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Several Sterilization Strategies Maintain the Functionality of Mucin Glycoproteins

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Mucin glycoproteins, the macromolecular components of mucus, combine a broad range of biomedically important properties. Among those is the ability of mucin solutions to act as excellent lubricants. However, to be able to use purified, endogenous mucin glycoproteins as components of a biomedical product, the mucins need to be sterile; this, in turn, makes it necessary to subject the mucins to quite harsh physical treatments, such as heat exposure, autoclaving, UV-, or γ-irradiation, which might compromise the functionality of the glycoproteins. Here, it is shown that mucins are indeed able to withstand most of those treatments without suffering significant lubrication impairment or structural degradation. Among those treatments, which left the mucins unharmed, γ-irradiation is identified to be the most powerful one in terms of inactivating microbial contaminations. The obtained results demonstrate a remarkable sturdiness of mucins, which opens up broad possibilities for them to be further processed into materials, e.g., as parts of biomedical products.

1. Introduction

Mucin glycoproteins are the macromolecular key component of mammalian mucus, the viscoelastic hydrogel that lines all mucosal surfaces, such as the respiratory tract and the gastrointestinal tract.[1] They have high molecular weights in the range of several MDa and comprise a long polypeptide backbone and a highly O-glycosylated central section.[2] The densely glycosylated protein core carries a large number of sialic acid residues and sulfate groups, which renders mucins highly hydrophilic and hygroscopic.[3] The mucin termini consist of sparsely glycosylated, cysteine-rich domains, are partially folded and contain hydrophobic residues.[4] The ensuing amphiphilic nature of the mucin molecule not only enables adsorption onto both, hydrophobic and hydrophilic surfaces,[5] but also results in an excellent lubricating performance.[6] Furthermore, due to the various different interaction sites provided by the complex microarchitecture of the mucin molecule, mucins constitute a unique barrier against bacteria and viruses, as they allow to trap or repel such pathogens.[7] In addition, when applied as passive or covalent surface coatings, mucins can prevent wear formation on corneal tissues and form stable antibiofouling layers on a broad range of medical polymer materials.[8] Owing to these highly interesting, beneficial properties, mucins have received increasing attention as an outstandingly versatile biomaterial for biomedical applications.[9]

Due to their high molecular weight (human gastric mucin MUC5AC comprises more than 5600 amino acids)[10] and the high complexity of the brush-like glycosylation pattern (which combines *O*-, *N*-, and *C*-glycosylation)[11] it is to date not possible to produce mucins synthetically, yet. Thus, the only possibility to obtain mucin molecules in significant amounts is to purify them from animal sources. One commonly used mucin variant is gastric mucin MUC5AC that can be purified from porcine gastric mucus. However, the harvested stomach mucus contains different microorganisms that can to a certain extent still be present in the purified mucin. This is of course in conflict with the requirements medical products must fulfill. Consequently, before using purified mucin molecules in real medical applications, they need to undergo a sterilization procedure. When selecting a suitable sterilization method, one needs to consider that the mucin glycoproteins need to maintain their functionality after sterilization. However, each of the commonly used sterilization methods, i.e., UV- and γ-irradiation, thermal treatment, or autoclaving, may have a negative impact on the physico-chemical and structural properties of mucins. Thermal stress, for instance, often results in the perturbation of intramolecular forces stabilizing a protein conformation, such as hydrogen bonds or van der Waals forces; very often, this results in the denaturation of proteins, i.e., changes in the secondary and tertiary structures of proteins. Similarly, also UV- and γ-irradiation can lead to protein damage, e.g., by inducing oxidation or the breakage of covalent bonds in the peptide backbone and in aromatic amino acid side chains.[12] A typical sterilization procedure for medical devices, such as syringes or catheters, is the treatment with ethylene oxide. However, previous studies[13] have indicated that even small amounts of this molecule readily modify methionine and cysteine residues in proteins, which in turn can affect their

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stability and agglomeration propensity. Hence, for this study, we decided to omit the investigation of this sterilization technique.

Here, we use rotational tribology to show that mucin macromolecules are capable of withstanding a certain degree of thermal or radiation treatments without suffering an impairment of their lubricity. Furthermore, by combining gel electrophoresis and a specific detection method for mucins based on antibodies, we demonstrate that those mucins, which maintained their lubricity after treatment, do not exhibit any detectable structural damages either. Among those treatments, which left the mucins unharmed, we identify γ-radiation to be the most efficient regarding inactivating bacterial or fungal contaminations of mucin samples.

