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Characterization of the Effects of Mucins on the Growth and Biofilm Formation of Pseudomonas Aeruginosa and Analysis of Cleaved Mucin Glycan Structures

JINNY L. LIU

Laboratory for Biomaterials and Systems Branch
Center for Bio/Molecular Science and Engineering Division

MATHEW THUM

Applied Concepts in Materials Section
Chemistry Division

KEITH WHITENER (FORMER NRL EMPLOYEE)

Surface Nanoscience and Sensor Technology Section
Chemistry Division

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Jinny L Liu, Mathew Thum, and Keith Whitener*

7. PERFORMING ORGANIZATION / AFFILIATION NAME(S) AND ADDRESS(ES)
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14. ABSTRACT
This report presents research conducted to characterize the effects of mucins on Pseudomonas Aeruginosa (PA01) cell growth and cell adhesion and also analyzed the cleaved glycan structure. We showed that the protein profiles and glycosylation extent of natural mucus and industrial mucins changed significantly after high heat denaturation by autoclave and these denatured mucins supported the growth of PA01, but had no effects on the cell adhesion that leads to biofilm formation. On the other hand, the native mucins, mucin IIf and mucin IIf, sterilized by filtration exhibited inhibition of cell adhesion; however the presence of native mucins had no effects on cell growth. Furthermore, the cleaved glycan structures were also analyzed using Mass spectrometry and ATRIR. Our results support the hypothesis that native mucins with intact glycans interact with bacterial cells, resulting in less biofilm formation. These findings show promise for in vivo inhibition of biofilm formation associated with wound infection and sepsis.

15. SUBJECT TERMS
mucin, glycans, PA01, biofilm, Carboxymethyl cellulose, MALDI, PAS.

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Jinny L. Liu	(202) 404-6052

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Characterization of the effects of mucins on the growth and biofilm formation of Pseudomonas aeruginosa and analysis of cleaved mucin glycan structures

This report presents research conducted to characterize the effects of mucins on Pseudomonas Aeruginosa (PA01) cell growth and cell adhesion and also analyzed the cleaved glycan structure. We showed that the protein profiles and glycosylation extent of natural mucus and industrial mucins changed significantly after high heat denaturation by autoclave and these denatured mucins supported the growth of PA01, but had no effects on the cell adhesion that leads to biofilm formation. On the other hand, the native mucins, mucin IIf and mucin IIf, sterilized by filtration exhibited inhibition of cell adhesion; however the presence of native mucins had no effects on cell growth. Furthermore, the cleaved glycan structures were also analyzed using Mass spectrometry and ATRIR. Our results support the hypothesis that native mucins with intact glycans interact with bacterial cells, resulting in less biofilm formation. These findings show promise for *in vivo* inhibition of biofilm formation associated with wound infection and sepsis.

I. Introduction

Mucins are heavily glycosylated, meaning that sugars and other carbohydrates are attached to certain amino acid residues within the mucin peptide backbone, resulting in 80% glycan content in the dry mass of mucins. Mucins are coated onto the inside surface of the animal organs, e.g. stomach and uterus, and show some protection from bacterial infection. It was shown that mucins derived from porcine gastric fluid can inhibit adhesion of microbial and mammalian cells to surfaces (Crouzier et al., 2013). In addition, mucins can also potentially disperse biofilms of *Pseudomonas aeruginosa*, a pathogenic bacterial species often found in infected skin wounds (Co et al., 2018). The mechanism for this biochemical activity is still not completely clear. Recent work has shown that much of the control that mucins exert over their microbial populations is mediated by the glycan (oligosaccharide) moieties that the mucins display. These glycans interact with external receptors on bacteria to modify an array of different biochemical pathways, changing the bacterial biochemistry and thereby its behavior. Thus, the active mucins should have the proper conformation and contents of glycans to initiate the interaction with bacteria. In this study, we characterized the mucins from different source and from different preparation procedures and to assess the effects of these mucins on Pseudomonas aeruginosa (PA01) growth and cell adhesion for biofilm formation. Our main objective is to study the cell effects imposed by different sources of mucins and understand the underlying mechanism.

Manuscript approved May 2, 2024.

