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Article

The effects of varied N-acetylcysteine concentration and electronegativity on bovine mucus hydrolysis

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SUMMARY

N-Acetylcysteine, more commonly known as NAC, is a modified amino acid that is a very common way to treat the blockage of viscous pulmonary mucus in the bronchioles due to chronic obstructive pulmonary diseases (COPDs). NAC is a common treatment for excess mucus because it can effectively dissolve the disulfide bonds within the glycoprotein Mucin 5AC. However, this hydrolyzation ability is not infallible, as it is only capable of loosening the mucus to a certain extent. This study aimed to increase the efficacy of NAC by first determining a metric to measure its capacity by using rheometry. We hypothesized that if N-acetylcysteine is chemically altered, its effect on the viscosity of bovine mucus will be measurable by rheometry. Once we obtained the method to comprehend data, we applied and manipulated variables of concentration and electronegativity to NAC to observe their effects, trying to produce the best results possible within this scope. Through rheological analysis, we determined that the standard dose of NAC has a very similar function as the elevated doses. We successfully manipulated the electronics of various cysteine variants and determined that of the variants we investigated, boc-cysteine was the most effective at breaking up and thinning mucus. This data may provide avenues for future research into COPDs and their treatments.

INTRODUCTION

Chronic obstructive pulmonary diseases (COPDs) are the sixth leading cause of death in the United States and third leading cause of death worldwide (1). Although COPDs refer to a broader category of diseases, there are certain similarities shared between them that signify the distinct characteristics of a COPD. One of the most prominent symptoms is mucus hypersecretion, which is what this research aims to address. Viscous and gel-like mucus hypersecretion is a phenotype that results in the upregulation of mucus production (2). The shift in physical properties can be attributed to mucins, which are high molecular weight glycoproteins that give viscoelastic and gel-forming characteristics to mucus (2). The molecular structure of secreted mucins are largely similar; they contain cysteine rich domains that are linked together covalently using disulfide bonds that form mucin dimers, resulting in the viscosity level typically found in mucus. These dimers are then glycosylated and multimerized to form long chains that compose the properties of the mucus gel layer. The specific type of mucin found in the airways of the lungs is (Mucin 5AC), whose genes encode for the production of viscous mucus (2). A COPD such as asthma or cystic fibrosis will cause the expression of Mucin 5AC to be unregulated due to multiple defects in the signal transduction pathway and other signaling processes that occur due to the diverse stimuli that provoke mucus production (2).

N-Acetylcysteine (NAC) is an amino acid (cysteine) with an acetyl protecting group attached to it. NAC is a thiol compound due to the presence of the sulfhydryl group. During mucolysis in which mucus gets dissolved by NAC, the compound uses its sulfhydryl group to break the disulfide bonds between the cysteine-rich domains in the mucin (2). Therefore, mucin dimers are unable to form, and the mucus will lose its gel-like property and viscosity (2). The ability to cleave disulfide bonds is what makes NAC an effective therapeutic in COPD treatment and mucus hypersecretion management (2). Research done by Sadowska, *et al.* revealed that two crucial pathological processes within COPDs lead to upregulated mucin expression: oxidative stress and inflammation (3). Oxidative stress refers to the imbalance of unstable atoms called free radicals and having an adequate amount of antioxidants to detoxify them; this condition can damage nearby cells and cause aging. Inflammation occurs in the bronchiole pathways in lungs; the surface of the bronchiole and epithelial cells swell in size, restricting air flow and increased mucus production. NAC can actively reduce both of these processes and mucus hydrolysis simultaneously. Ultimately, it is the combination of these functions that provides relief to patients suffering from a COPD (3).