2. Experimental Section

If not stated otherwise, all chemicals were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

2.1. Mucin Purification and Reconstitution

For the purification of porcine gastric mucin MUC5AC, a protocol previously outlined in Schömig et al.[14] was used. In brief, crude mucus was manually harvested from the tissue surface of pig stomachs (Schlacht- und Viehhof, München, Germany). The obtained mucus was diluted 1:5 in sodium phosphate buffer (10×10^{-3} M, pH 7.0) containing 170×10^{-3} M NaCl and 0.04% w/v sodium azide, and then it was stirred at 4 °C overnight. To remove cells and impurities, two centrifugation steps (30 min at 17590 × g and 45 min at 158306 × g) were performed at 4 °C each. To further separate the mucins from other mucus constituents, size exclusion chromatography was conducted using an ÄKTA purifier device (GE Healthcare, Chicago, IL) equipped with an XK50/100 column packed with Sepharose 6FF resin (GE Healthcare). The obtained mucin-containing eluate was dialyzed against ultrapure water and concentrated by cross-flow filtration using an ultrafiltration hollow fiber cartridge with a molecular weight cut-off of 100 kDa (Xampler Ultrafiltration Cartridge, GE Healthcare). After lyophilization, the concentrate was stored at –80 °C. To reconstitute the mucins, lyophilized MUC5AC was dissolved to the desired concentration in ultrapure water or in an adequate buffer at 4 °C for 2 h while shaking at 750 rpm.

2.2. Treatments

All treatments were conducted with both, lyophilized and solubilized mucin samples. The mucin concentration and buffer of the solubilized samples were chosen according to the respective requirements of the experiments conducted with them.

2.2.1. Thermal Treatment

For the thermal treatment, the samples were filled into small Eppendorf tubes (volume: 2 mL; Eppendorf GmbH, Hamburg, Germany). The closed tubes were then placed into an oven that was preheated to the desired temperature (60, 80, 100, or 120 °C) and left there for 1 h. Those temperatures were chosen to systematically study the resilience of mucin molecules against thermal stress up to 120 °C, which approximates the typical temperature level used during autoclaving. For most proteins, temperatures above ≈40 °C are already sufficient to induce denaturation. However, from previous experiments, it was suspected that the (rather unfolded) mucin might be more resistant to heat than the average protein.

2.2.2. Autoclaving

The samples were filled into small Eppendorf tubes (Eppendorf), and the lids of the tubes were only slightly closed so that a small gap remained (to allow the generated steam to exit the tube thus avoiding overpressure). The tubes were then placed into an autoclave (Varioklav, HP Medizintechnik GmbH, Oberschleißheim, Germany), and a liquid standard sterilizing process (121 °C, 20 min) was started. After finishing the process, the tubes were removed from the autoclave and closed immediately. To compensate for lost water due to vapor generation (thus restoring the initial mucin concentration), sterile filtrated water was added to the solubilized samples. Lyophilized samples were recovered from the autoclave and left as they were until further usage, i.e., they were reconstituted into mucin solutions when needed.

2.2.3. UV Irradiation

For sterilization via UV irradiation, the samples—either lyophilized or solubilized MUC5AC—were filled into Eppendorf tubes (Eppendorf). The closed tubes were then placed into a sterilization chamber (BLX-254, Vilber Lourmat GmbH, Eberhardzell, Germany) and exposed to UV irradiation (254 nm, 5 × 8 W) for different time periods (i.e., 10, 30, or 60 min) as indicated in the respective figure.

2.2.4. Treatment with γ-Rays

For gamma sterilization, the lyophilized and solubilized mucin samples were filled into Eppendorf tubes (Eppendorf AG). The tubes were then closed and placed into a paper cryobox. The whole box was then treated with γ-rays using a dose of 25–50 kGy by applying a commercial standard process available at the company steripac GmbH (Calw, Germany).

2.3. Tribological Experiments

Friction measurements were performed on a commercial shear rheometer (MCR 302, Anton Paar, Graz, Austria) equipped with a rotational tribology setup (T-PTD 200, Anton Paar) as described previously in Boettcher et al.[15] In brief, steel spheres (Ø 12.7 mm, Kugel Pompel, Wien, Austria) and Polydimethylsiloxane (PDMS) pins (Ø 5.5 mm) were used as material combination in a ball-on-3-pins geometry. The PDMS cylinders were fabricated by mixing PDMS prepolymer and cross-linker

in a 10:1 ratio (PDMS, Sylgard 184, DowCorning, Wiesbaden, Germany), degassing the mixture in a vacuum for 1 h, filling it into custom made molds and conducting a final curing step at 80 °C for 4 h. Before each measurement, three pins were cleaned with 80% v/v ethanol and ultrapure water and inserted into the sample holder. For each tribological test, 600 µL of lubricant solution (0.1% w/v mucin in 20×10^{-3} M HEPES buffer (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid buffer), pH 7.3) were applied; this ensured that the PDMS cylinders were fully covered with lubricant. During the measurements, the temperature control was set to $T = 20$ °C. A normal force of $F_N = 6$ N was used, which resulted in a contact area of ≈8.1 mm^2 and thus (according to Hertzian contact theory as in Käsdorf et al.)[16] a contact pressure of ≈0.35 MPa. Friction coefficients were documented for sliding velocities from 1000 to 0.01 mm s^{-1} (logarithmic speed ramps, 10 measuring points per decade) using an acquisition time of 10 s per data point.

