FINAL REPORT (MARSHALLPLAN)

IDENTIFICATION AND CHARACTERIZATION OF FUNCTIONAL RECEPTORS

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1 Identification and characterization of functional receptors

1.1 Abstract:

Biophysical methods in the field of medical diagnostic need continuous improvement to become highly sensitive, easy to apply and fast. In particular, the decoding of the genetic code in the 1960s and the solvation of the human genome was a major milestone. However, to predict the extent to which genetic disorders affect the physiology requires more information. For example, many genetic defects in cell membrane receptor genes cause in most organisms non-viability. However, in a few cases – depending on which and how many receptors are affected – this means a shortened life span. Currently, there is no high throughput or general applicable quantitative analysis available to determine the number of affected cell membrane receptors. Here, we intend to quantify the number of altered receptors within the cell membrane and more importantly, to predict the mutation of the receptors using atomic force microscopy / force spectroscopy. Specifically, we are studying receptors whose dysfunction leads to familial hypercholesterolemia. Depending on the severity of the malfunction, persons between 5 and 50 years of age die from premature onset of atherosclerosis, heart attacks, and strokes. Studying the interaction between low density lipoproteins and low density lipoprotein receptors gives insight into the disease where these receptors fail to work accordingly to their purpose. Single molecule force spectroscopy with functionalized tips provides a method to analyze this interaction and is used here. Our results show a detection of specific bonds between tip and cells which can be related to the low density lipoprotein interaction.

1.2 Familial Hypercholesterolemia (FH):

The cell membrane plays a crucial role in vital processes, but also for the genesis of diseases (e.g. uptake and interaction of bacteria and viruses with cells). It acts as protective gate and decides whether molecules or whole particle can enter or are to be averted. Thus, several thousand various receptors are facilitating targeted interaction of chemical compounds with the cell membrane. Our research contributes significantly to the functional characterization of receptor modifications triggered by the genetic metabolic disease FH. Thus, it will help to understand its genetic effects on the cellular level. Risk of cardiovascular disease is increased when a defined range of total cholesterol (low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides) is exceeded. If no correlation is found between the person's elevated cholesterol levels and his or her lifestyle (e.g. body weight, diet, exercise), its cause is probably a genetic disorder caused predominately by mutations of the LDL receptor[1,2] (i.e. detection of familial hypercholesterolemia (FH) in the affected individual[3]). Depending on the homozygous or heterozygous genetic variant and type of molecular defect, cardiovascular disease is expected early in life and life expectancy drops significantly – mostly as a result of premature onset of heart attacks, strokes and peripheral atherosclerosis. A homozygous genotype can lead to complete LDL receptor defect. Thus, the question arises, how these people survive even for several decades, when only the cell's own cholesterol biosynthesis is available. To study this medical issue, a broad spectrum of innovative biophysical methods has been applied. At the University of Applied Sciences,

receptor distribution and lipoprotein particle interaction will be studied using single-molecule-sensitive fluorescence microscopy (SMFM).

My research at ASU focused on applying force spectroscopy and atomic force microscopy (AFM) measurements to characterize the binding force between single Low Density Lipoprotein Receptors (LDLR) and its ligand (i.e. Low Density Lipoprotein (LDL) particle). The aim is to determine the single molecule binding characteristics to apply the generated knowledge and assign interactions of specific interacting partners. Here we used this technique for the first time to quantitatively estimate the cell surface receptor density as function of disease.

Prof. Robert Ros (ASU) is a well-known specialist in the field of atomic force microscopy and will support the expansion of this technique for new biomedical questions. Up to now the analysis of such cell-force curves was not accomplished for this approach. However, Prof. Steve Presse (ASU) develops models to describe the curves quantitatively. With the help of different scientific groups and their specific expertise, a new method shall be developed to specify and to determine the number of functional receptors at the cellular membrane. [4, 5, 6, 7, 8]

1.3 Research aim:

Several genetic diseases are based on certain disorders of cell membrane receptors structure. In general, physicians arrange for a genetic screening if distinct symptoms are observed. If specific mutations are indeed detected, a diagnosis and treatment plan can be made. The extent a specific disorder affects the quality of life or even the overall lifespan is usually predicted from empirical values. But, the lack of appropriate techniques does not allow the quantification of cell receptor alterations and how this dysfunction influences cell viability.

In close cooperation between the ASU and the University of Applied Sciences Upper Austria (FH OÖ), we intend to establish a new method for the identification of altered cell receptor density/affinity. Furthermore, this new method will be applied to a fundamental medical issue: a disease called FH. The collaboration between ASU and FH OÖ has been previously established. The focus of my research was to evaluate if the predicted approach facilitates the expected outcome. Thus, I applied Single Molecule Force Spectroscopy (SMFS) between single LDL particles and distinct receptors on the living cell membrane in the lab of Prof. Ros at ASU. This approach allows to estimate kinetics and forces between these molecules. Additionally, it was planned to use Single Cell Force Spectroscopy (SCFS) to quantify the interaction of whole cells. For this purpose, surfaces are coated with different concentrations of LDL particles and the interaction with living cells is measured. The Ros lab at ASU has long time experience in SCFS and SMFS in biological systems. Having observed distinct forces between various ligand densities and cell membrane receptors, the measured data set shall be analyzed in close cooperation with Prof. Steve Presse (ASU), who is an expert in mathematical simulation and modelling. The project is a logical broadening of the thematic focus of all collaboration partner and is based methodologically on the model systems developed recently. [9, 10, 11, 12, 13]

1.4 Specific Aims (SA):

In SA 1 we intended to measure and characterize interaction between LDL particles and distinct cell surface receptors. Therefore, we covalently bind LDL particle to AFM tips and

measure several thousands of force-distance-curves on different cells with their individual receptor density. Positive (cell line GM01386 – healthy control) and negative (cell line GM00701 – no functional LDL-receptor LDLR) control experiments were seeded for comparable results in respect to the binding of the LDL particle with its receptor. To estimate the binding force between receptor (LDLR) and ligand (LDL) particles, SMFS measurements were performed. Several areas on each cell were measured to explore intercellular differences/ similarities. All results were compared to negative control experiments and will be further analyzed with already developed and established verification programs.