The metric that was used to establish and quantify data comes from a rheometer, which is a device capable of applying different types of forces to a liquid or gel substance to determine its viscosity. This experiment applies oscillatory shear force to a mucus sample, and the machine measures its response, quantifies it, and graphs the data using a software called Trios that is integrated with the rheometer. The measurements collected were categorized into three components: storage modulus (G'), loss modulus (G''), and complex viscosity. The G' represents the energy stored in a substance and the subsequent amount of mechanical force needed to distort it. The G" refers to the viscous and liquid-state behavior of a substance, and characterizes the energy dissipated as a substance when the viscosity changes. The complex viscosity measures a substance's resistance to flow through a mechanical force and is a ratio of stress to strain. Analyzing these components in conjunction led to a further understanding of how mucus responds to stressors and is a reliable indicator of how mucus moves in a high-pressure environment such as inflamed bronchioles (4). Previous studies have shown the connection between the various

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rheological hallmarks of mucus and its purpose in the body, most notably for lubricating the vocal folds to ensure proper oscillation and speech (5).

The goal of this research was to be able to investigate methods to cleave apart the mucus in a more efficient manner. We hypothesized that tuning the nucleophilicity of the sulfur in the NAC compound will strengthen its ability to hydrolyze mucus due to the mechanism of nucleophilic substitution that is required to cleave mucus disulfide bonds and thus decrease their viscosity. NAC derivatives adorned with various protecting and functional groups were utilized to test our hypothesis. Boc-cystine and fmoc-cysteine were chosen to undergo this process. These are two variants of NAC. Boc-cystine is a dimer (connected through a disulfide bond) of the cysteine amino acid; it also has a tert-butyloxycarbonyl on its N-terminus. Fmoc-cysteine is cysteine with a fluorenylmethoxycarbonyl protecting group on its N-terminus. We also reduced boc-cystine, producing the boc-cysteine monomer, to electronically modulate the molecule and observe its effect on mucus hydrolysis. Through these experiments, we determined whether the electronegativity of a compound can affect mucus. The data presented here may open up potential avenues to develop therapies for COPD.

RESULTS

The first objective considered when conducting this research was to determine how to synthesize the mucus that would serve as the control group. Using the methodology from Celli *et al.* led to the successful creation of mucus in vitro (6). We mixed a bovine submaxillary mucin (BSM) in phosphate buffered saline (PBS) solution (75 mg/mL), which served as the control group. The mucus mixed in these proportions exhibited cohesive properties evidenced by the long strand of mucus formed when we placed a sterile loop into the mucus then raised it upward, suggesting the chains of mucin dimers had formed. Like human mucus, the chosen sample—while being fully dissolved--also had areas that were highly concentrated despite thorough mixing, which is

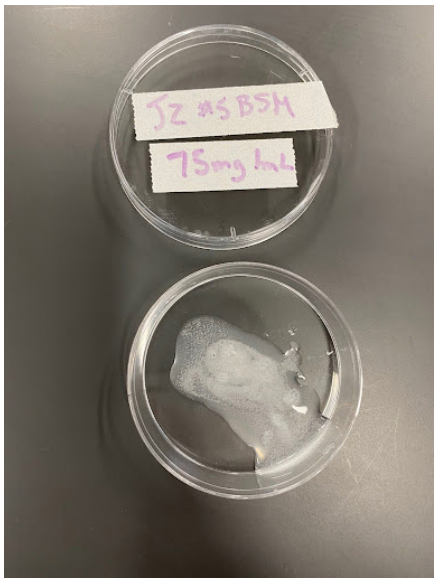

Figure 1: Rehydrated mucus sample. Dehydrated bovine mucus rehydrated in PBS (75 mg/mL).

visual support of the disulfide bonds between the cysteine rich domain.

We collected data for our various experiments in terms of the graphical representations of G', G", and complex viscosity via our properly equipped rheometer (**Figures 2-5**). The first experiment that we carried out on the rheometer, of just mucus alone, we used as a positive control of sorts for the general arrangement of G', G", and complex viscosity lines on a plot (**Figure 2**). The numerical measurements display that as the frequency increases from a scale of around one rad/s to 100 rad/s, the internal pressure of the complex moduli (in MPa) increases from 10^{-7} MPa to 10^{-4} MPa. Since the complex moduli ultimately represent resistance to deformation, it is plausible that a higher frequency results in an increase of MPa because they are resisting the force enacted upon them (4). On the other hand, the complex viscosity is measured using pascal-seconds (Pa·s) and decreases from around 0.6 Pa·s to 0.1 Pa·s (**Figure 2**).