2.4. ELISA

An indirect enzyme linked immunosorbent assay (ELISA) was conducted to evaluate the integrity of the hydrophobic C-termini of the mucin molecules. Therefore, after gently rinsing the wells of a 96-well plate (Corning CellBIND surface, Corning, New York) with DPBS-Tween (1 mg mL^{-1} Tween 20 in Dulbecco's phosphate buffered saline (DPBS), pH = 7.3), 200 µL of a 0.1% w/v mucin solution in DPBS were added to each well. Incubation was allowed at room temperature (RT) for 2 h. Afterward, the mucin solution was removed, and the wells were washed with DPBS-Tween before incubating them with blocking buffer (5% w/v milk powder dissolved in DPBS-Tween) at 4 °C overnight. After overnight incubation, all wells were gently rinsed with DPBS-Tween before adding 300 µL of blocking buffer containing a specific antibody for MUC5AC detection (1:400 v/v, ABIN966608, antibodies-online GmbH, Aachen, Germany). The well plate was incubated on a shaker (75 rpm) at RT for 1 h. After incubation, the wells were again rinsed with DPBS-Tween. A horse radish peroxidase (HRP) conjugated goat antimouse (murine) IgG antibody (ABIN237501, antibodies-online GmbH) was used for secondary antibody staining. Therefore, the antibody was diluted 1:5000 v/v in blocking buffer, and 200 µL of this solution were added to the wells. Antibody incubation was allowed to take place on a shaker (75 rpm) at RT for 2 h. After washing the wells in pure DPBS (from this step on, no Tween is used anymore since Tween tends to interfere with the solutions used later), 150 µL of QuantaRed Working Solution were added to each well. This solution consists of 50 parts QuantaRed Enhancer Solution, 50 parts QuantaRed Stable Peroxide, and one part of QuantaRed ADHP Concentrate (QuantaRed Enhanced Chemifluorescent HRP Substrate Kit 15159, Thermo Fisher Scientific, Waltham, MA). Since the Working Solution is light sensitive, direct light contact was avoided. After incubation at RT for 30 min, the fluorescence signal created by the converted substrate was measured with a multilabel plate reader (Viktor3, PerkinElmer, Inc., MA). Fluorescence was quantified at a wavelength of 570 nm using a data acquisition time of 0.1 s.

The primary anti-MUC5AC antibody used in this study binds to a recognition site located in the mucin C-terminus. Of course, since this antibody does not target the N-terminus of mucins, unambiguous conclusions about the presence/integrity of this specific motif cannot be drawn. However, it is reasonable to assume that both termini are equally susceptible to damage by the sterilization treatments. Thus, for sterilized samples which return a reduced fluorescence signal, It was assumed that both, the C-terminus and the N-terminus, were cleaved or structurally modified. Vice versa, for samples, which return a fluorescence signal equally strong as untreated (and structurally intact) mucin, it was assumed that both termini are present and structurally unaltered.

2.5. SDS–PAGE and Coomassie Staining

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), 20 µL of each mucin solution (1% w/v mucin in ultrapure water) were first mixed with 2x sample buffer (0.4 M triethanolamine (TEA), 2% w/v SDS, 20% v/v glycerol, 2×10^{-3} M ethylenediaminetetraacetic acid (EDTA), 0.02% w/v bromophenol blue; pH 7.8) in a ratio of 1:1. After boiling for 5 min at 95 °C, 20 µL of each mixture were then loaded onto a precast polyacrylamide gel (4–20% TruPAGE Precast Protein Gels, Sigma-Aldrich) in separate lanes. Additionally, 4 µL of a protein marker solution (Marker Precision Plus Protein Kaleidoscope Standards, Bio-Rad Laboratories Inc.) were loaded onto the gel in a separate lane. The gel electrophoresis was run in TEA-tricine-SDS buffer (60×10^{-3} M TEA, 40×10^{-3} M tricine 0.1 % w/v SDS; pH 8.3) at 120 V for 1 h.

For protein detection, the gels were stained in a Coomassie staining solution (0.1% w/v Coomassie R250, 10% v/v glacial acetic acid, 40% v/v methanol) for 1 h while shaking at room temperature. Afterward, the gel was destained in 10% v/v acetic acid overnight and then imaged.

