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Technical challenges in immobilizing MUC Glycoproteins for Atomic force microscopy (AFM) analysis

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Abstract: *Mucins are gel forming extensively glycosylated, high molecular weight glycoproteins. Sugar molecules in glycoproteins are highly diverse in structure and generally conserved. Sometimes these sugar molecules stimulates certain types of chemicals and send messages in to the nucleus and interferes the genes and causes effect of disease in cells. Glycosylation plays a key role in antibody recognition. Glycosides are quiet heterogeneous in structure, generally carbohydrates play major role in protein folding, structure of cells etc., when antibodies are introduced to them and they turn out their function. By understanding the glycobiology of mucin proteins can lead to development of drug. So it becomes an attractive factor in drug design. Nanotechnology is highly emerging field that allows us to visualize individual structures at nanoscale and manipulating those individual structures at atomic and molecular level. Obtaining three dimensional structure of a protein is quiet hard due to their amino acid sequences. Atomic force microscopy (AFM) helps us to obtain three dimensional surface morphology of mucin MUC proteins. When we know the size of proteins we can take them to next step process to search all possible conformations and their desired target structure. However there are technical challenges to immobilize glycoprotein for AFM analysis. In this paper we will be discussing about factors that affected protein immobilizations. This study was carried out to understand the how MUC proteins regulated in airways and how their structural change varies using in vitro cell models. Their mechanisms were studied using epithelial cell lines and analyzed them using atomic force microscopy (AFM).*

Keywords: *Glycoproteins, Mucin, Proteins, Glycobiology, Atomic force microscopy (AFM), Immobilization, Nanotechnology*

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I. Introduction

Finding human genome contains fewer genes than previously predicted might be compensated for by combinational diversity generated at the level of post translational modification of proteins (J.C.Venter, et al., science, 2001). In human the proteins are encoded by genomes and modified in very complicated and diverse ways to create biological complexity by predicting and counting the genes in genomes and modifications of those proteins are we called as post translational modifications. One of the most complicated post translational modifications of proteins are attachment of sugar molecules and that process is called glycosylation (Term glycol means sugar in greek). In fact they reside at the surface of the cells generally called as sugar modified proteins (glycoproteins). The sugar part of the glycoproteins and the glycolipids are called as glycans.Glycoproteins changes as the cell function changes, during diseases (Varki A, et al., 2009). Glycans at the normal cell is completely different from glycans in COPD and cancer cells. If we see the glycan changes in human body, we might be able to detect diseases. Glycans are the products of metabolic pathways and begins by uptake of simple sugars into the cells and these simple sugars are called as monosaccharide. Inside the cell the monosaccharide building blocks are processed by enzymes, eventually those building blocks are sent into endoplasmic reticulum and the golgi compartments, where complex glycans are constructed from simple monosaccharide monomers. The glycans developed in endoplasmic reticulum are attached to proteins or lipids and called as glycoproteins and glycolipids are delivered to plasma membrane.

Glycans are essential for the stability and function of proteins they affect enzymatic activity, half life and receptor binding among other biological processes. Proteins can be modified with different types of carbohydrates namely N-glycans and O-glycans are the most common in eukaryotes. N-glycans are attached to aspargine residues of the proteins, N-glycans have a common biological synthesis pathways which reflects intheir common structure of core. N-glycans can be high manose, which means all the component monosaccharides are manose residues with GlcNAc residues. N-glycans are complex in structure their manose branches are extended with other monosaccharides such as GlcNAc, galactose, sialic acid and fucose. O-glycans structure starts with GalNAc residues to serine or threonine. O-glycans can form long chain with addition of different monosaccharides and resulting in a longer chain (Davril et al., 1999).

Sugar molecules in glycoproteins are highly diverse in structure and generally conserved. Sometimes these sugar molecules stimulates certain types of chemicals and send messages in to the nucleus and interferes the genes and causes effect of disease in cells. Glycosylation plays a key role in antibody recognition. Glycosides are quiet heterogeneous in structure, generally carbohydrates play major role in protein folding, structure of cells etc., when antibodies are indrocuded to them and they turn out their function (Lamblin, 1999). By understanding the glycobiology of mucin proteins can lead to development of drug. So it becomes an attractive factor in drug design (Yarema KJ, et al., 1998).