Our first additive, N-acetylcysteine is commonly used in medicine to hydrolyze mucus. Initially, we recorded qualitative observations when 0.6 mg of NAC were added to the mucus sample. Almost immediately, the mucus visibly thinned out and became less viscous, adopting a more water-like consistency. When a sterile loop was placed into the sample and lifted, the film that arose was filmy and broke quickly, unlike the control mucus sample. After its preparation, we loaded the sample for rheological analysis. The complex moduli increased from about $10^{-7.5}$ MPa to around $10^{-4.5}$ MPa, which is similar to what the control mucus sample yielded (**Figure 3A**). Considering the range from multiple trials, we concluded that there was no change on the complex moduli, which was expected to occur. Even though the structure of the complex moduli may have been different due to NAC, energy of the mucin persisted, perhaps due to many of the mucin disulfide bonds remaining intact. The complex viscosity decreased from about 0.4 to 0.1 Pa·s over the course of the rheological experiment with the NAC additive (**Figure 3A**).

From there, we increased the concentration with one group receiving 1.5 mg of NAC. In the graph (**Figure 3B**) the complex moduli increased a similar amount from 10^{-7} MPa to about 10^{-4} MPa, indicating a resistance to deformation at higher angular frequencies. Similar to the standard dose NAC trial, the complex viscosity decreased from about 0.6 to 0.1 Pa·s demonstrating that the increased concentration of NAC has only negligible effects on the hydrolysis of mucus; the presence of the standard dose was enough to dissolve the viscosity of the mucus sample.

In order to vary the electronegativity of the compound, we wanted to acquire cysteine with a more electronegative protecting group, tert-butyloxycarbonyl (boc), which is a compound known as boc-cysteine. Since the sulfhydryl group that cleaves the disulfide bond between the cystine-rich domains in mucus originates from cysteine, boc-cystine and related compounds should be able to carry out its hydrolysis on mucus as effectively, if not more than NAC. We performed two reactions with boc-cystine to determine whether electronegativity was an essential factor in mucus dissolution: a reduction and a deprotection. We reduced boc-cystine in order to give the compound increased electron density around its potential sulfur nucleophile and observe its effect on mucus. Additionally, the boc-cystine underwent boc-deprotection to produce a more sterically unincumbered

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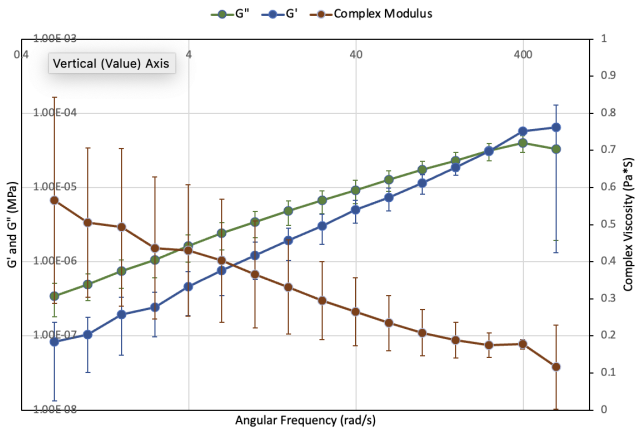

Figure 2: Averaged untreated-mucus rheological graph. Graph shows general outline of what can be expected from mucus-containing rheological samples: positive slopes for G' and G" and negative slope for complex viscosity (2 replicates completed).

cystine product.