The tasks in SA 2 deal with the development of methods and were included in SA1 if progress was without problems. In fact, some hurdles had to be overcome in SA1 (see chapter "Cell culture – Problems during cell culture"); in this respect, the contents in SA2 could be considered purely theoretically. However, the data obtained in SA1 show a positive development and thus a promising possibility of a successful method development in SA2, which will be further worked on in other research projects. In particular, in SA 2 the process should be swapped by functionalizing the surface with different LDL particle densities and attaching the cell to the tip. This enables quantification of how active the receptor and LDL particles react with each other. After applying these steps, spatial force maps can be generated with the same method as mentioned above. Cells, either lacking or overexpressing the corresponding receptor will be attached via the lectin concanavalin A to a tip-less cantilever. Again, force-distance-curves will be generated on surfaces with varying densities of covalently linked LDL particles. First, to quantify unspecific interaction the force between cell and a bare glass slide will be determined. In the second step, a glass surface – homogeneously covered with LDL particles – will be measured. Thus, the maximum force between cell and surface is determined. More precise, non-specific as well as specific receptor-ligand interactions are detected.

Timetable:

At first, I had to go through a complex enrollment procedure which included several classes and tests to be eligible to access all laboratories. Taking these instructional courses and introduction into the workflow of Ros´s lab took roughly 2 months. During this introductory procedure I already started working in the cell culture lab. I shadowed one of Dr. Ros´s employees and studied all procedures needed to start my own cell culture. Fortunately, while shadowing Dr. Ros´s employee, my cells arrived and in cooperation with him I was able to start cell passaging. We ran into issues on our first try to passage them, probably due to wrong solution concentrations and after one week the cells died. Reordering the cells led to a time loss of 2 weeks in which I focused on tip functionalization. After that, I was able to establish a cell backup bank to ensure a sufficient number of cells available for the measurements. This took roughly 4 weeks since every cell line had to be passaged and frozen for backup to prevent further setbacks. Once the cell backup bank was established, I started prioritizing my work on tip functionalization whilst learning to use the local AFM correctly for my purposes. Gaining this knowledge and creating enough tips took 3 months. After this I could solely focus on creating force-distance-curves using the AFM, which was performed until the end of my stay at ASU. These measurements required parallel work of cell culture and tip functionalization which was optimized to ensure maximum work progress. Analysis of

the obtained data is currently in progress and will be addressed in my master thesis. Around 12.000 curves are currently analyzed in cooperation with both universities. The following timetable (Table 1) shows the invested time over the course of my internship (6 months). The darker colors indicate a primary focus of the task, while the lighter colors indicate a parallel work or introductory work into the Materia. E.g. cell culture in August and September was the main focus of my work, while in the months of October until January my time investment into cell culture reduced.

	August	*September*	*October*	*November*	*December*	*January*
Cell culture						
Tip funct.						
AFM meas.						

Table 1 shows the timetable of the work focused on in their respective time period. For example, cell culture was performed over the entire period, the lighter color from October to January indicates, that the cell culture was performed parallel to another work. The darker color in August and September indicate a focus of work in cell culture.

SA 2 was not executable during my stay at ASU due to the prolonged time investment required for cell culture. Nevertheless, the cooperation will continue and will lead to another graduate project. There are several interests in continuing this project and to being able to quantify the receptor densities on living cells. A basic research project is currently being submitted to the FWF. As this method of research is capable of being expanded to other molecular interactions it opens up multiple ways to expand this research.

1.5 Cell culture

Fibroblasts, the cells used for this research, turned out to be more difficult in handling regarding their culturing and general usage. The protocol used in this research is taken from "Coriell institute" official website specific for Fibroblast culture. Getting familiar with cell culture protocols and learning the processes took more of my time than expected. With nearly no work experience in a biosafety cabinet class 2, I had to learn the workflow independently. Dr. Ros's lab strongly emphasizes on independent work, which helped me grow throughout my time there.

The ASU lab is specialized in cancer cells. Fibroblasts are not as robust and require a more careful handling. Two cell-lines were used for SMFS, one as positive and the other one as negative control. GM00701 is a fibroblast cell type taken from a human with the heritable disease FH. It is published as showing receptor negativity in the uptake of LDL particles. The receptor activity lies below 1% compared to the value obtained in normal cells (see Coriell´s official website). Therefore, we assume the cell-activity regarding uptake of LDL particles is nearly zero and this cell-line serves as negative control. We also use GM01386 cells where the receptors are unaffected, and the blood of the donor shows normal cholesterol levels. Here, functionalized tips should interact with these cells yielding our expected outcome of attraction/binding forces between receptor and LDL particles. Depending on the activity, one can further perform SMFS measurements, where cells are attached to the tip and force measurements are tested on different surfaces with different densities of LDL particles. Based on these tests, quantifications can be made, and assumptions or further information can be taken from the degree of binding processes. To get deeper insight into this matter, another cell line is required. There, the detected binding events are related to the LDL-receptor or the

binding forces might originate from other membrane interactions. I was not able to realize quantification and single cell force spectroscopy at ASU.