2.6. Contamination Tests

2.6.1. Bacterial and Yeast Cultivation and Mucin Inoculation

To test for bacterial contamination, mucin samples were inoculated with either *Escherichia coli* (*E. coli*) DH5-Alpha pro (kindly provided by Urartu Seker, Bilkent University/UNAM, Turkey) or *Saccharomyces cerevisiae* (*S. cerevisiae*) (Dr. Oetker, Bielefeld, Germany) before subjecting them to a heat treatment, UV-exposure or γ-irradiation. In brief, bacteria were cultivated by inoculating of liquid Luria/Miller LB-medium with the respective frozen bacterial/glycerol stock. Yeast was cultivated by inoculating liquid extract peptone dextrose with a small amount of yeast powder. After incubation at 37 °C (and 30 °C for yeast, respectively) with 90 rpm in a shaking incubator (Sartorius, Göttingen, Germany) for 16 h, the bacterial/yeast cells were counted, and a certain volume corresponding to 10^9 cells per inoculated mg mucin sample was separated. Two washing steps separated by a centrifugation step (4 °C, 10 min, 1950 x g) were performed with DPBS to remove the cultivation media from the cell solutions. Then, lyophilized mucin was dissolved in DPBS, and for each mg of mucin, 10^9 bacteria/yeast cells were added to the mucin solution. The inoculation of mucin samples with 10^9 cells mg^{-1} mucin was

chosen to allow to determine the level of sterility with a sufficient reliability and to be able to assess whether or not sterility as defined by the U.S. Food & Drug Administration (FDA)[17] (only 1 out of 10^6 microorganisms survives) is met. After gentle mixing, the samples were lyophilized and stored at −80 °C until they were subjected to a thermal/UV/γ-treatment as described above.

2.6.2. Test for Bacterial Contamination and Fungal Growth after Treatment

To test contaminated mucin samples for remaining, viable bacterial or fungal cells after thermal/UV/γ-treatment, 1.5% w/v agar plates were prepared. Here, the agar layer was enriched with either 2.5% w/v LB-medium supplemented with 1% v/v glycerol and 100×10^{-6} M manganese(II)sulfate (LBGM-medium) or with 2.5% w/v Becton Dickinson Sabouraud agar supplemented with chloramphenicol (BD-medium, pH 5.6). The media-enriched agar was autoclaved at 121 °C and ≈1 bar for 20 min, poured into petri dishes and allowed to solidify under a sterile hood. Until further usage, the agar plates were stored at 4 °C.

Both, treated and untreated mucin samples were dissolved in sterile DPBS at a concentration of 1 mg mL^{-1}. Then, 200 µL of each solution were plated onto LBGM- (*E. coli*) or BD-agar (*S. cerevisiae*) by homogeneously distributing them with a Drigalski spatula. After incubation at 37 °C for 24 h (in case of *E. coli*) or at 25 °C for 5 d (in case of *S. cerevisiae*), the generated colonies were counted.

2.7. Statistical Analysis

Normal distribution of each sample was tested with a Lilliefors test, and a two-sample *F*-test was employed to test for equal variances. For normally distributed samples, a two-sample *t*-test was applied when homogeneity of variances was met, whereas a Welch's *t*-test was performed for unequal variances. For samples that were not normally distributed, a Wilcoxon–Mann–Whitney-test was performed. All statistical analyses were performed using Matlab (version R2019a, MathWorks, Natick, MA) applying a *p*-value of 0.05.

3. Results and Discussion

In a first set of experiments, we assess the lubricity of mucin solutions and compare the behavior of treated mucins to those of untreated mucins. Our rational for starting with tribological experiments is as follows: previous experiments have demonstrated that the lubricating potential of mucin solutions sensitively depends on the molecular integrity of the mucin glycoprotein. When using a steel-on-PDMS material pairing, the loss of the hydrophobic termini of the macromolecule,[16] severe damage to its glycosylated central region[3b] and even minor modifications, such as the removal of a single type of anionic residue (such as sulfate or sialic acid groups)[6b] each leads to a significant and easily detectable loss of lubricity.

When gastric mucin MUC5AC is subjected to elevated temperatures, i.e., via incubation of either mucin solutions or lyophilized mucin powder at 60, 80, 100, or 120 °C for 1 h each, we find that the lubricity of 0.1% w/v solutions generated from those thermally treated mucins is not compromised in any case (**Figure 1**a,b). For all mucin samples we find excellent lubricity, and the friction curves obtained with thermally treated mucins are virtually identical to those obtained with untreated mucins. This suggests that this thermal treatment does not induce any major damage in the mucin structure. Although such a behavior is not typical for proteins (which often tend to denature when exposed to temperatures above 40 °C), the high glycosylation density of mucins and low content of folded peptide sequences may be responsible for the unusual high sturdiness of this glycoprotein toward heat.