From analyzing the rheological graphs of all trials utilizing electronically- and sterically-manipulated boc-cystine (**Figure 4**), it seems that boc-cysteine managed to dissolve mucus samples the most compared to the other compounds. This is supported by the data from the complex moduli and viscosity. To start, the G' of the mucus treated with cystine starts initially at roughly $10^{-4.6}$ MPa and finishes at 101 MPa as oscillations increase (**Figure 4A**). When looking at the data for the mucus treated with boc-cysteine, the G' begins at approximately $10^{-6.7}$ MPa and ends at about 100.6 MPa (**Figure 4B**). Since the G' represents the amount of energy in a substance and the force needed to distort it, boc-cysteine starting and ending with a lower MPa value signifies that the substance is holding less energy and therefore less applied force is needed to change its shape. As the G' also correlates directly to the structural integrity of a substance, having a lower MPa value is sufficient evidence that boc-cysteine is more effective than NAC at effectively thinning the mucus. The same conclusion can be drawn by performing a comparative analysis of the two graphs' complex viscosities, characterized by the resistance shown by a substance when moving through a mechanical force. The cystine-exposed mucus sample starts at around 50 Pa·s and ends around 0.3 Pa·s while the boc-cysteine is initially at 2 Pa·s and finishes also at 0.2 Pa·s (**Figure 4A** and **Figure 4B**). Even though they ended around the same measurement, the complex viscosity of the reduced sample (boc-cysteine) is much lower, showing lesser resistance whilst traveling through a force.

Along with boc-cystine, we carried out a deprotection with fmoc-cysteine and the corresponding rheological analysis of the cysteine product's effect on mucus (**Figure 5**). As seen, the G' and complex viscosity of the mucus treated with deprotected fmoc-cysteine conclude at 100.9 MPa and 0.6 Pa·s compared to the boc-cysteine's 100.6 MPa and 0.2 Pa·s (**Figure 4B**), respectively. The lower numerical value of the mucus's measurements treated with boc-cysteine once again supports the claim that boc-cysteine was the electronically modified amine most efficient in mucus dissolution.

In analysis that goes hand-in-hand with the visual analysis of the rheological graphs, we calculated the *t*-scores for G' and G'' average trendline slopes in the 0.6 mg of NAC experiments and all other additives (**Table 1**). A *t*-score is the number of standard deviations a number is from an average. In other words, it is a measure of how different two quantities are. The larger a *t*-score is, the more different measurements are considered to be. For ease of analysis, we averaged the G' and G'' *t*-scores for each comparison so that we had one (albeit arbitrary) measure of difference between the mucus additives and the standard 0.6 mg NAC additive. It is important to note that the overall simplified difference between the slopes produced *p*-values that were not statistically significant ($p > 0.05$), thus this treatment of the data was only used as a portion of our analysis and would be strengthened if time and resources allowed for more trials to be conducted. We found that the biggest calculated composite *t*-score was that which compared the 0.6 mg NAC additive to the boc-cysteine additive at $t = 1.4149$ (**Figure 4b**). The other composite *t* scores comparing our standard 0.6 mg dose of NAC and our other additives ranged from the most similar (1.5 mg of NAC) at $t = 0.8946$ (**Figure 3a**), to $t = 0.9708$ (cysteine, **Figure 5**), to $t = 1.3556$ (cysteine, **Figure 4a**).