As mentioned, cells were ordered from Coriell institute and after arrival they had to be sub-cultured. Fibroblasts were transported in tissue flasks with slow growth medium. This medium assures that the confluency of the cells doesn´t exceed a monolayer during the transport process. As cells shall be sub-cultured as soon as they reach total confluency, this media also increases endurance of the cells, since during transport they will be temperature variations. For sub-culturing, the cells were transferred into T25 tissue culture flasks. Living cells have to be kept under special conditions in an cell incubator. For human cells, these conditions are 37° C and 5% CO_2. CO_2 in the incubator should counteract against the cells natural carbon metabolism. Cells create CO_2 in several ways, one of them being the production of ATP. It dissolves in water and forms carbonic acid H_2CO_3, and subsequently dissociates into hydrogen ions and bicarbonate ions. When high hydrogen levels are produced through the metabolisms, the pH decreases and this leads to a more acidic environment. In contrary, the pH level increases when less H^+ ions are present. The incubator provides 5% CO_2 to the chamber which helps creating the correct conditions for the cell's metabolism in combination with the bicarbonate buffer system. We used a New Brunswick Galaxy 170 S incubator (sterile conditions with CO_2 regulator) to produce the desired 37° C and 5% CO_2. After leaving the newly arrived cells in the incubator over night, I sub-cultured them according to the protocol given by Coriell. It provides a standard protocol for seeding of living cells and has been tested previously by our colleagues in Linz. The cells were confluent and ready to be transferred to the biosafety cabinet. We used a class 2 Biological Safety Cabinet (Baker Company, Sterilgard). A laminar airflow provides safety in regard of contamination from outside the hood while keeping cells inside the hood. When operated correctly, it prevents the user and the cells from potential hazards. The biosafety cabinet uses a sterile working area to reduce the risk of contamination from airborne particles and aerosols like dust or spores. To ensure sterile conditions it has to be free from storage items and solely contain equipment used for the experiment. Additionally, every item placed in the hood has to be sterilized or at least wiped with 70% ethanol. Sterilization can be done in an autoclave for medium and materials. Before and after every work cycle ultraviolet light was turned on for 30 min to sterilize the air and exposed work surface in the biosafety cabinet. Users should always wear gloves and disinfect them with 70% ethanol and further a lab coat is mandatory to reduce contamination risks through skin while providing self-protection. After surfaces and all objects placed in the hood were disinfected with 70% ethanol, the old media was aspirated from the flasks. After that, 3 mL of 0.53 mM EthyleneDiamineTetraacetic Acid (EDTA) in Hanks Buffered Salt Solution (HBSS) was added to the cells. As cells attach to surfaces by using adhesion molecules and integrins expressed on their cell membrane, a trypsin-EDTA solution is used to remove these adhesives cells from the surface. EDTA binds calcium and magnesium ions required for integrin activity which allows cells to attach to the surface. Trypsin is a proteolytic enzyme, which cleaves peptides on Lysine and Arginine. All steps have to be done in a timely manner, so cells do not die throughout the processThe EDTA solution was created before each sub-culturing using solid EDTA mixed in HBSS to create a 0.53 mM solution. The cells were left in the solution for up to 10min, depending on the development of their morphology. Therefore, they were checked frequently with a 20x magnification phase

contrast microscope. The solution has to be removed through aspiration before the trypsin solution is added. Then, 3 mL of 0.04% trypsin + 0.53 mM EDTA in HBSS have been added to the flask. The cells are incubated in this solution for up to 7 min. During this time, the cells are places into the incubator as the temperature increase enhances the enzyme activity. A microscope was used to check the morphology of the cells. This step is highly time reliant since all cells should detach, while no cells should disintegrate due to the trypsin treatment. To stop trypsin activity, we added 5 mL Fibroblast growth medium (Minimum Essential Medium Eagle) to the 3 mL trypsin solution containing the cells. This medium contains Fetal Calf Serum (FCS) which competes with the cell membrane proteins for the enzymatic activity of trpysin (i.e. competitive inhibition). [14, 15, 16, 17]

A hemocytometer (Daigger scientific, Neubauer chamber) was used to count the number of cells per volume (i.e. density). It contains two identical chambers into which a small volume of cell suspension (10 µL) is pipetted. Each chamber is divided into a grid pattern consisting of nine large squares. The depth of the chamber is 0.1mm and all squares have a dimension of 1 mm x 1 mm and thus they contain 10^{-1} µL of suspension. A further separation of these squares is given for each square, as for example, all corner pieces are divided into 16 smaller squares of equal size (0.25 mm x 0.25 mm). The middle square divides into dimensions as small as 0.05 mm x 0.05 mm. Here each square contains 0.25 nL. Trypan blue is added which allows distinguishing between living and dead cells. This dye penetrates the membrane of dead cells and thus they appear blue when examining them through the microphone – on the contrary, living cells appear undyed. We used 100 µL trypan blue mixed with 100 µL cell suspension; our diluting factor is 2. To determine the number of viable and non-viable cells, I counted the cells in every "odd-number" squares (1, 3, 5, 7, 9) and calculated the average number of viable cells over the five squares. Finally, to calculate the number of living cells in the total volume of the tissue flask, I multiplied the average count of cells per square volume with the dilution factor (in our case 2) and again multiply it with 10^4 to get the cell count per mL. Coriell suggests to seed 250.000 cells for sub-culturing in a T25-flask. After pipetting the corresponding volume of cell suspension into the flask, additional fibroblast media was added to a final volume of 8 mL and the flasks were put in the incubator. [18]