Nanotechnology has the potential to change the world in many positive ways. It's believed that it will improve the potential of products which we are using. It allows us to visualize individual structures at nanoscale and manipulating those individual structures at atomic and molecular level. It has ability to provide insight into structural features of biological systems such as cells, tissues and proteins as well as to develop medicine for their diagnostics, therapeutics and tissue regeneration. mucin proteins are complex in structure and it is hard to study their structure and size, the advancement in nanotechnology makes that simpler to obtain their structure and helps to finding out their target structure which will helps to therapeutics and development of vaccines or antibodies for the deadliest diseases. Atomic force microscopy (AFM) helps us to obtain three dimensional surface morphology of mucin proteins. When we know the size of proteins we can take them to next step process to search all possible conformations and their desired target structure. Using AFM we studied the structure and size of MUC proteins. However there were technical challenges faced during immobilization of protein over gold substrate. In this paper we will discuss about the factors that affected protein immobilization during AFM imaging process.

II. Materials and Methods

Materials list for cell culture

1. F-12 K Medium
2. 10% Fetal bovine serum
3. Pen-strep (penicillin – streptomycin solution)
4. Phosphate buffered saline (PBS)
5. T25, T75, T225 tissue culture flasks
6. Trypsin
7. Pipettes (1, 5, 10, 25, 50 ml)
8. Centrifuge tubes
9. DMSO (Dimethyl sulfoxide)
10. Microscope
11. Centrifuge machine
12. Micro pipettes
13. Incubator

Cell culture

Cell lines derived from human lung, A549 human lung adenocarcinoma epithelial cells were used and stimulated by Lipopolysaccharide **(**LPS) and Tumour Necrosis factor- Alpha (TNF-α). First the cells were cultured, passaged and randomly divided into three different groups which were designated as control, LPS treated and TNF-α treated. After incubating for 24 hrs, the expression of MUC5AC and MUC5B secreted by cell line at mRNA and protein level in the groups were determined by real-time quantitative PCR and enzyme-linked immunosorbent assay (ELISA). After incubating cells for certain period passaged cells were treated with serum free media before protein extraction.

Protein Extraction

Cells are cultured and we treated with inflammatory agents for mucin hyper secretion, and then after a day we separated the concentrated protein by ultrafiltration and immobilized them in substrate for AFM imaging.

Surface preparation

The proteins were separated and centrifuged from the cultured mucin mediums control, TNF alpha treated and LPS treated. The concentrations of separated proteins were unknown, so we tried to immobilize over the substrate as it is. In this project we immobilized protein over the nano porus gold substrates (NPG) and allowed them to stick over EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) functionalized surface.

Atomic force microscopy analysis

The AFM imaging was performed in both taping and control mode with bruker nanoscope control. But mostly carried out in tapping mode to scan soft sample to avoid damage that could be caused by tip, A picoforce z-scanner and a silicon nitride (SNL 10) cantilever tip with a cantilever constant of 0.24N/m were used. Frequency of the tip is 40-75 hertz. Imaging was performed in tapping mode. Calibration was carried out in x, y, and z direction to get three dimensional surface image. Imaging was performed at room temperature. During scanning the tip oscillates as the sample is scanned. The amplitude of oscillation is used as feedback signal. The proteins were measured quantitatively using cursor profiles. The experiment was repeated with different batches until required results were obtained.

III. Results

Human lung carcinoma A549 cells were cultured and passaged at 85-95% confluency throughout the experiment. We never know mucins were secreted in cells during treatment. (Marina G.S, et al., 2000 and Koen JP.V, et al., 2013) and several other articles proved that TNF- α and LPS induces hyper secretion of mucins in cell. So our role was to identify those secreted mucins from cell line, we used amicon ultra centrifugal filter device to extract proteins. Samples were transferred to centrifuge tube containing device, during centrifugation process the proteins with greater density were trapped into the device. Later trapped solutes were recovered from the tube by same process by placing the device upside down in a clean centrifuge device. One of the major technical challenge during extraction process was we do not know the exact concentration of mucins which were secreted during treatment. So we tried to increase their concentration by adding them with PBS.