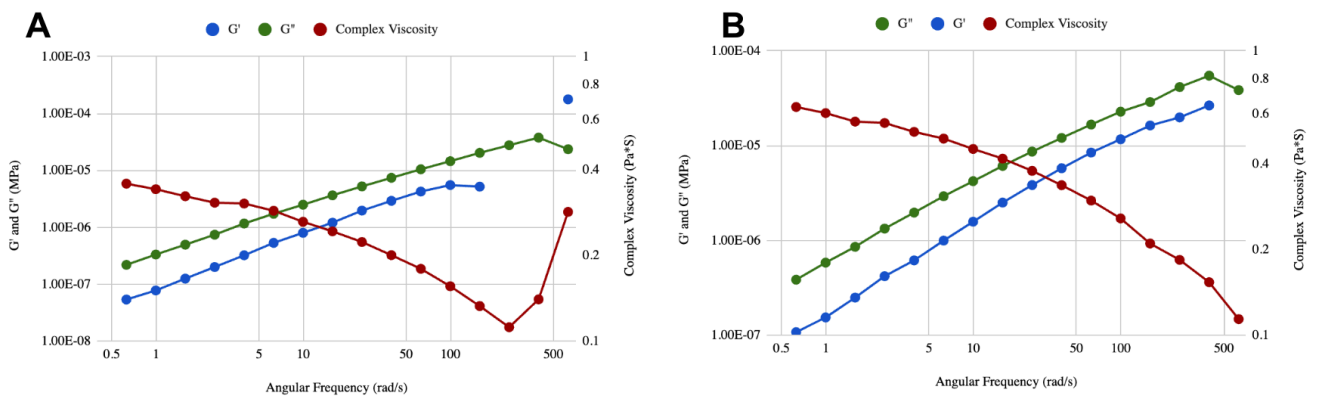

Figure 3: Rheological graphs of mucus with standard 0.6 mg NAC dose applied (A) and increased 1.5 mg NAC dose applied (B). The *t*-score comparing 0.6 mg NAC to 1.5 mg NAC is 0.8946. (A) shows an artifact in the complex viscosity and G' graphs that indicates a gap in the mucus coating on the rheometer plates. This artificial jump was not included in the analysis.

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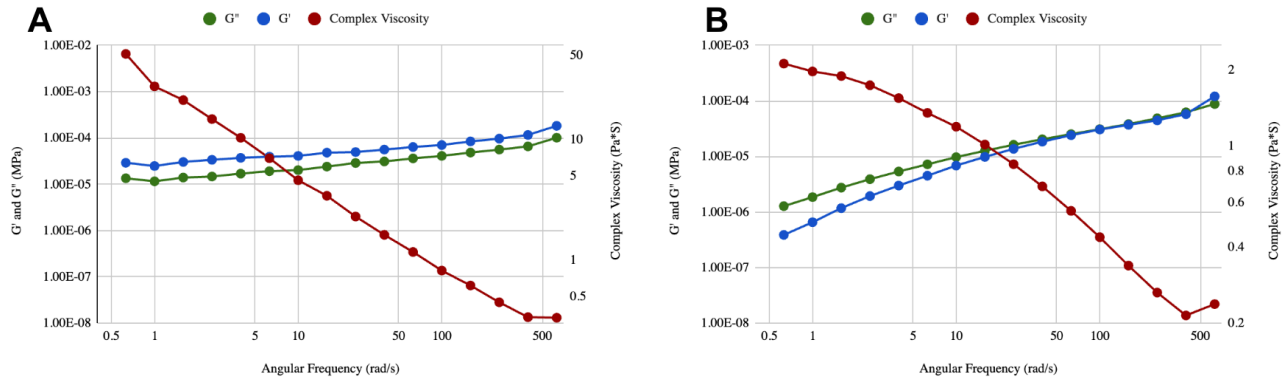

Figure 4: Rheological graphs of mucus with cystine (A) and boc-cysteine (B). The *t*-score comparing 0.6 mg NAC to cystine is 1.3556. The *t*-score comparing 0.6 mg NAC to boc-cysteine is 1.4149.

DISCUSSION

The goal of this study was to discern the difference between NAC variants and their effect on the hydrolysis of mucus. NAC at two different dosages (0.6 mg and 1.5 mg) along with cystine, boc-cysteine, and cysteine were all added to rehydrated bovine mucus and the rheological effects were observed and measured. Both with and without additives, we observed that the complex modulus increases because the structural components of the mucus are resisting deformation, which leads to more pressure as a whole on the substance (4). The complex viscosity decreases because an increase of oscillation frequency does not allow mucus to flow through resistance, which is why the Pa·s is decreased (4).