In general, confluency was reached after 7 days and cell media was replaced every 4-5 days to ensure a proper environment for the cells. Enough flasks were produced to generate a backup for any malfunctions, an aliquot of these backup cells was frozen for long-time storage. The freezing cell protocol is similar to the sub-culturing protocol up to the trypsinization-step. Once growth medium was added to stop trypsin activity, the cell suspension was centrifuged at 80 x g for 10 min at 8 °C. Centrifugation will yield a cell pellet at the bottom of the tube and cell-free liquid above the pellet. Now we remove as much as possible of the culture media supernatant. Freezing solution is a mixture of cell pellet and freeze media. Freeze media is 10% mixture of glycerol with 90% growth media (Eagle´s MEM). The pellet is resuspended with freeze media, diluted to 5 x 10^5 cells per mL and aliquoted into cryovials. To freeze them, first they were placed into a "Mr. Frosty" overnight. This container provides for the successful cryopreservation of cells required cooling rate of 1° C/min. All vials are stored in the gas phase of a liquid nitrogen container after the frosting procedure.

For measurements, these cells had to be prepared differently in order to prepare samples able to fit onto the AFM stage, whilst providing a reachable surface for the probe. Thus, petri dishes were used. The protocol follows the same principle as explained before, only a much lower concentration of cells is used to provide single cell density. Also, we only left the petri dishes in the incubator for around 24 hours maximum, otherwise the confluency posed a challenge. [14, 15, 16, 17, 18]

Problems during cell culture

As already mentioned, studying cell culture took more time than assumed. Apart from learning the handling of a class 2 biosafety cabinet, the procedure had to be studied in self-study in cooperation with my colleagues in Linz. This created a great opportunity for me to get insight into how to approach a situation with hardly any prior knowledge. At first, the aim was to create a cell bank of frozen cells for backup if something malfunctions and the currently processed cells die. Unfortunately, the media taken to process the newly received cells was created with a false concentration and the cells died after sub-culturing. After several days showing no reaction according to cell growth, we assumed the cells were completely contaminated or simply dead, approximately due to the wrong concentration in the media. Therefore, another set of cells had to be ordered. Upon their arrival, the first sub-culturing step was a success and cells were healthy and thus growing. This led to further sub-culturing and to freezing of the cells. After several sub-culturing steps, a sufficient cell bank of frozen cells was created, and sub-culturing of fewer cells began resulting in creation of cells grown in petri dishes. I discovered that if the number of sub-cultured cells (250.000 cells/mL) was exceeded, the next sub-culturing process had to be done prior than the 7-day mark. If the number was below the desired cell count, the cells might not reach confluency at all. Therefore, seeding density turned out to be the most crucial parameter of the sub-culturing protocol. Another issue occurred by contamination of other cell lines in the incubator. Although reasons are unclear to why contamination occurred in the incubator, it led to disposal of all cells within the incubator. Each time cells had to be disposed of, thawing of cells from the cell bank was required and to assure safety, new cells had to be frozen back into the cell bank. However, these flaws could all be detected and prevented for further extractions during my time at ASU to provide cells for AFM measurements. Providing the right amount of cells to seed onto petri dishes also took some experiments. AFM measurements shall only measure one cell at the time to prevent any overlapping effects or bulking effects due area shortage. Another effect occurs when not enough cells are provided at the surface. The cells then fail to grow on the petri dishes over time, we assume they are damaged in the transport procedure due to too small pipette tips. Cells when pipetted have to be handled with care – too small pipette tips may cause damage through rupture effects.

1.6 Single Molecule Force Spectroscopy (SMFS) experiments

AFM offers a variety of techniques to characterize surface properties and interactions in biomolecular systems in the nanoscale range. In general, AFM became an appropriate tool to provide valuable information on biological materials at the nanometer scale. It is applied for topographical imaging [20,21], measuring forces between interacting molecules [22,23], protein unfolding [24,25,26] or elasticity [27,28] of biological samples. SMFS can be used to measure and physically describe binding forces between individual partners, whereas single-cell-based

force spectroscopy is used to examine the entire range of binding possibilities. It can provide 3-dimensional views of biological systems in real time. While maintaining these benefits it also allows biological systems to stay in the desired liquid environment at a regulated temperature while measuring the surface/structure. Apart from making use of highly sensitive methods to image nanoscale structures, it also enables force spectroscopy showing interactions with cells/surfaces and their molecular interactions. This technique opened up the possibility to analyze biomolecular systems and manipulation of such through the exceptional signal-to-noise ratio in sub nanometer scale. AFM uses a sharp probe (cantilever) to probe a surface of interest. In SMFM, the cantilever is moved down to the surface and retracted again. Depending on the attraction forces between surface and tip, different force curves as function of the distance from the surface are measured. If there are interactions between surface and cantilever, the cantilever bends towards the surface. There will be a significant drop of the force at tear-off and the cantilever will oscillate back to its equilibrium state (i.e. no bending). Based on this information and the cantilevers spring constant, we can calculate the rupture force. With functionalized tips attached these rupture forces will give insight into specific bindings. Rupture length will vary according to the interaction between tip and surface, leading to a reliance to the tip's properties.