In contrast, a significant loss of functionality is observed when mucins are thermally treated in an autoclave (Figure 1c). Here, after completion of a standard treatment cycle, the lyophilized mucin sample is not even soluble anymore. Such a loss of solubility is a hallmark for a protein denaturation event and renders those lyophilized mucins fully unusable for any further testing.

The treated mucin solution remains a homogeneous liquid and still provides a reasonable level of lubricity—at least at medium and high sliding speeds, i.e., in the mixed and hydrodynamic lubrication regime. At low sliding speeds, however, where boundary lubrication dominates, the lubricity of these autoclaved mucin solutions is clearly compromised, and the measured friction coefficient is about one order of magnitude higher than what we obtain for solutions reconstituted from untreated mucins.

An even more pronounced decrease in lubricity is observed for MUC5AC that was γ-irradiated in the solubilized form (Figure 1d). Here, the obtained friction curve is quite similar to that obtained with simple buffer (devoid of any mucins), which suggests that the molecular structure of mucins was severely compromised by the γ-rays. The finding is supported by the fact that the solution showed discoloration after γ-irradiation. This outcome, however, is in marked contrast to the results we obtain with γ-treated mucin powder. Here, the lubricity of the reconstituted mucin solution is very similar to the result obtained with untreated mucins. This suggests that γ-irradiation–although harmful for solubilized mucins–seems to leave the lyophilized mucin macromolecule intact.

A similar picture emerges for mucin samples that were subjected to UV-irradiation (Figure 1e,f). Mucins still provide excellent lubricity independent of the treatment time when they were treated in their lyophilized state (Figure 1e), whereas solubilized mucins (Figure 1f) appear to be more vulnerable to UV-treatment. Here, a 30 min treatment with UV light leads to compromised lubricity, whereas shorter treatment times do not.

From this first set of experiments, we conclude that autoclaving is not a suitable treatment procedure. Moreover, in their lyophilized state, mucins appear to resist thermal and radiation/UV-treatment more efficiently than when they are treated as solutions.

As the tribology experiments showed impaired functionality for some of the treated mucin samples, we next assess if any of the treatments induced structural damages to the mucin. First, we use an ELISA which probes the presence and accessibility of the mucin C-terminus.[18] At this point, it is important to recall