We observed that the addition of NAC showed a marked difference in the viscosity of the mucus compared to the mucus alone as we observed a lower complex viscosity compared to the mucus alone. When the internal disulfide bonds of mucus are broken, it takes on a more liquid form and loses parts of its three-dimensional structure allowing it to flow through highly constricted spaces easier, quite like the bronchioles of an asthma sufferer. We also observed that the addition of boc-cysteine to mucus visually thinned the mucus solution. Boc-cystine was reduced in order to increase the electron density around the sulfhydryl functional group. When we collected the rheological data for the mucus with boc-cysteine added, we noticed that the G" was greater than the G' from the start of the graph (at 0.1 Hz) until they become coincident at the end (at 100 Hz). When G'' is greater than G', the substance exhibits properties of a viscoelastic liquid and its liquid behaviors dominate. The crossing of the lines represents the gel-point, the transition from a liquid-like to a solid-like state or vice versa. In this experiment, it is desirable to produce results with a G' greater than the G'' (8). Analysis of the boc-cysteine data shows that the G" is indeed greater for the majority until the gel point at the end. We also saw that *t*-scores, with necessary caveats, support the assertion that the mucus treated with boc-cysteine was the most different from the mucus treated with the standard 0.6 mg of NAC. These results suggest that the mucus treated with the boc-cysteine is constantly in a liquid or gel-like state with no solid-like behaviors, which will make it easier for the treated mucus to be expelled from bronchioles and expunged from the body.

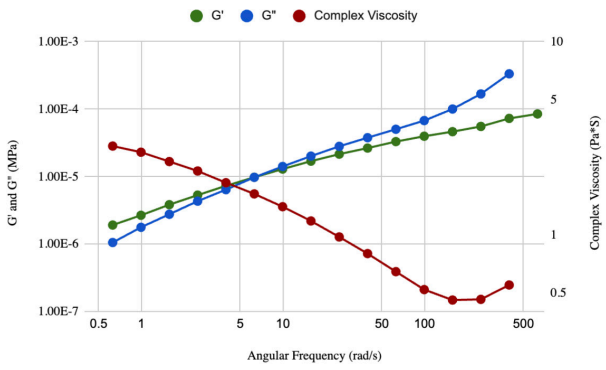

Figure 5: Rheological graphs of mucus with cysteine. The *t*-score comparing 0.6 mg NAC to cysteine is 0.9708.

In our view, the data suggests that changing the electronics and sterics of an NAC additive has effects on its ability to hydrolyze mucus and, of the variants we tested, boc-cysteine had the most marked positive effect – that is it thinned our simulated mucus based on our rheological and qualitative analyses. Our experimental design would be strengthened by additional trials and rheological experiments, something that can be elusive given the time and resource constraints of doing research science in a high school setting. This would allow us to more conclusively lean on statistical data to better distinguish the NAC-variants and their effects from one another. This said, having information on the potential efficacy of NAC variants in dissolving mucus has the potential to provide useful information about how chronic obstructive pulmonary disorders are treated in the future.

MATERIALS AND METHODS

Mucus Solvation

To begin experimentation, mucus was artificially synthesized by combining a high molecular weight glycoprotein with a solvent. In this case, BSM (Thermo Fisher Scientific) is the glycoprotein of choice, while PBS was determined as the appropriate solvent. Four samples of mucus ranging from 25 mg, 50 mg, 75 mg, and 100 mg of BSM to 1 mL of PBS were prepared. The 25 mg/mL sample was too viscous and would not hold its form when probed with a sterile loop, the 50 mg/mL samples could create a mucus film but could

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File Name	Storage Modulus Trendline Slope	Loss Modulus Trendline Slope	Complex Viscosity slope Trendline Slope
Mucus Trial 5 NAC added excel	1.67E-07	5.41E-08	-1.58E-04
Mucus Trial 6 w NAC (0.6 mg)	-3.76E-08	1.03E-07	-9.51E-04
Mucus Trial 8 w NAC (0.6 mg)	2.54E-07	9.75E-08	-2.25E-04
Mucus Trial 11 w NAC (1.5 mg)	2.77E-07	1.02E-07	-5.53E-03
Mucus Trial 13 w NAC (1.5 mg)	-4.60E-08	8.27E-08	-7.54E-04
Mucus Trial 15 (deprotected boc-cys)	2.27E-07	1.32E-07	-3.05E-02
Mucus Trial 16 (deprotected boc-cys)	2.57E-07	1.14E-07	-2.65E-03
Mucus Trial 17 (reduced boc-cys)	1.72E-07	1.36E-07	-2.55E-03
Muscus Trial 18 (reduced boc-cys)	1.57E-07	1.07E-08	-6.36E-04
Mucus Trial 19 (deprotected fmoc-cys)	4.88E-07	1.36E-07	-3.25E-03
Mucus Trial 20 (deprotected fmoc-cys)	9.44E-05	2.64E-05	0.0922