To get specific interaction between LDL particles and its receptor, the tips have to be specifically functionalized. Lipoprotein particles are thereby covalently linked to the tip, which is an established procedure in our labs. Interaction forces are measured by performing force-distance-cycle experiments on glass seeded with positive- and negative-control cells represented by healthy and genetically mutated cells lacking the LDL receptor totally. Thus, the cells are cultured in petri dishes. To ensure that single cells are measured, these petri dishes have to be cultured a day prior to the measurements. Any longer culturing would lead to an overlapping of the cells since the seeded concentration is large compare to the given surface. For tip functionalization reactive sites have to be generated on the tip through amino functionalization – either by using APTES in gas or liquid phase. At ASU the gas phase approach has been used. This provides functional amino groups ideal for linker chemicals to attach. Coupling to the linker groups occurs through amid bond formation of linkers with the amino reactive group on the tip and a reactive one on the linker. These linkers have an N-hydroxysuccinimide (NHS) ester function which leads to coupling to amino groups on the tip surface to form the stable amide bond (this is shown in Figure 1/b. in red), while the other side acts as a free-tangling end. This end can serve as attachment for sensor molecules like maleimide which has been used for this research purpose (Figure 1/b. in blue). Maleimide molecules on linkers use thiol groups to form a robust bond. LDL contains free thiols which enable binding events between maleimide and particle. Another possibility is binding to the cysteines included in LDL particles, although this is not necessary when free thiols are already provided for the functionalization. We used a flexible tether between tip and probe molecule provided by a Poly Ethylene Glycol (PEG) chain within the linker to ensure higher probability for binding of the probe molecule onto the target molecule/surface via its free rotatability and flexibility. Its basic structural form is shown in Figure 1 (a.). In Linz a different approach has been used, one where either aldehyde or acetal linkers were used. Figure 1/c. shows the complete AFM-tip functionalization with all its necessary binding partners. For readability the LDL particle was not included, and a simple cysteine molecule was used to visualize the effect.

In reality the cysteine is replaced by the LDL particle and binding occurs through a free thiol group. Once this functionalization is completed, the tip can be used to detect specific binding forces between LDL particles and its receptor. Dr. Ros and his group have long-lasting experience with these linkers and helped me to create a protocol to assure the correct attachment of the LDL particles. In principle, SMFS measurements require additional blocking experiments. These are performed to ensure the specificity of the binding. Typically, the receptor bonds to be investigated are saturated with antibodies on the cell. Latter studies showed that the sensitivity of available antibodies is not sufficient for single molecule studies. Therefore, no such experiments are currently planned. One of the cells used in our experiments (GM00701) have no receptors on the cell surface and thus represents the desired blocking experiment anyway. In order to also characterize unspecific interactions generated by the chemical functionalization, control experiments on glass surfaces were performed. For this figure we used a simplification of the bond by showing solely the thiol bond in a cysteine rather than showing the complex structure of the APOb particle used in our experiment. The binding event works in the same way as shown on the cysteine molecule. [29, 30, 31, 32, 33, 34, 35, 19]

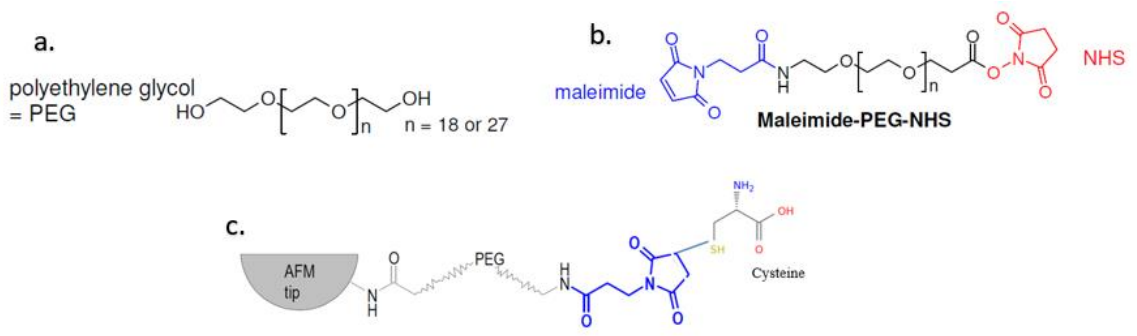

Figure 1 (a.) shows a polyethylene glycol (PEG) chain used as linker for functionalization of AFM tips. It is a flexible tether between tip and probe molecule to ensure a higher probability for binding of the probe molecule onto the target molecule/surface; (b.) shows a PEG linker with maleimide reactive group (in blue) and NHS ester function (in red). After amino functionalizing, the tip can bind to the NHS ester to form an amid bond. The free maleimide end leads to a coupling to thiol groups; (c.) Tip functionalization using a Maleimide PEG-NHS linker and further linkage to thiol groups. Free thiol groups are available in the LDL particle.

The AFM we used was an Asylum Research MFP 3D which is in constant use in Dr. Ros´s lab. As a side experiment we tried to image cells using an AFM. Before and after performing SMFM measurements the cells can be inspected visually through an inverted microscope. We set the AFM on a cell and imaged a large area to see different areas of the cell. Goal is to detect, where the cells membrane lies and where the nucleus is detected. Here we tested contact mode and tapping mode as modes of operation for imaging. Not long until we discovered, that contact mode destroys the membrane and moves the cell with the tip. Therefore, we continued with tapping mode. Once several images were taken and they were compared to the visual presumption it showed that it is possible to detect the nucleus visually to a certain degree. Our SMFM measurements ideally are made in areas far from the nucleus. The softer area away from the nucleus provides a longer time before the tip retracts again leading to higher chances of LDL interaction with the LDLR. Also, deformation due to surface roughness created from the nucleus would change the cantilevers contact angle.