In order to identify the extracted mucins, we used AFM to identify proteins and we did by immobilizing them on a gold substrate made up of nano gold particles which have specialized EDC/NHS surface properties than ordinary gold particles. We used AFM to identify the proteins immobilized on the substrate. AFM is an advanced tool to image and identify the proteins at their molecular level. AFM is an integrative tool for structural studies where we can get three dimensional topography of immobilized protein and understanding the structure and size of proteins.

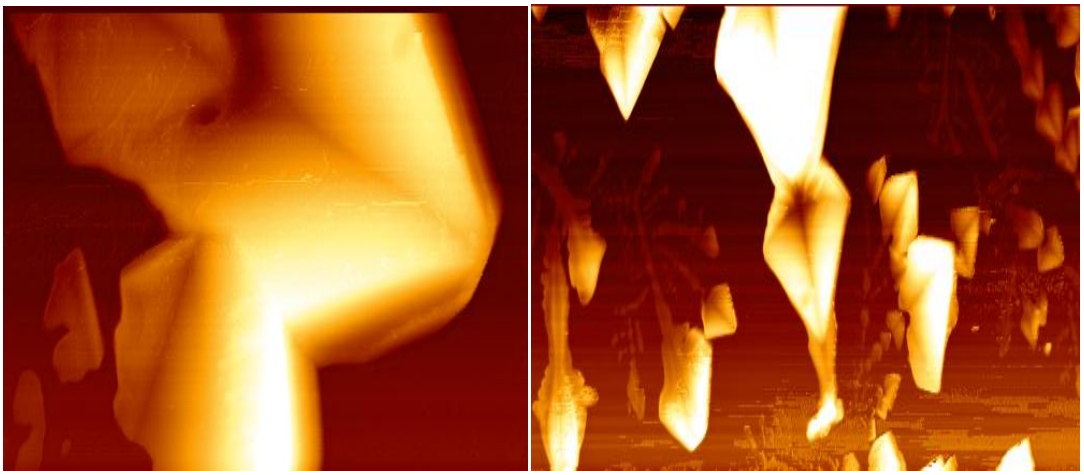

Figure 1.a (left), 1.b (right) immobilized samples in gold substrate coupled under EDC/NHS mechanism and imaged under contact mode (100 μm x 100 μm scanning area)

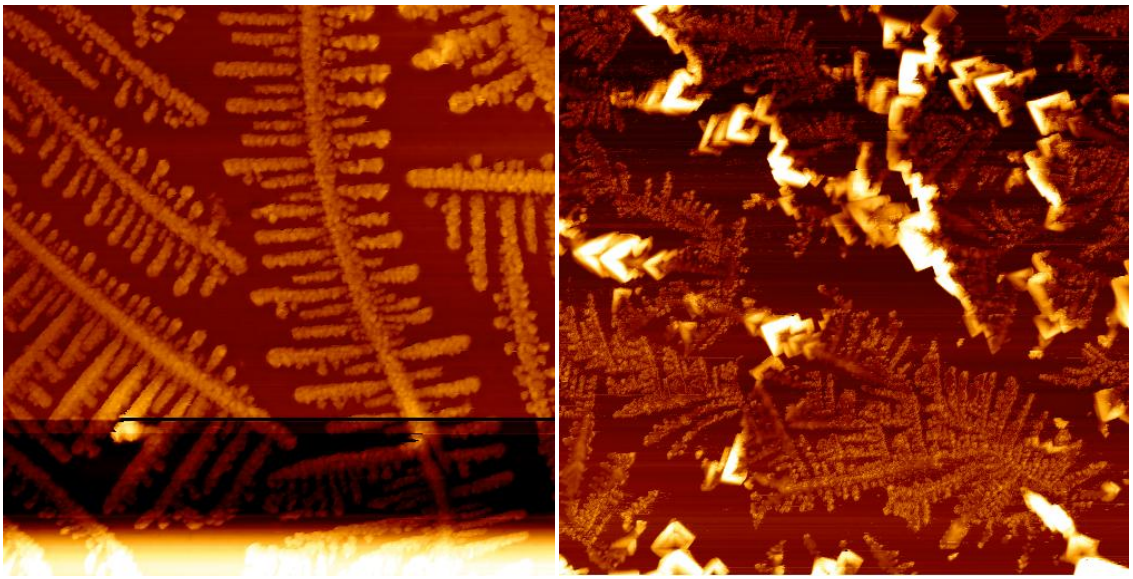

Figure 2.a (left), 2.b (right) Protein samples immobilized immobilized samples in gold substrate coupled under EDC/NHS mechanism and imaged under contact mode (100 μm x 100 μm scanning area)