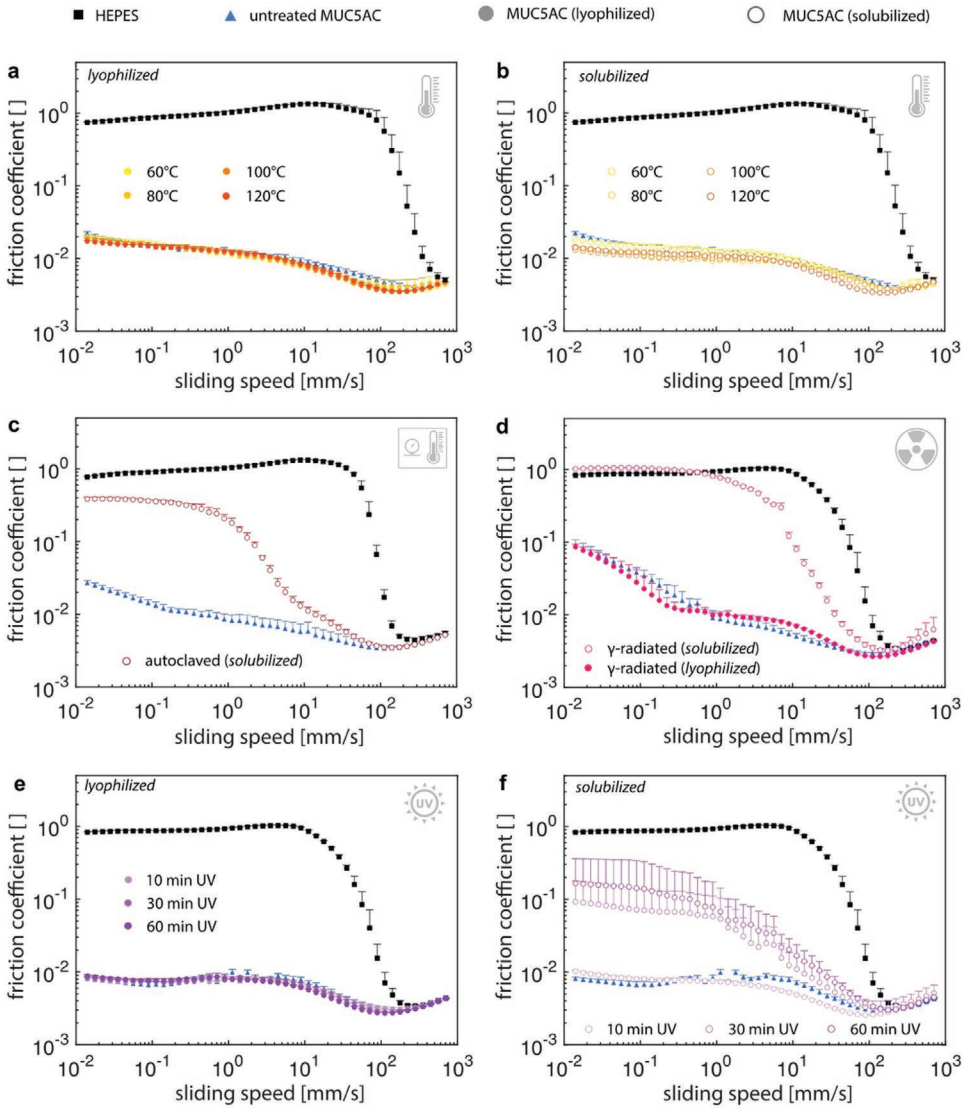

Figure 1. Lubricating behavior of differently treated mucin samples. The Stribeck curves shown were obtained for solutions of mucins that have been exposed to heating/autoclaving, UV- or γ-irradiation treatment, respectively. For all data, a steel-on-PDMS material pairing was used in a rotational tribology setup. For data shown in (a,d,e), the treatment procedure was conducted with lyophilized mucin powder. For data shown in (b,c,d,f), the treatment was conducted with a mucin solution. Error bars denote the standard error of the mean as obtained from $n = 3$ independent measurements per condition.

that most of the mucin structure comprises unfolded, heavily glycosylated regions. Thus, it is mostly the hydrophobic termini of the mucin glycoprotein that could be vulnerable to denaturation. Since those termini are critically involved in the surface adsorption of mucins (which is mandatory for mucin lubricity), their integrity is crucial for many mucin functions. Owing to the amphiphilic character of the mucin molecule, it can adsorb onto both, hydrophobic and hydrophilic surfaces. As the used well plate surfaces are hydrophilic, the mucin is expected to adsorb via the densely glycosylated central region. In this case, the C-termini of the mucins can be expected to be freely accessible for antibodies targeting this recognition site. Structural damages within those terminal regions of the mucin molecules will be visualized by decreased fluorescence intensities when compared to intact protein samples, since insufficient integrity or accessibility of the C-terminus of the mucin molecule reduces the efficiency of the antibody binding reaction; hence, those molecules will not be recognized as well by the assay as untreated mucins.

Importantly, for all mucins (with the exception of autoclaved mucin, of course, which was not soluble anymore) that were treated in their lyophilized form, we find no decreased ELISA signal compared to the untreated control sample (**Figure 2**). This confirms the results from the tribology tests, which did not indicate any perceivable loss of functionality for those mucin molecules.

Also for solubilized samples that were subjected to either a thermal treatment up to 120 °C or to a short, 10 min UV-exposure, the ELISA test returns virtually identical intensity values as for the untreated control; again, this is consistent with the

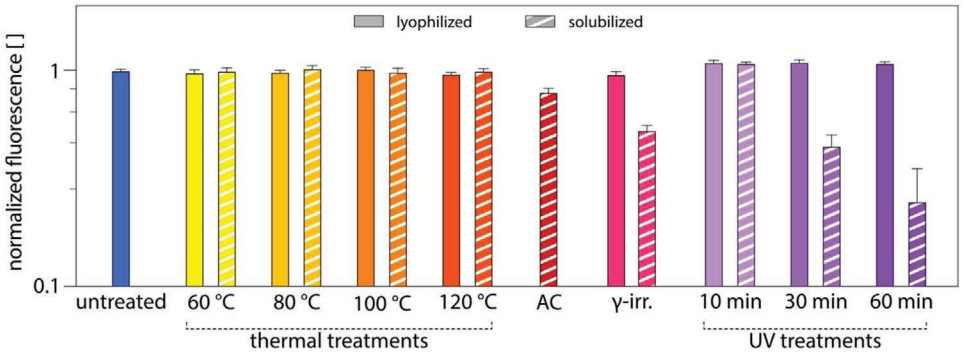

Figure 2. Detection of adsorbed mucins via specific antibodies. The normalized fluorescence intensities depicted in this graph were obtained for mucin solutions that have been exposed to heating/autoclaving (AC), γ- or UV-irradiation treatment, respectively. The intensity of an untreated sample was used as a reference and set to 1. Signals obtained for mucins that were treated in their lyophilized state (full bars) are compared to results obtained for mucin samples that were treated as solutions (striped bars). The error bars denote the standard error of the mean as obtained from $n = 3$ independent samples.

results from tribology. In contrast, for the solubilized mucins that underwent an autoclaving procedure, longer UV exposure (i.e., for 30 or 60 min), or γ-irradiation treatment, we detect a decreased ELISA signal. Also, this is in agreement with the results obtained from tribology and supports our notion that, for those treated mucin variants, the integrity of the hydrophobic peptide termini is compromised.