II. Materials and methods

Protein profile measurements. Natural mucins in raw mucus extracted from pig stomach (provided by code 6100) and commercial mucins purchased from MilliporeSigma, type II and type III (fractions purified from type II) were separated on native agarose gels and 4-12% gradient denatured polyacrylamide gels (SDS-PAGE), followed by staining gels with coomassie blue dye (Thermo Scientific). Additionally, denaturing gels were stained with Periodic Acid-Schiff (PAS) method to identify glycosylated protein according to the manufacturer's protocol (Thermo Scientific). In brief, gels were fixed in acetic acid, oxidized and stained with glycosylation stain. The glycosylated protein appeared as magenta bands when reduced.

Mucin preparation. 10% mucin stocks were prepared by dissolving industrial purified mucin powder in water/ water-based culture media shaking at 250 rpm at room temperature overnight. Next day, mucin solutions were spun down at 10,000xg for 10 min and their supernatants were then filtered through 0.2 μm membrane. Mucin solutions were also autoclaved at 121 °C for 30 min at 15 lb pressure, followed by centrifugation to remove the insoluble precipitate. Each sterile mucin solution was then diluted for 10 times (1E-1) with corresponding culture media (M9GC or NB). Natural mucins were scraped from the lining of pig stomachs and resuspended in 2M NaCl solution, then separated via gel permeation chromatography using Sepharose CL-4B as a solid support and eluting with 2M NaCl. Fractions that tested positive for carbohydrates via PAS staining were collected, the material was desalted on a pre-packed Sephadex G-25 column, and 0.01% sodium azide was added as a preservative. The material was dried on a rotary evaporator via toluene-assisted azeotropic distillation and stored at -20°C.

Cell growth inhibition assay. Pseudomonas aeruginosa (PA01) purchased from ATCC was streaked out onto a LB agar plate incubated at 37 °C overnight. The next day, a single colony was inoculated into 2 mL of LB culture media shaking at 250 rpm at 37 °C overnight. The following day, PA01 cells were diluted (1:200) in M9GC medium (0.5% glucose and 0.5% Casa amino acid in minimal M9 medium) and mixed with mucins to reach final mucin concentrations at 0, 0.01, 0.05, 0.1, 0.15, 0.5 and 1%. 140 μL of mucin and PA01 were dispensed into each well including the control without mucin in a 96-well plate. The plate was placed in 30 °C shaker at 225 rpm for 18 hrs. Next day, A600 was obtained from each well using a Tecan Spark Multimode microplate reader (Baldwin Park, CA).

Cell adhesion inhibition assay. A standard microtiter plate biofilm assay developed by Merritt et al., (2005) was modified and used in assessing cell adhesion inhibition. In brief, a serial 2 fold dilution of mucins was mixed with the diluted stationary PA01 cells and subsequently grown in microtiter dishes for 48 hrs in nutrient broth (NB). Then the wells were washed with water to remove planktonic bacteria. Cells remaining adhered to the wells were subsequently stained with crystal violet (0.1%) to visualize the attachment pattern. This surface-associated dye was then solubilized by acetic acid for semi-quantitative assessment of the biofilm formed by measuring A590.

Glycan Cleavage. The reaction was performed on purified 10% mucin stock according to the following procedure (Huang et al., 2001). Glycans were analyzed using Matrix Assisted Laser Desorption Time of Flight Mass Spectrometry (MALDI/TOFMS).

MALDI/TOFMS. Was performed on a Bruker autoflex maX. A matrix solution of 20 mg/mL of 2,5-dihydroxy benzoic acid (2,5 DHB) in TA30 was used. The instrument was run in negative ion mode with a mass range of 400-5000. First, the matrix solution was deposited onto the metal plate and allowed to dry. After drying, 2 µL of glycan solution was deposited on top of the dried matrix. After deposition, the samples on the metal plate was allowed to dry in air for 10 min before analysis.

