Rheology Measurement

**Objective**

**Purpose of the experiment**
Test the viscoelastic properties of pig gastric mucin (PGM) samples with and without mucus degradation.

**Background information**
Mucus is a highly multifunctional network of heavily glycosylated proteins called mucins covering the bodies' inner surface epithelium that builds a slippery and viscous gel extremely potent in trapping and preventing foreign particles and pathogens from entering the body. Thus, it serves as immunological protection layer between environment and body.

Here, we wanted to deglycosylate our PGM samples with the GlycoProfile β-Elimination Kit to test the change in the mucus properties caused by the degradation.

One of our advisors, Thomas Crouzier, prepared and provided the purified mucin samples that he extracted from pig stomach. This procedure is conducted following current regulations and is approved by "Jordbruksverket" (The Swedish Board of Agriculture).

**Experimental setup**
We performed a rheology measurement on untreated pig gastric mucin (PGM) sample to establish a baseline of the visco-elastic properties of the mucus sample. Thereafter, we tested the mucus degradation effect by degrading the mucus sample with the GlycoProfile β-Elimination Kit (Sigma-Aldrich) which deglycosylated the sample in a non-reducing manner by efficiently cleaving the O-linked glycans with minimal protein or glycan destruction. For time reasons, we were not able to test the effect on PGM of our self-produced Sialidase and Beta-Endo-galactosidase.
Procedure - 1

Method
Rheology

Instruments and parameters
Machine: Discovery Hybrid Rheometer (DHR2), TA Instruments
Geometry: 25mm parallel plate, stainless steel
Gap: 150μm
Instrument settings:
   Frequency Sweep
   Temp: 20°C
   Stress: 0.1Pa
   Angular Frequency: 0.1 to 100.0 rad/s
   Points per decade: 5

Materials and properties
Mucus sample: 1% (1mg/mL) PGM, pH6
Mucus sample consist of purified MUC5AC mucins extracted from pig stomach.
(PGM sample was provided from our Advisor Thomas Crouzier)

Protocols
Rheology experiment was run with the untreated PGM sample (2%, pH 5.5)
(Daniel Hult, PhD student, School of Chemical Science and Engineering from KTH helped with the handling of the machine and execution of the experiment).

Results
Conclusions
The mucus sample is to water like. We have to increase the concentration and decrease the pH to obtain more gel like sample. Redo testing with changed properties.
Evt. 40mm geometry would help to get better results as well, but this entails a larger sample volume.

Procedure - 2
Method
Rheology

Instruments and parameters
Machine: Discovery Hybrid Rheometer (DHR2), TA Instruments
Geometry: 25mm parallel plate, stainless steel
Gap: 150μm
Instrument settings:
  Amplitude Sweep
    Temp: 20°C
    Frequency: 1.0 Hz
    Torque: 0.1 to 10.0 μNm
    Points per decade: 5
  Frequency Sweep
    Temp: 20°C
    Torque: 0.15 μNm
    Frequency: 0.01 to 100.0 Hz
    Points per decade: 5
  Flow Sweep
    Temp: 20°C
    Torque: 0.1 to 100.0 μNm
    Points per decade: 5

Materials and properties
Mucus sample: 2% (2mg/mL) PGM, pH5.5
Mucus sample consist of purified MUC5AC mucins extracted from pig stomach.
(PGM sample was provided from our Advisor Thomas Crouzier)
Protocols
Rheology experiment was run with the untreated PGM sample (2%, pH 5.5).
(Daniel Hult, PhD student, School of Chemical Science and Engineering from KTH helped with the handling of the machine and execution of the experiment).

Results
Graph 1: Amplitude sweep

Graph 2: Frequency sweep
**Discussions**

The first rheology measurement was done with a mucus sample of 1% MUC5AC and pH6, and a 25mm geometry. The results showed that the mucus properties were too water-like. Thus, we increased the concentration to 2% and reduced the pH to 5.5 leading to stiffer (more gel-like) mucus. The results were promising, but the stable baseline range was rather too small.

**Graph 1: Amplitude sweep**

The storage modulus is higher than the loss modulus indicating gel-like properties of the mucus sample. Unfortunately, the graph is only stable in the initial part. Afterwards the storage modulus decreases indicating a transition into more water-like state.