Of course, the termini of the mucin glycoprotein cannot only be damaged in terms of their conformation (i.e., folding pattern) but they could also be cleaved from the mucin by hydrolysis. In this case, mucin fragments should be detectable in the treated samples. Testing for a fragmentation of mucins is important as other biopolymer fragments, e.g., those from the extracellular matrix component laminin, have been shown to be cytotoxic even though the full biopolymer is not.[19] Thus, we here subject the sterilized mucin samples to a gel electrophoresis under denaturing conditions and conduct a Coomassie staining (see the Experimental Section); this procedure allows for separating the (protein) components of the mucin samples according to their molecular weight and visualizing the different subpopulations. If mucin fragmentation occurs, we should be able to detect additional protein bands in the band pattern, which are not present in the pattern of an untreated reference sample. However, we do not detect any such additional bands (**Figure 3**). Given that the antibody, which targets the mucin C-terminus, returns a signal for each of the mucin samples that have been treated in the lyophilized state in the ELISA, this suggests that none of these treatments induced fragmentation of the lyophilized mucin.

We and others have already reported that, owing to its high molecular weight, lab-purified porcine gastric mucin hardly enters the matrix of a polyacrylamide gel,[6b,20] and indeed we find the majority of all samples in the pockets of the gel (Figure 3). These pronounced bands in the loading pockets of each channel indicate large amounts of high-molecular weight MUC5AC molecules. However, for mucin that was γ-irradiated in its solubilized form, we find that the intensity of this MUC5AC band is reduced. Since this mucin also failed to provide lubrication, this confirms the ELISA result which already indicated that this mucin variant was somehow damaged by this treatment. In addition, for almost all samples, we observe weaker bands in the range of 10–250 kDa; there are only few exceptions where those additional bands appear to be absent, i.e., when mucin was autoclaved or subjected to γ-irradiation—either in its solubilized or lyophilized form. This suggests that, here, smaller protein impurities within the samples might have been broken down by thermal hydrolysis into such minuscule fragments that they are not detectable anymore.

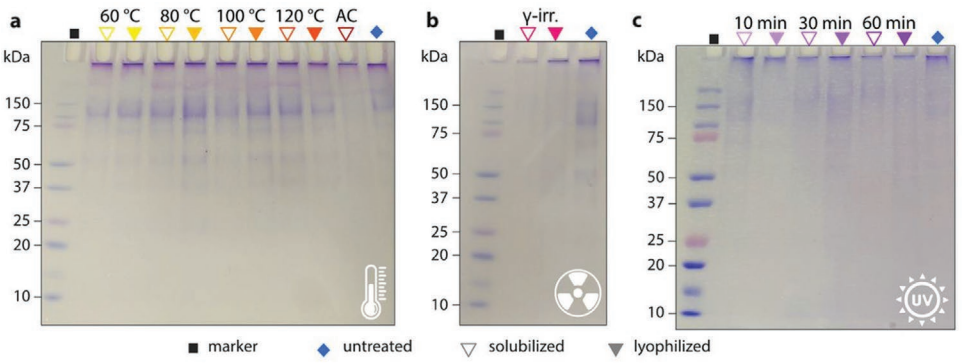

Figure 3. Molecular mass distribution of proteinous sample components as visualized by an SDS-Page. Electrophoresis gels are displayed for thermally treated a), γ-irradiated b), and UV treated c) mucins. The blue signal originates from a Coomassie staining, which shows the presence of different polypeptides.