Synthesis of Azide-modified Carboxymethyl cellulose (CMC). Synthesis was performed according to the following procedure (Filpponen, et al., 2012). In brief, CMC (200 mg, DS = 0.7, M_w 25000 g•mol^{-1}) was dissolved in 200 mL of 10 mM NaOAc buffer (pH = 5). To this, EDC (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride, 920 mg, 5.04 mmol), 1 eq of NHS (N-hydroxysuccinimide), and 0.8 eq of 11-azido-3,6,9-trioxaundecan-1-amine in 20 mL of buffer were added and the reaction was allowed to stir at RT for 48 hrs. After the desired period of time, ethanolamine (4 mmol) was added and the mixture was dialyzed (MWCO = 30000 g•mol^{-1}) against distilled water for 72 hrs. The final product was recovered after removal of the solvent under reduced pressure as 178 mg of a slightly yellow solid.

Fourier Transform Infrared Spectroscopy. Structural characterization was investigated through attenuated total reflectance Fourier transform infrared (ATR-IR). Spectra were collected using a Thermo Scientific Nicolet iS50-FTIR spectrometer equipped with an iS50 ATR attachment and Ge crystal. Background and sample spectra consisted of 128 scans averaged together with 4 cm^{-1} resolution at a scanner velocity of 10 kHz.

III. Results and discussion

Assessment of glycosylated mucins. The protein staining indicated that mucins from multiple sources have different extent of glycosylation. Frozen raw mucus scraped from pig stomach's lining exhibited a wide range of high molecular weight (MW) proteins (smear) as shown in native gel (Fig. 1A) and denaturing

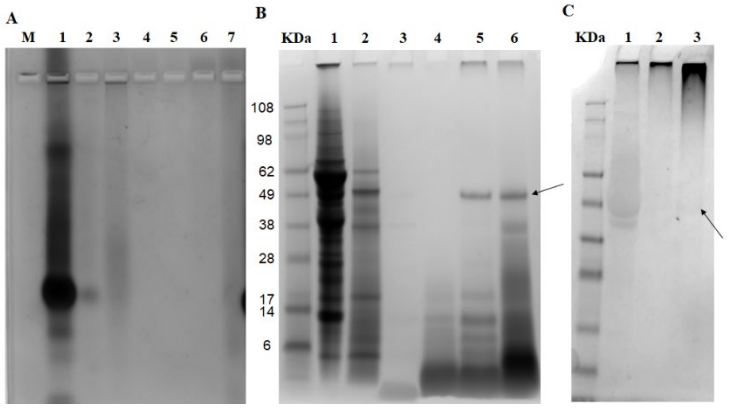
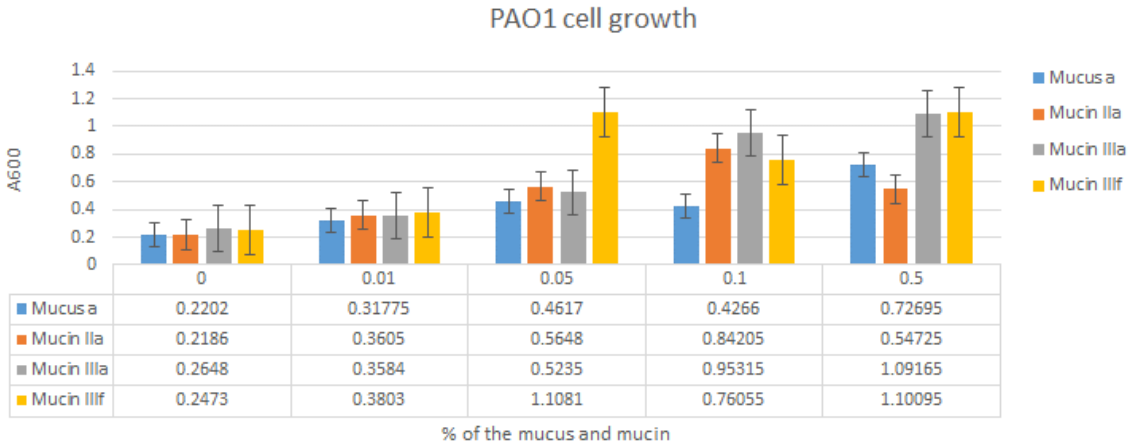