Table 1: Slopes of all rheometry trials in terms of complex moduli and complex viscosity. Data points were exported via TRIOS software and organized in Google Sheets. Graphs were created from the data and a line of best fit was generated. Slopes were recorded in the table above.

not sustain it, and the 100 mg/mL exhibited the exact same qualities as the 75 mg/mL, so it was disregarded to maximize the use of resources available. Literature procedure detailed the combination of BSM and PBS via vortex mixer (6). The specified amounts of glycoprotein were combined with 1 mL of PBS and vortexed until there were no visible clumps (6).

Rheometer Usage

Rheology was used as a metric to quantify its viscosity and other properties. Before rheometer use, the device was serviced by Waters, LLC. Activation of the machine should be accompanied by an air pressurizer, a system to dispense cooling liquid, and a device with the TRIOS software installed. For proper usage, regard the following: switch on the air pressurizer and wait for equilibration to occur, turn the valve so that the air can flow through, and remove the spindle lid before finally switching on the rheometer. Air pressure should remain above 30 psi for the entire duration of usage. The geometry gap was also set to 100 nm. Conduct standard rheological calibrations to ensure accuracy and precision when gathering data. Enter the Set Point temperature as 30 ˚C and use the oscillation frequency setting to conduct surveys from 0.1 Hz to 100 Hz (standard conditions for all rheometer trials conducted) (6).

N-Acetylcysteine Application

NAC compound was directly applied to the mucus by gently sprinkling it on. The given dose of NAC to patients is 600 mg/L of mucus. Since the sample size is only one mL, the ratio was sized down by dividing both sides by 1000 to yield 0.6 mg per 1 mL.

Electronic Manipulation of Cysteine and Application

Boc-cystine (SigmaAldrich) was reduced to gauge how a more naked sulfur nucleophile impacted mucus dissolution. DTT was used as the reagent for this process. A 1 M stock solution of DTT in ethanol was made and added to boc-cystine in a ratio of 1 mM boc-cystine to 10 mM DTT. The aqueous mixture was left to incubate for 30 minutes at room temperature and 0.6 mL (~0.6 mg of boc-cysteine) was extracted to be added to the mucus. The aliquot of boc-cysteine dissolved in ethanol was transferred, as a solution, to dry down in the mucus-reaction vessel. Mucus sample was then added (7).

The deprotection reactions for both amines required various reagents. To deprotect boc-cystine, trifluoroacetic acid (TFA) and DCM were utilized; 2.11 g of boc-cystine was reacted with 20 mL of TFA and 80 mL of DCM mixed with a stir bar overnight. The solution was transferred to a separatory funnel and shaken for the organic and aqueous layers to emerge. The organic layer was then removed, inverting the funnel multiple times throughout to prevent emulsion, and washed with sodium bicarbonate and dried over sodium sulfate (9). The filtered organic layer was then allowed to evaporate to reveal our solid cystine. This white solid (0.6 mg) was then added and mixed into our mucus solution.

To deprotect fmoc-cysteine, 2.5 mL of triethylamine, 5.86 g of fmoc-cysteine, and 1 mL of [Bmim][BF4] were combined and stirred for 10 minutes at room temperature. The organic layer was separated and the aqueous layer was washed with 2 mL of diethyl ether three times. The combined organic solution was dried over sodium sulfate and concentrated to produce solid cysteine (10). Cysteine (0.6 mg) was then added and mixed into our mucus solution.

Statistical Analysis

T-scores and *p*-values were calculated using an online calculator (11). Values were calculated comparing G' and G" slope values for all experiments against those of the experiment where 0.6 mg of NAC were added to mucus. G' and G'' *t*-scores were averaged for each comparison so that one composite measure of difference between the mucus additives and the standard 0.6 mg NAC additive was available for ease of comparison.

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