**Graph 2: Frequency sweep**

The graph shows again that storage modulus is higher than the loss modulus indicating gel-like properties. Although, at raw phase value 150 (blue line increasing tremendously) the data is not reliably measured anymore. It would be preferable to have higher Hz values than just up to 1Hz before the data get unreliable. “Stiffer” mucus and bigger geometry (40mm) could help. We have contacted the company of the instrument provider (TA instruments) to receive a bigger geometry which allows to have more fine-tuned measurement. We received them.
Graph 3: Flow test
Focusing on the blue line we can observe a small plateau around the 5th and 8th measurement point. After this we have the shear thinning effect, meaning that with increased shear rate the viscosity is reduced. We obtained a zero-rate viscosity value of 635 Pa*s.

**Conclusions**
The results were better than the measurement of the 13.06.2017.
- The amplitude sweep revealed a Torq value of 0.15 μNm. This is rather at the lower detection limit of the apparatus but okey.
- We observe reliable data in the frequency sweep until 1Hz. Possibly we should be able to have reliable data up to 10Hz.
- We saw a Newtonian behavior of the mucus sample in the flow sweep in a small range around the share rate of 10^-3 * 1/s. Zero rate viscosity: 635, 070 Pa*s

=>Test the mucus sample, 2% MUC5AC and pH5.5 with 40mm geometry.

**Procedure - 3**

**Method**
Deglycosylation of PGM
Rheology

**Instruments and parameters**
Machine: Discovery Hybrid Rheometer (DHR2), TA Instruments
Geometry: 40mm parallel plate, stainless steel
Gap: 250μm
Instrument settings:
- **Amplitude Sweep**
  - Temp: 20°C
  - Soak Time: 0.0s
  - Frequency: 1.0 Hz
  - Torque: 0.1 to 10.0 μNm
  - Points per decade: 5
- **Frequency Sweep**
  - Temp: 20°C
  - Soak Time: 0s
  - Torque: 0.15 μNm
  - Frequency: 0.01 to 100.0 Hz
  - Points per decade: 5
Flow Sweep
- Temp: 20° C
- Soak time: 0.0s
- Torque: 0.1 to 100.0 μNm
- Points per decade: 5
- Max equilibration time: 180s
- Sample period: 30s

**Materials and properties**
Mucus sample: 2% (2mg/mL) PGM, pH 5.5
Mucus sample consist of purified MUC5AC mucins extracted from pig stomach.
(PGM sample was provided from our Advisor Thomas Crouzier.)
Microcentrifuge, Centrifuge
GlycoProfile β-Elimination Kit (Sigma-Aldrich). It contains a non-reducing reagent mixture that efficiently cleaves the O-linked glycans with minimal protein or glycan destruction.
  - Collection tubes + Microcon centrifugal filter unit, YM-10 membrane, (NMWCO 10 kDa)
  - β-Elimination Reagent, Sodium hydroxide solution, 5.0 M

**Protocols**
Rheology experiment was run with the untreated PGM sample (2%, pH 5.5).
(Daniel Hult, PhD student, School of Chemical Science and Engineering from KTH helped with the handling of the machine and execution of the experiment).

Deglycosylation of PGM:
GlycoProfile β-Elimination Kit (Sigma-Aldrich) Protocol

**Day1**
For each 200 μL of sample (≤10μg/μL of glycoprotein) we need 40μL of β-Elimination. End concentration of treated PGM sample should be 2% (for rheology 40mm plate):
400μl * 2% = 8mg
Glycans weight is 60% of the total molecular weight (→ multiply by factor 2.5)
8mg*2.5 = 20mg
Prepare Beta-Elimination Reagent Mixture just before use:
- We have 5 tubes à 400ul PGM sample -> we need 5x80ul β-Elimination Reagent Mixture
- Pipette 376ul of β-Elimination Reagent and 24ul of 5M Sodium hydroxide solution into an Eppendorf tube and mix (store kit components at room temperature, mixture has limit stability and should not be stored longer than 2 hours before use)