Fig. 1. Protein profiles for mucins separated on native gel electrophoresis (A) and denaturing PAGE (B). Mucins from different sources were separated in 1% agarose gel (A) and 4%-12% gradient SDS-PAGE gel, followed by coomassie blue staining (B) and PAS-Schiff staining (C). M in A represents 1 kb plus DNA size marker. KDa represents protein size markers in B and C. Lanes 1-7 in A represent frozen mucus from pig stomachs, empty, autoclaved mucus, mucin II filtered (IIf), autoclaved mucin II (Iia), filtered mucin III (IIIf), autoclaved mucin III (IIIa), respectively. Lanes 1-6 in B represent frozen mucus, autoclaved mucus, Iif, Iia, IIIf, and IIIa, respectively. Lanes 1-3 represent raw mucus, IIf and IIIf, respectively. Arrows indicate the mucin bands.

polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B). Autoclaved mucin followed by centrifugation removed a great portion of the high MW and other proteins (lanes 3 in Fig 1A and 2 in Fig. 1B). The high temperature apparently denatures most protein structures along with associated glycans. The protein profile in filtered mucins indicate that the filtration preserved more proteins than autoclave sterilization as indicated in lanes 4 and 6 in Fig. 1B. The estimated size of mucin without much glycosylation is around 50 kDa (arrow in Fig. 1B), which is present in both autoclaved mucus and mucin III in Fig. 1B. However, the band does not appear in mucin II, possibly due to the insoluble aggregation of mucins removed by centrifugation during the preparation. Industrial mucin II and III exhibited very low solubility in water and water-based buffers observed during the preparation. Raw mucus and filtered mucins separated on denatured SDS-PAGE and stained with PAS-Schiff staining showed the similar size of mucin band as in fig. 1B and high molecular weight proteins on the top of the gel (Fig. 1C).

Mucin effect on PA01 cell growth. Cell growth effect was measured at multiple concentrations of gastric mucus, soluble mucins (0-1% w/v) mixed with Pseudomonas aeruginosa (PA01), as indicated in Fig. 2. Our results indicated that no growth inhibition for mucins and gastric mucus



	0	0.01	0.05	0.1	0.5
Mucus a	0.2202	0.31775	0.4617	0.4266	0.72695
Mucin IIa	0.2186	0.3605	0.5648	0.84205	0.54725
Mucin IIIa	0.2648	0.3584	0.5235	0.95315	1.09165
Mucin IIIf	0.2473	0.3803	1.1081	0.76055	1.10095

Fig. 2. PA01 cell growth assay with mucins. PA01 cells were mixed with different concentrations of mucins and A590 was measured after 18 hrs. Mucus a represents autoclaved mucus. IIa and IIIa represent autoclaved mucin II and III and IIIf represent fil d i IIIf

was observed, but enhanced the bacterial growth, likely due to the use of mucins as nutrient by PA01 (Fig. 2). The enhancement is consistently across from autoclaved raw mucus to filtered mucins (Fig. 2). Since 80% of dry mucin mass is glycan, mainly sugars and polysaccharides, it is likely that the bacteria use mucins as a good source of carbons.

Mucin inhibition effect on PA01 cell adhesion. Previous studies demonstrated that the purified native gastric mucins dispersed established PA01 biofilms by confocal microscopy (Wheeler et al., 2019; Co et al., 2018). However, its effect on the cell adhesion is still unknown. Here, we used a standard 96 well format developed by Merrit et al (2005) to assess the mucin effects on cell adhesion for biofilm formation. PAO1 cells were mixed with the varied concentrations of filtered mucin II and III for 48 hrs and the adherent cells were then stained with crystal violet and

quantified by absorbance at 590 nm. Our results indicated that 0.5% (2 fold dilution) and 0.125% (4 fold dilution) mucin IIf (0.5% and 0.125%) inhibited cell adhesion as indicated in Fig. 3A, while only 0.5% mucin IIIf inhibited the adhesion of PA01 cells (Fig. 3B). The data showing that filtered mucin II and III can inhibit PA01 cell adhesion in higher concentrations suggest that these two type of mucins still contain some native mucins to interact with PA01 cells to prevent the cell adhesion onto the solid surface. Since mucin III was a fraction of mucin II, the content of native mucins should be less than mucin II. Thus higher concentrations of mucin III was needed for inhibiting cell adhesion. (Fig. 3B). We did not see the similar inhibition effect from autoclaved mucus and mucins (data not shown), suggesting there is no native mucins left after high heat denaturation by autoclave. Our results are consistent with the finding that only purified native mucins could disrupt the biofilms by interacting with the PA01 cells (Wheeler et al., 2019). Native mucins containing proper glycan conformation and contents have the ability to bind to PA01 cell surface protein to disperse biofilms. However, there is still a lack of information on what are the proper glycan conformation (N-link vs O-link) and contents required to exert the inhibition effects on cell adhesion.