1.7 Results

The overall objective of my research work included the qualitative investigation of the specific receptor interaction between LDL particles and the associated receptor LDLR. Based on the fact that people suffering from FH disease have different mutations or even lack the corresponding receptor, two cell lines of corresponding individuals were examined, either those classified as clinically healthy (GM01386) and those where the specified receptor was completely absent (GM00701). It can be assumed that in addition to the specific interaction, there are other interactions between LDL particles and other receptors, such as the Scavenger Receptor Class B Type 1 (SR-B1). Furthermore, based on current studies[36,37,13] receptor independent interactions occur, such as a direct fusion between cell membrane and LDL particle. My studies should show which interactions can take place, as well as the specificity of the corresponding receptor. Specifically, the absence of the receptor in one cell line (GM00701) will be revealed from the measurements and the proportion of other interactions will be described. Therefore, more than 12.000 force-distance curves were measured and analyzed. All following results and interpretations are principal examples not based on statistical analysis. The final goal is to separate binding events between tip and membrane through statistical analysis, which will be further studied in my master thesis and through colleagues in Linz. When looking at the results, three main binding features can be detected. Different forces act between the LDL particle-functionalized measuring tip and the cell membrane. They are separated in the tear-off curves and consequently the cantilever (measuring tip) returns to its rest position (i.e. ground state). With the help of this technique smallest electrostatic interactions between measuring tip and surface can be characterized. In this respect, this technique requires an isolated environment (acoustic and electronic). Often disturbances cannot be prevented and therefore some measurement curves have to be eliminated during the evaluation. Figure 2(a) shows the three most frequently observed binding events, with the curves in (a) and (b) suggesting a simple binding interaction and the curve in (c) a two-stage interaction. All curves were taken from the cell line (GM01386) (clinically healthy). When tips functionalized with LDL particles are brought into contact with a cell membrane, we expect a rupture event that is specifically mediated by LDL receptor activity. A closer look at Figure 2 (a) and (b) reveals individual rupture curves which can be assigned to a specific bond. Basically, the shown bond separations differ in their force and tear-off length. Considering that the selected linker system is about 10 nm (PEG length , taken from their official website) long and the cell has a certain elasticity, a separation of the binding between the measuring tip and the cell membrane must be greater than 10 nm. It can be assumed that the interaction between receptor and ligand is in the range of 20 - 200 pN [38]. The shown curves (a) and (b) differ in force by about 20 pN with almost the same break length – break length is around 25 nm and would basically meet the expectations of the selected interaction partners. Similarly, the detected forces are in the expected range of 70 pN. As described above, curve (c) shows two interactions. The first interaction (right) is very short-range in the range of a few nanometers with a force of about 10 pN. Basically, these short interactions are usually associated with adhesion, but they must be included in the analysis of the slope of the curve. Here the gradient of the tear-off force differs and is therefore not based on adhesion. Based on published data, a direct (receptor independent interaction) interaction between lipoprotein particle and cell membrane can also take place –

the particle fuses directly with the membrane. In this respect, a very close interaction would be expected and correspond to the first interaction in terms of force and distance. The second interaction is around 50 nm away from the contact point with a force of about 20 pN. Either one assumes that this is also a specific LDLR and LDL-particle binding, or other receptors (e.g. SR-B1) are involved. Only by a more detailed static examination of the measurement data and evaluations, a qualitative and finally quantitative statement can be made. Another reason for two breaking forces could be multiple binding events between tip and cell. Since our tip contains several LDL particles, it can be assumed that multiple binding events between LDL particle and receptor(s) can occur. Fracture length and breaking force can give information about which case occurred within which curve.

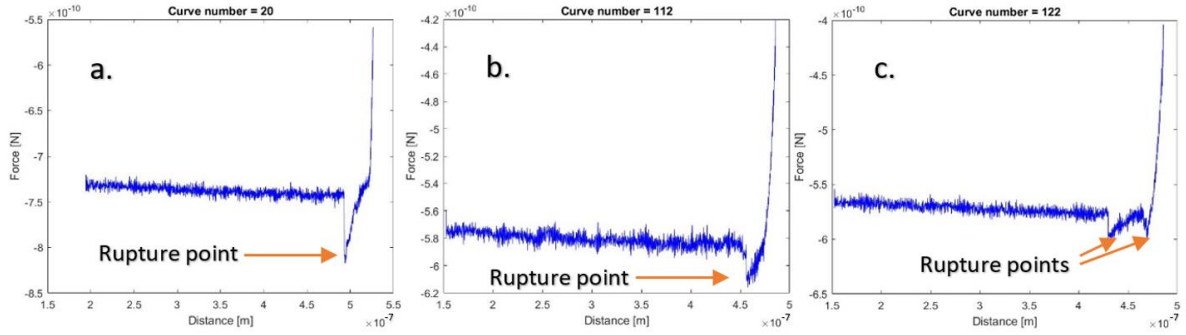

Figure 2 Force-distance curves representing different binding events for GM01386 cells (healthy cell-type); (a) shows a single rupture event. This can be a representative rupture event for LDL and LDL-receptor interaction or another specific binding event (rupture distance: 25 nm; rupture force: 30 pN); (b) shows another single rupture event. This event differs from the one in (a) in the rupture force needed (rupture distance: 25 nm; rupture force: 70 pN) to detach the tips; (c) pictures two binding events, one of which could be a depiction of an uptake of the LDL into the cell membrane without the specific LDL-receptor bond and the second one potentially showing the specific LDL bond (due to the parabolic behavior we assume so). The right rupture point has a distance <10 nm and a force = 20 pN; the second rupture point has a distance = 35 nm and a force = 25 pN