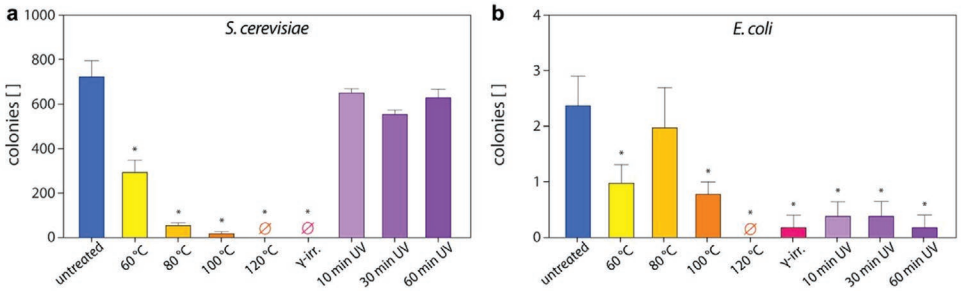

Figure 4. Bacterial and fungal colony counts after different treatments. The number of grown colonies after different sample treatments is displayed for *S. cerevisiae* a) and *E. coli* b) cells. The error bars denote the standard error of the mean as obtained from $n = 5$ replicates. Asterisks denote statistically significant differences between the untreated reference and the respective treated sample (based on a p-value of 0.05).

So far, our experiments have shown that—in their lyophilized form—mucin glycoproteins are very resilient against various treatments that can be considered as disinfection or sterilization methods. Thus, in a last step, we now assess the sterilization efficiency of the respective methods toward two microbiological model organisms representing a bacterial (*E. coli*) or fungal (*S. cerevisiae*) contamination. For this purpose, the mucin samples are inoculated with the respective microorganism, lyophilized, and then (in this dry state) subjected to the different treatments that we have already discussed above (see the Experimental Section).

For samples inoculated with *S. cerevisiae*, we observe a strong reduction in the number of viable (= colony forming) cells after both, thermal treatment or γ-irradiation (**Figure 4**a). In fact, after a thermal treatment at 120 °C or a γ-irradiation treatment, we cannot detect any formation of yeast colonies anymore. In contrast, the efficiency of the UV-treatment is rather low here, as the numbers of yeast colonies is only slightly lower here than in the untreated control sample (Figure 4a). These findings agree with previous studies which had demonstrated a high heat-sensitivity[21] of this kind of haploid fungi yet a good resistance toward UV radiation.[22]

When we analyze the treatment efficiency for *E. coli* contaminated mucin samples, a different picture emerges: here, not only samples exposed to γ-irradiation or heat treatment (for temperature levels of 100 °C and above), but also those exposed to UV light show a significant reduction of colony counts (Figure 4b). The latter finding is consistent with previous studies that link bacterial cell death to a UV-induced dimerization of thymine and uracil bases within DNA or RNA strands,[23] and such a cell death in response to DNA/RNA-damage is more likely to occur for the rapidly dividing bacteria compared to the more slowly proliferating eukaryotic yeast cells. However, another, equally likely explanation for the ability of yeast cells to survive the UV treatment could be that yeast cells are able to form very sturdy and resistant spores.[24] If we consider that a lyophilization step (similar to the one that is typically performed during the mucin purification process) was applied during the preparation process of the microbially contaminated mucin samples, it seems possible that the yeast cells have formed such resistant spores that endured the UV exposure and started to germinate again as soon as they encountered favorable growth conditions on the agar plates. In contrast, *E. coli* can be expected to be rather vulnerable to the extreme conditions present during the lyophilization process, which would explain the rather low numbers of *E. coli* colonies we find for the "untreated" reference samples (Figure 4b).

4. Conclusion

The ability to endure high temperatures and different types of radiation (such as UV-light or γ-rays) is essential for materials to be used in biomedical applications: they not only need to be able to undergo different fabrication processes but also have to withstand sterilization methods. Here, we observed that the biomedically very interesting mucin glycoproteins are able to endure thermal treatments (at least to a certain degree), UV-exposure and γ-irradiation without suffering detectable structural damages or functional losses. Hence, we conclude that mucins should be stable enough to be further processed for different biomedical applications that might require heating steps or UV curing with (relatively) short curing times.

Regarding their potential to sterilize contaminated mucin samples, we here identified γ-irradiation and thermal treatments with 120 °C to be the most suitable among the tested treatments. Gamma rays can permanently damage DNA and exhibit a high penetration efficiency across biological matter; thus, they can not only inactivate bacteria and yeast cells but also (very sturdy) fungal spores can be efficiently killed. With the low numbers of remaining microorganisms we obtain for γ-irradiated and thermally (120°) treated mucins and the high inoculation density of microbes chosen here, we achieve—for those two treatments—mucins, that are both, functional and sterile (according to FDA regulations).[17]

Based on the data we obtained so far, it seems that autoclaving mucin samples are only possible with mucin solutions, and even this comes with a certain loss of mucin functionality. Whether or not mucin-based materials (e.g., when the glycoprotein is used as a surface coating or as a constituent of a multicomponent bulk-material) are able to withstand such an autoclave treatment, will have to be tested in future experiments.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

ionizing radiation, lubrication, microbial contamination, temperature, UV

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