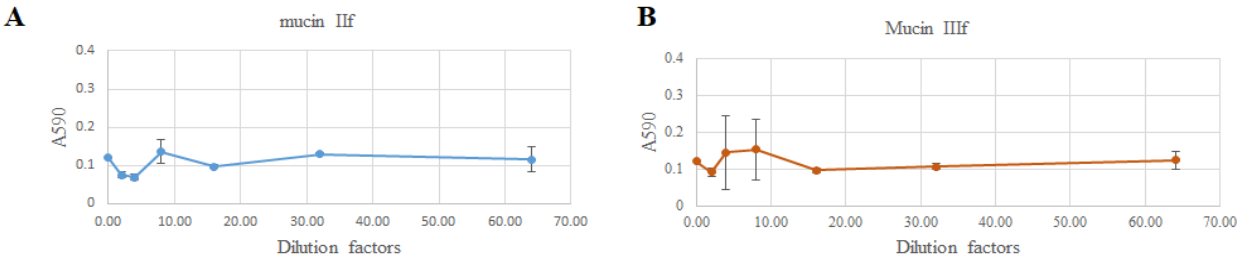


Fig. 3. PA01 biofilm adhesion inhibition assay. PA01 cells were mixed with a series of concentrations of mucin IIf (A) and mucin IIIf (B) to assess the inhibition of cell adhesions after 48 hrs. A590 for each well was measured.

Glycan Cleavage. Glycans (a type of oligosaccharide) are crucial to the function and survival of an organism, however, any specific role is difficult to describe due to significant structural variations in the polymer sequence due to differences in environmental conditions (Varki, 1993). Because of their presence surrounding a core protein structure, glycans

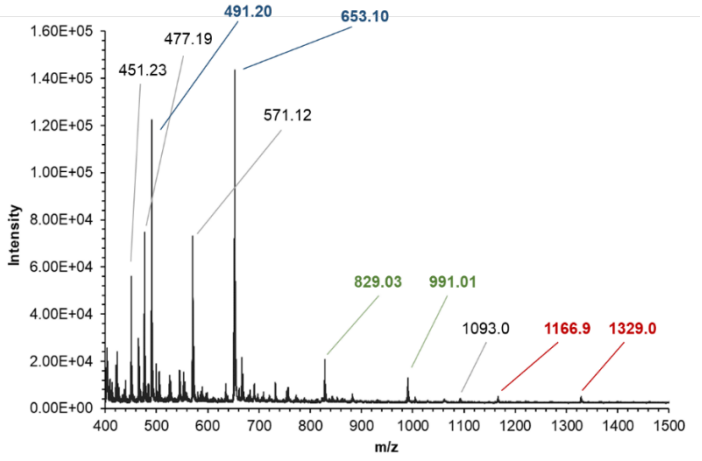


Fig. 4. Mass spectra from cleaved glycans. Negative ion mode with a mass range of 400 -5000 g. Mass units in corresponding colors represent the cleavage of a single sugar unit.

mainly function in the recognition events and modulation of biological processes. Due to the heavy degree of glycosylation of mucin, they are understood to play a major role in recognition processes that drive some of mucin's desirable properties. To characterize glycans, first they must be cleaved from the protein backbone (Wilkinson and Saldova, 2020). Of the many glycan cleavage mechanisms, ammonia-based β-elimination has become popularized due to its mild reaction conditions and scalability (Huang et al., 2001).