All curves solely show the retraction of the cantilever. Therefore, considering the time, the figures are read from right to left. In these plots the points of interest are the contact and rupture point. We created a control experiment to assure a visual difference between rupture lengths and adhesive forces. Force measurements on glass shall provide force curves, where no specific binding is observable. After contact point all adhesive forces start to act therefor, we can detect the bending from the tip towards the cell. The distance between these two points is called the rupture length. According to the rupture length, the kind of binding is determinable. Figure 3 shows the difference between a force-distance curve on glass with no interaction (a) and on a cell with specific binding action (b). In Figure 3(a) the cantilever immediately reaches its equilibrium state after retraction from the surface. There is no rupture event detectable and barely any attractive force. This curve has been taken with a functionalized tip in cell media to ensure comparability to the other image. On the contrary, in Figure 3(b) one can detect a rupture point (red arrow) and adhesive forces after the contact point. The attractive forces begin to act after the contact point (green arrow) and they act until the rupture point (red arrow). To be more precise, these forces are should no longer be called attractive forces, more the specific binding force between tip and surface. Depending on the specific binding acting on the cantilever, the rupture point appears at a shorter/longer distance with less/more force for rupture. The parabolic behavior after the contact point until the rupture point indicates a specific bond related to the LDL interaction.

The force is around 40 pN and the rupture length is 25 nm. The combined length of the linker with the particle shall provide this specific length for each rupture event. Since the linker is around 10 nm, a rupture length of 25 nm seems realistic.

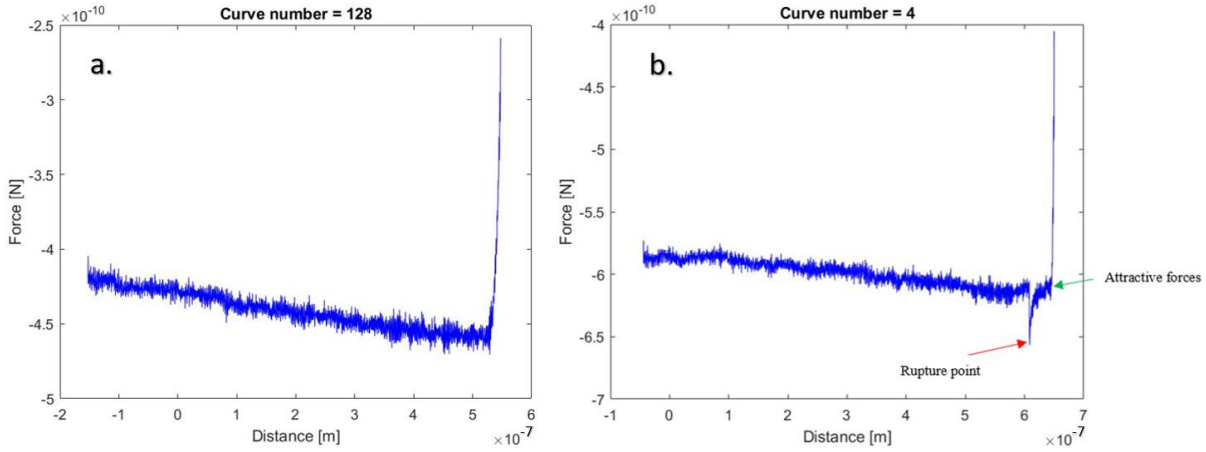

Figure 3 shows two images to describe the difference between force-distance curves on glass (a) and on cells (b); (a) is a force-distance curve with a LDL functionalized tip on a glass surface. This image shows no rupture event or specific adhesion to the surface. It was also taken in cell-media environment to provide a comparable image; (b) shows a force-distance curve on a cell with a specific rupture event. The green arrow signals the point of contact and the start of the adhesive/attractive forces which act on the cantilever. The red arrow shows the rupture point, where the attractive forces stop, and the cantilever abruptly moves back to its equilibrium state

After statistical analysis we assume to achieve specific bindings between LDL and LDL-receptor. Apart from that, when inspecting some samples other specific bindings have been detected. This leads to the assumption, that these also have a specific pattern and can be analyzed and quantified. Challenging will be the separation of these events. Based on linker length and the size of LDL and LDLR, one can make assumptions on whether a rupture length belongs to LDL-LDLR binding. Another possible outcome could be the detection of cell membrane fusion. Figure 4 shows the interaction between cantilever and cell membrane. In (a) we can see the equilibrium state where the cantilever is not in contact with the membrane. The LDL particle (grey circle with red counterpart for interaction) is attached to the cantilever tip via a Mal-PEG linker (black line) and serves as functional counterpart to the LDLR (green and yellow) embedded in the cell membrane. This serves as positive control and leads to a binding event (Figure 4(b)), which can be detected on the force-distance curve. In (c) we can see the negative control procedure, where no binding events regarding the LDL and LDLR interaction can occur.