The gold substrates were incubated with EDC and NHS to form a self assembled monolayer to immobilize protein for imaging. After incubating with EDC/NHS layer we exposed the protein samples and incubated them for one hour. To make sure proteins immobilized on the gold surface it was taken to AFM for imaging. The substrates were imaged in AFM under contact mode and Figure 1.a and 1.b were generated. During imaging we observed that large crystal like structures found over the substrate. The crystals were results due to using water to wash substrate. During washing some micro water droplets may stay over the substrate and induces crystal formation

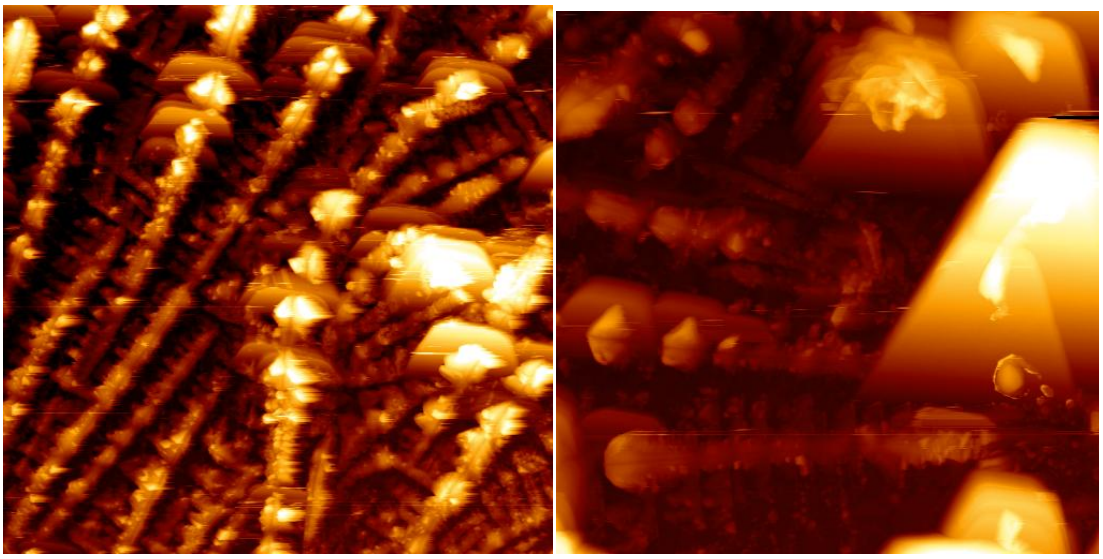

Figure 3.a (left), 3.b (right) TNF- α and LPS induced Protein samples immobilized immobilized samples in gold substrate coupled under EDC/NHS mechanism and imaged under contact mode (100 μm x 100 μm scanning area)

Protein immobilization depends upon its concentration and incubation time (Tan YH, et al., 2011). As we do not know the exact concentration of protein we tried to increase their incubation time for better immobilization. Generally control samples have less concentration than treatment samples, so we tried to immobilize treatment samples on substrates. Substrates were cleaned and prepared incubated with self assembled monolayers for immobilization. Then the substrates were carefully washed with PBS and prepared treatment samples (LPS and TNF- α treated samples) were immersed over the substrate. This time in spite of incubating for 60 minutes we tried to let them dry over the substrate. The protein immersed substrates were carefully placed inside the desiccators for 3 hours. Desiccators is an apparatus generally used by chemists to dry

samples from moisture and protecting them from environment. When we took the substrates from desiccators for AFM analyzing the whole substrates were covered by crystals which were easily visible under naked eyes. As we tried to immobilize by drying the liquid phase of sample gets solidified slowly and precipitated over the substrate. We did not image these samples, because we can clearly see the crystallized surface and no use in imaging them under AFM. Also its waste of time and resource to image crystallized surface, as our main goal is to get the structure and size of proteins. However these crystals were not an excellent tool to obtain three dimensional structures. Imaging them may cause tip breakage as the crystals were in irregular pattern so we decided to go with immobilizing under different technique.