In this work, glycans were cleaved from purified mucin using ammonia-based β-

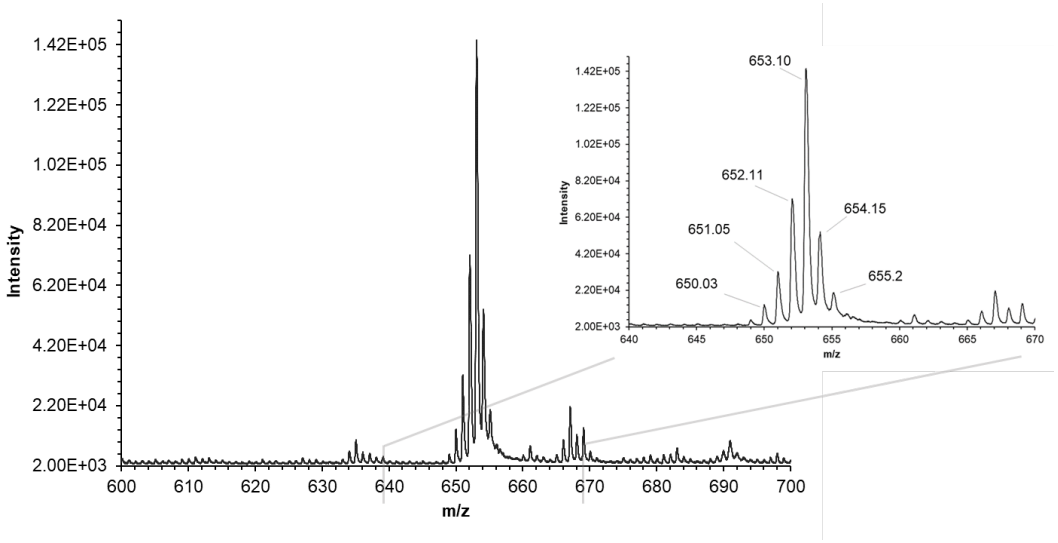


Fig. 5. An expanded mass spectra from cleaved glycans to show the splitting of a single peak.

elimination and were subsequently analyzed using MALDI TOF/MS (Figures 4 and 5). Cleavage of a single sugar unit (mannose or galactose) can be seen in the spectra as a change in m/z of 162 as highlighted in red, green, and blue. The complicated splitting pattern is illustrated in Figure 5. Although the exact structure of the glycans could not be determined, the mass spectra confirmed cleavage of intact glycans from the protein backbone for future work.

Glycan Attachment to a Synthetic Polymer Backbone. Despite the antibacterial and antifungal properties of gastric porcine mucus, it has rarely seen broad applications due to difficulties in physically employing the mucus in real-world conditions. Given that the glycan surface of glycoproteins drive recognition, and thus some biological functionality, we propose to synthesize a mucin mimic by attaching cleaved glycans to a polymer backbone. If the functionality of the glycans are retained, their functionality can be imposed onto a more-robust form factor (polymer) that can be employed in various applications. For this application, carboxymethyl cellulose (CMC) was chosen due to it easy of synthetic tunability, and its many uses in medicine. There are two proposed methods for the attachment of glycans cleaved from mucin to a polymer backbone (Figure 6). Our approach involves the use of copper-based "click" chemistry, which has been widely adapted for nucleic acids (Fantoni et a l., 2021), as a way to covalently attach the cleaved glycans to CMC in an aqueous environment. CMC was modified using EDC coupling to have a terminal azide (Figure 7). The presence of the amide, and azide moieties were

confirmed using ATRIR (Figure 8). After the coupling reaction a strong amide stretch can be seen at ca. 1600 and 2900 cm^{-1} and the azide stretch can be seen at ca. 2200 cm^{-1}.