Sample treatment:
- Pipette 400ul PGM sample into each of the 5 Eppendorf tubes
- Add 80ul Beta-Elimination Reagent Mixture to each of the 5 Eppendorf tubes (mixture volume = 480ul (400ul + 80ul))
- Overnight incubation at 4°C (15hours)

Preparation for Day2:
- Put filter units into tubes and add to each 500ul diWater (to wash the glycerol out of the membranes)
- Centrifuge at 14000g for 10min.
- Discard flow through
- Add again 500ul of diWater to each filter unit and let stand until you start day2 (that the membranes do not dry out)

Day2
- Centrifuge the pre-prepared filter units filled with water at 14000g for 10min
- Discard flow through
- Add the β-Elimination Kit treated PGM sample (approx. 480ul) to each respective filter unit
- Centrifuge at 14000g for approx. 30min
- discard flow through
- add up to 500ul with diWater and centrifuge at 14000 for approx. 30min (pipette a bit up and down to dissolve the pellet before centrifuging, but don't destroy the membrane)
- discard flow through
- add up to 500ul with diWater and centrifuge at 14000 for approx. 30min (pipette a bit up and down to dissolve the pellet before centrifuging, but don't destroy the membrane)
- discard flow through
- add up to 500ul with diWater and centrifuge at 14000 for approx. 30min (pipette a bit up and down to dissolve the pellet before centrifuging, but don't destroy the membrane)
- discard flow through
- invert filter units and put them into new collective tubes
- centrifuge for 3min at 1000g
- add 100ul diWater and dissolve sample remnants stuck to filter unit by pipetting up and down
- centrifuge the inverted filter units again for 3min at 1000g
- pipette the content of all 5 tubes into one tube
- put sample in fridge and let them stand

Here, Thomas Crouzier continued for lyophilization and dissolving samples to 2% (2mg/ml)
- After lyophilization, the sample weighed 13.2mg (initial 20mg)
  -> 34% mass removed
For the rheology testing we dissolve 8mg in 400ul -> 2% PGM sample
The untreated PGM sample also has 2% PGM

Note: In theory, the glycans make up around 60% of the mucin dry weight. We have removed 34%, thus there is potential for optimisation that might even entail bigger impact on rheology.

**Day3**

Run the rheology experiment with the untreated and treated PGM sample (both 2%, pH 5.5)
(Daniel Hult, PhD student, School of Chemical Science and Engineering from KTH helped with the handling of the machine and execution of the experiment)
Results

native PGM sample (2%, pH5.5)

Graph 1: Amplitude sweep

Graph 2: Frequency sweep
Graph 3: Flow sweep

Deglycosylated PGM (2%, pH 5.5)

Graph 4: Amplitude sweep
Graph 5: Frequency sweep

Graph 6: Flow sweep
Graph 1: Storage modulus is bigger than the loss modulus which suggests a gel-like behavior of the sample. PGM stays stably linear with increasing applied force.

Graph 2: Rheometer measures reliably until an angular frequency of approximately 1 rad/s.

Graph 3: Stable baseline in the initial part of the viscosity, then abrupt shear thinning effect.

Graph 4: Initially, storage modulus is bigger than the loss modulus which suggest a gel-like behavior of the sample (as graph 1, native PGM). But graphs shows, that compared to native PGM, shear thinning occurs earlier with increasing applied force (stable line of storage modulus starts dropping).

Graph 5: Rheometer measures reliably until an angular frequency of approximately 1 rad/s. Compared to native PGM (graph 2), storage and loss modulus starts at much lower level and slope of storage modulus line is flatter.
Graph 6:
sample dried out and machine had issues measuring accurately
We did a Cox-Merz transformation to transform frequency sweep data into flow sweep data (graph 7)

Graph 7:
At low shear rate (comparable to no-movement), the deglycosylated PGM has a slight higher viscosity and more stress needs to be applied on structure to obtain the same shear rate. With increasing shear rate, the viscosity of deglycosylated PGM drops faster than the native PGM. Also, less stress needs to be applied on deglycosylated PGM to achieve the same shear rate as native PGM. This also indicates that the shear thinning effect is higher in the deglycosylated PGM compared to the native PGM