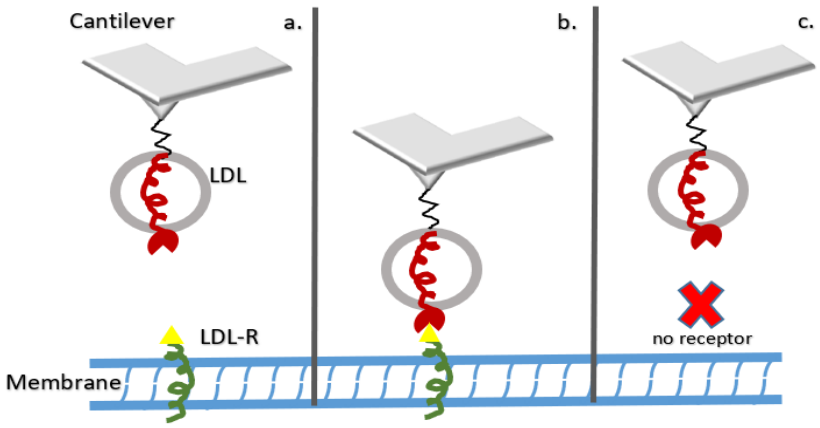

Figure 4 Scheme of binding event of LDL and LDLR; (a) shows LDL (grey circle with red counterpart for the receptor) attached to the cantilever tip through a Mal-PEG linker (black line). The binding partner of the LDL (in red) acts as counterpart to the LDL-receptor (green with yellow counterpart) located in the cell membrane (light blue). (b) shows the interaction between LDL and LDLR. (c) is the corresponding negative control, where no receptors are present in the membrane.

There are also events occurring in the genetically mutated cellline GM00701, even though no binding shall occur. To visualize, what is happening when specific adhesive forces occur, we can inspect (Figure 5). It addresses two major events detected by AFM, apart from the LDL interaction with its respective receptor. At first in a. and b. we see once again the approach of the cantilever tip onto the cell membrane. The only alteration to Figure 4 is the receiving receptor embedded in the membrane. Which specific receptor interacts with the LDL is unknown, yet there are specific interactions apart from the expected bond to LDLR. Separation of these specific binding events shall be given through different rupture lengths and forces. Figure 5(c). shows the fusion of LDL with the cell membrane. It is known that the cell membrane can uptake particles without the need of the LDL-receptor. Typical for these force curves are two rupture points, where the first one signals the exit of the LDL out of the cell membrane (after fusion into the cell membrane), and the second one signals a specific binding event occurring additionally.

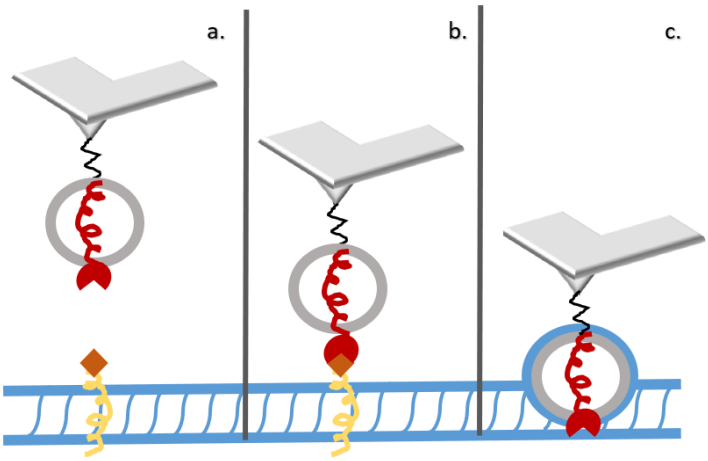

Figure 5 binding events not related to LDL and LDLR interaction; (a) shows the approach of with LDL functionalized tip to the cell membrane. The receptor embedded in the membrane is not an LDL-receptor, but it still reacts with LDL;(b) pictures this interaction. The receptor part interacts with LDL due to the similar shape; (c) depicts the fusion of LDL with the cell membrane. Here no specific binding occurs, an uptake into the membrane takes place. Here it is likely to see two rupture points: one being the exit of the particle out of the membrane after fusion, the other being the rupture of a specific bond.

1.8 Discussion

Our results show that functionalized tips can be used to detect binding events between particles and membrane receptors. Although we cannot yet determine which of the cases is specific to the LDL interaction, it is clear, that interactions occur and can be analyzed. Having established the fundamental part for the quantification provides a good base for future research regarding this topic. My master thesis will therefore address statistical analysis comparing different binding events and give insight about how often LDL interactions with the receptor occur. In these analyses we will try and compare 12.000 curves from the ASU lab with the several 10.000 curves taken at the FH OÖ lab by AFM experienced employees. Another upside to our cooperation with ASU is the different analysis programs. Comparing the two programs will point out any unexpected errors or miscalculations, thus resulting in a more reliant examination of the curves. When looking at the results it is clear to see different binding types with certain pattern. The explained statistical approach can give insight into whether the three different types are present. Also, these other specific bindings or the fusion with the membrane can give further insight into the membrane's behavior to other biological and chemical problems. Many interactions can occur using functionalized tips, therefor examining these experiments can give several information outside the expected outcome.

This technique is successfully applied to study FH disease. However, this approach can be applied to nearly all membrane related diseases, like for example insulin resistance or specific uptake of pharmaceuticals. In case of insulin resistance – diabetes 2 – the genetic modification of the insulin receptor causes insulin to be unable to bind and thus glucose cannot be taken up into the cell. Again, in the case of pharmaceuticals, one wishes to have directed delivery to specific target cells. This requires that only those cells which express exactly the desired receptor predominantly ensure the uptake. Functionalized tips in these cases open up the possibility to inspect all activity and give information that can help further treatment in a medical aspect. AFM functionality keeps growing and we intend to help contribute to that growth in SMFM.

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