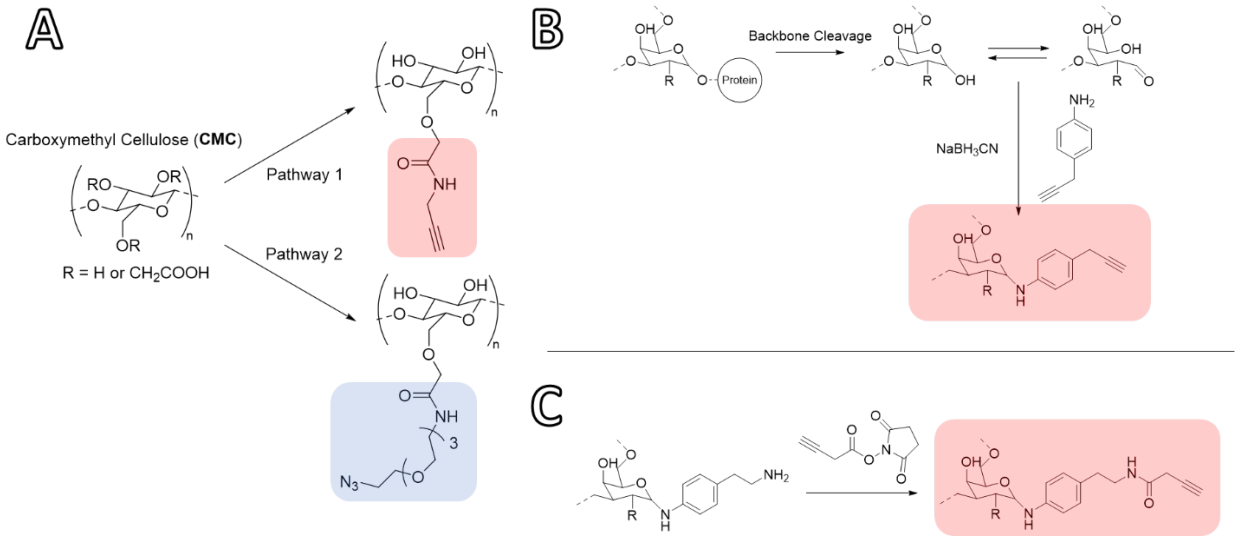


Fig. 6. A) Modification of CMC to include and alkyne (pathway 1) or azide (pathway 2) moiety to directly attach to cleaved glycans. A) Cleaved glycans can modified with an alkyne during the cleavage process. C) Using terminal amines present on cleaved glycans to

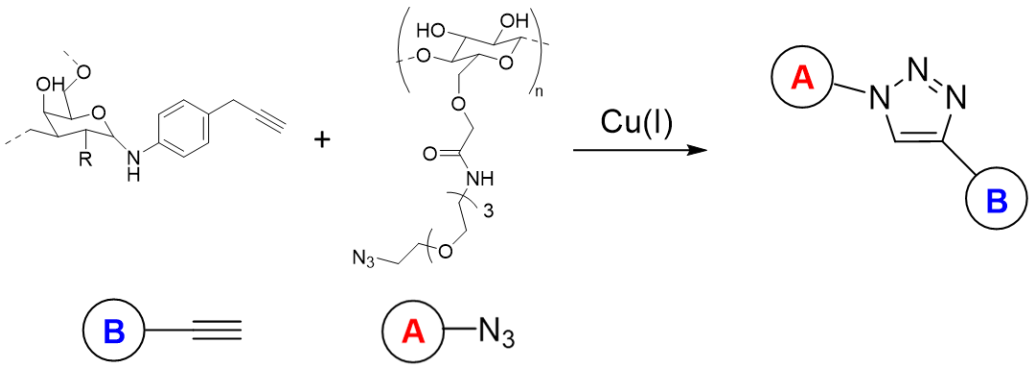


Fig. 7. Alkyne-modified glycans and azide modified CMC can be covalently attached using copper "click chemistry.