IV. Discussion

Protein immobilization is not an easy process the immobilization process is a time dependent process. Also there are several difficulties during immobilization process, environmental conditions plays a key role during this process. pH, selection of buffer, deionised water are the factors governing the process. This factors may interferes and affect the samples polarity and acidity. The adsorption of protein on substrates is always influenced by physical and chemical characteristics of the sample. Changes in pH, ionic strength or contamination cause desorption of immobilized proteins (Frenderix et al., 2004).

Immobilizing proteins the solid substrates for AFM imaging is a complicated and problematic procedure due to various factors mentioned above, these processes can be overcome by formation of self assembled monolayer such EDC-NHS layer on the substrates and immobilizing proteins by covalently coupling them (Frenderix et al., 2004). However using of buffer solution and deionised water makes more challenging and compromising.

From figure 1.a, 1.b, 2.a and fig 2..b its clear that there heavy formation of crystals on the substrate, this was the major technical challenge. The crystal formation is due to using deionised water to wash the substrate after every procedure during immobilization protocols (Mark Schnea, 2005; Craciun D, et al., 2012). We cannot wipe off the water during immobilization process, it may cause damage to monolayer formation, also immobilization protocol is a time dependent factor and when we allowed exposing the protein sample for further more time (Tan et al., 2012), the deionised water which we used for washing may settled in porous area of the substrate induces crystal effect. There is no proper evident to prove that proteins may present underneath the crystals.

Also when we do not know the exact concentration of protein and trying to immobilize on a substrate you have to carry out various steps to immobilize them. So next step we used TNF- α sample and LPS sample to immobilize them on a substrate by increasing their exposure time and letting them to dry over the substrate. We thought it was worth to try than doing nothing after 3 hours when we tried to look at the substrate still there is a huge crystal formation than any other previous results (figure 3.a, figure 3.b). Generally deposition and immobilization of proteins requires a reductive environment as long as we exposed protein with water droplets, that may settled in substrate undergoes various changes and a result huge crystal formation. And it is more clear and evident that deposited protein droplets should allowed to dry over the substrate, water droplets are unstable and when they are exposed to longer time and allowing them to contact with air there will be a thermal differences occurs between the gas phase and substrate turned into change in droplet volume and it's difficult to achieve protein immobilization.

The adsorption of protein molecules on solid- liquid interface is a damaging procedure because it includes aqueous environment on one side of molecules reacting with solid substrate. The water molecules were buried in porous membrane of the substrate and when we expose protein along with that cause conformational changes. Also it is dangerous to scan substrates with irregular crystal formation because the exact scan rate for scanning is unknown. Scanning on them causes tip breakage and damage.

Controlling crystallization process was the major concern. Water droplets will remain in porous area of the substrate and initiate quicker crystallization due to mobility of the water molecules. So it is important remove the water droplets from the substrate or to dissolve crystal to check whether the proteins where immobilized beneath under their surface. Hong et al. 2005 article suggests that using of butanol can dissolve or avoid the crystal formations. Also it is necessary to immobilize protein in particular area of the substrate for easier imaging process. When we were incubating samples throughout the substrates it is difficult to locate particular area during imaging and it would be like trial and error method to locate exact area. This can be overcome by isolating proteins at central region of substrate.

V. Conclusion

Proteins are incredibly important they play countless roles throughout the biological world. The main aim was to develop AFM to visualize the overall structure of respiratory mucins once secreted, as a collection of molecules. The resolutions were too low to determine the structure of the single molecule. This is incredibly hard process to find out their structure in three dimensional due to heavy crystallization over the substrate. The

crystal formations were due to using of deionised water, when we wash our substrates with deionised water and PBS, we do not wipe the surface of substrate so the water droplets stays in substrates and induces crystal formation proteins might trap under them. For every immobilization process we wash our substrate with Piranha solution (mixture of sulphuric acid and hydrogen peroxide). Constant use of piranha solution causes damage to substrate it is found out that, there was a channel formation in the substrate which was caused by higher order crystallisation from previous steps and we used piranha solution for washing them. Piranha is a very concentrated solution employed to wash off residues present in substrates. We do not have an enough substrates and we used same substrates for all through our project so this leads to formation of channels in substrates. Crystal formation can be avoided by incubating the substrate with butanol for 5 minutes after immobilizing the sample with EDC-NHS self assembled monolayer.

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