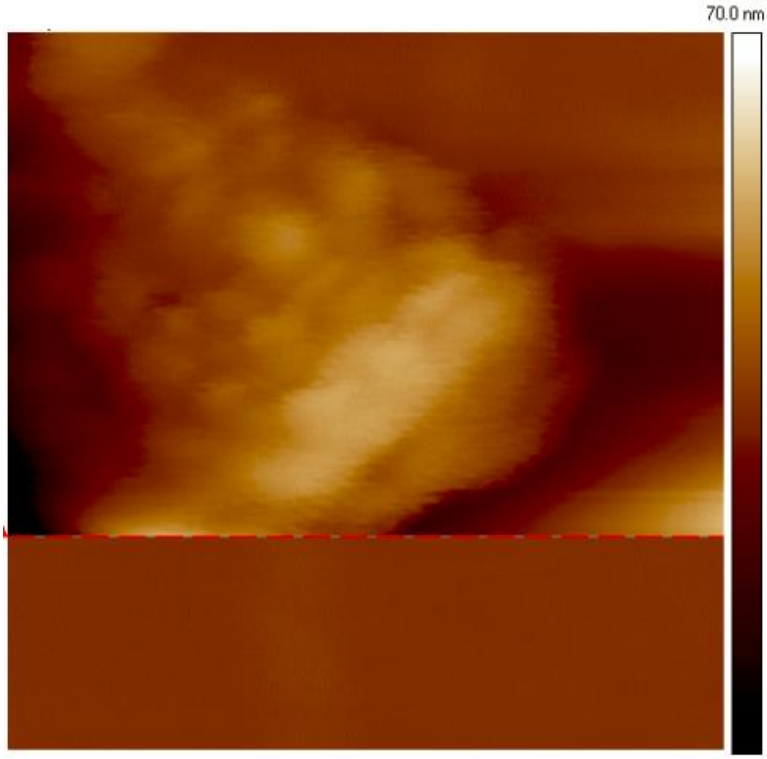

Figure 23 AFM scan of the gold surface without the protein on it.