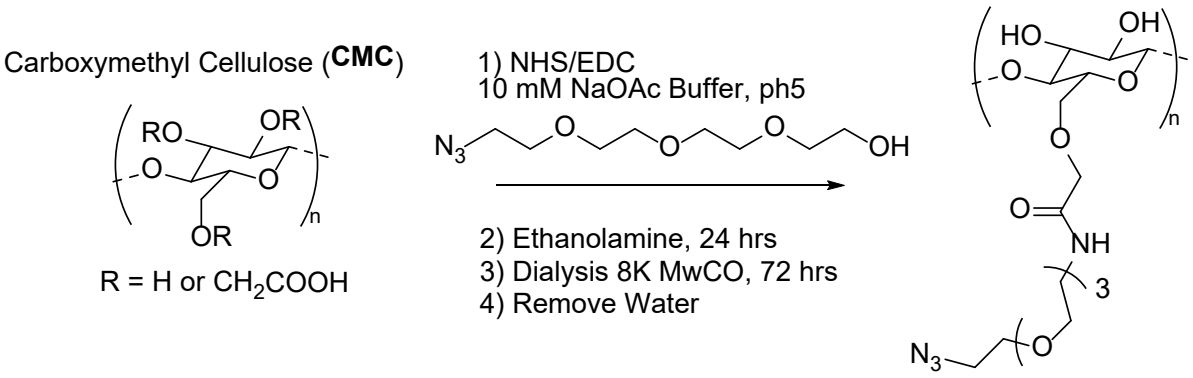


Fig. 8. Synthesis of azide-terminated CMC.

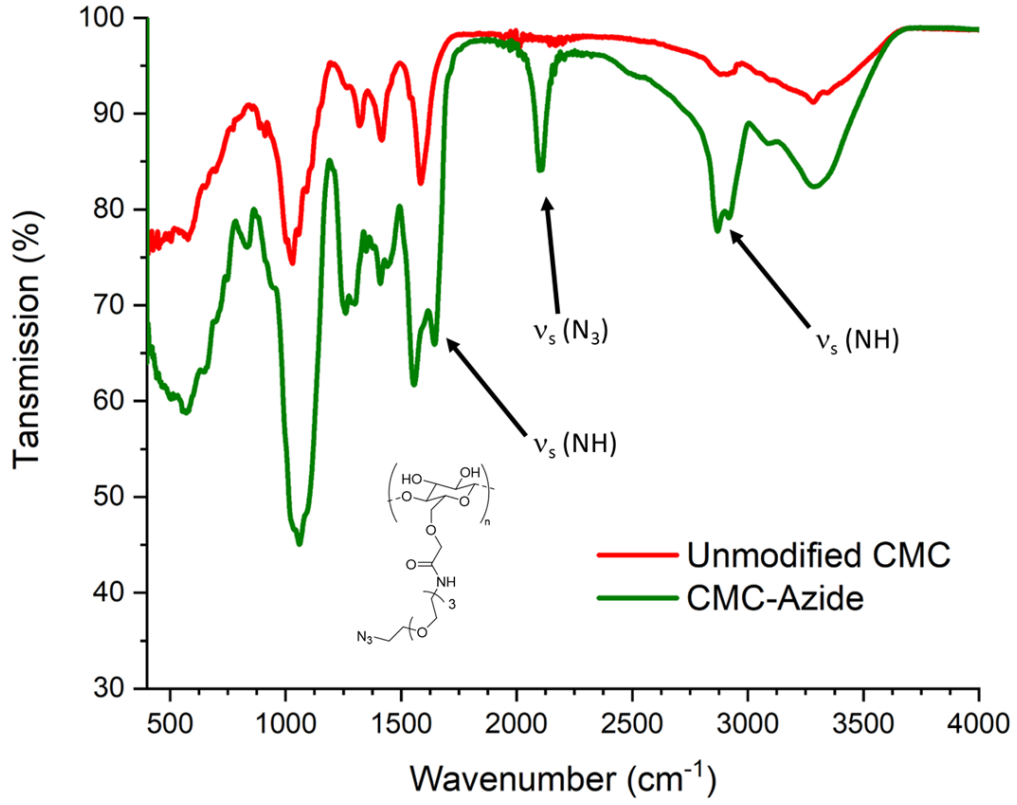


Fig. 9. ATRIR spectra of azide-terminated CMC.

IV. Conclusions

The glycoprotein profiles were different among the autoclaved (denatured condition) and filtered mucins. The autoclaved mucins do not have native mucin with proper glycan conformation and

tend to aggregate with no effects on cell adhesion. On the other hand, filtered industrial mucins contain native mucins that can inhibit the adhesion of PA01 onto cell culture plates. All mucins can be used as carbon source for bacterial proliferation and show enhancement of bacterial growth. We believe the information from this study and the assays established in this study will be very useful for future study on other biofilms including marine biofilms to prevent anti-fouling on ship hulls. Furthermore, we have demonstrated cleavage of intact glycans from glycoproteins in mucin and presented a methodology for the attachment of cleaved glycans onto a robust polymer framework (CMC).

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