Optimization of Alcian blue pH 1.0 histo-staining protocols to match mass spectrometric quantification of sulfomucins and circumvent false positive results due to sialomucins

Sulfate detection with Alcian blue versus mass spectrometry

**Keywords:** Alcian blue/ mass spectrometry/ mucin/ O-glycosylation/ sulfation.

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Supplementary materials: Supplementary Table I-II

Supplementary results

Supplementary Figure S1

Materials and methods

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Abstract

Sulomucins are in some body-locations and species a normal occurrence, whereas in other situations a sign of pathology. Sulomucin content on histological sections and isolated material is frequently analyzed with Alcian blue staining at pH 1.0. However, since the stain detects the charge, a high density of other charged molecules, such as sialic acids has potential to impede specificity. Here, we compared the outcome from four staining protocols with the level of sulfation determined by liquid chromatography-tandem mass spectrometry analysis (MS) on samples from various tissues with variable sulfation and sialylation levels. We found that a protocol we designed, including rinsing with MetOH and 0.5M NaCl buffer at pH 1.0 eliminates false positive staining of tissues outperforming commonly recommended solutions. In tissues with low to moderately sulfated mucins (e.g. human stomach and salmonid epithelia) this method enables accurate relative quantification (e.g. sulfate scoring comparisons between healthy and diseased tissues) whereas the range of the method is not suitable for comparisons between tissues with high sulfomucin content (e.g. pig stomach and colon).
Introduction

Goblet cells are a dominant feature of many epithelia and they continuously secrete mucus, which is mainly composed of heavily glycosylated mucins (Quintana-Hayashi, M.P., Padra, M., et al. 2018). Sialylation and sulfation are terminal modifications of mucin O-glycans that differ between species, individuals, epithelia within each individual and health status as determined by MS and summarized in Supplementary Table 1 (Benktander, J., Venkatakrishnan, V., et al. 2019, Padra, M., Adamczyk, B., et al. 2018, Quintana-Hayashi, M.P., Padra, M., et al. 2018). For example, Atlantic salmon mucin glycan structures are highly sialylated (Benktander, J., Padra, J.T., et al. 2020, Jin, C., Padra, J.T., et al. 2015) while human and porcine gastric mucins have comparatively low sialylation (Jin, C., Kenny, D.T., et al. 2017, Padra, M., Adamczyk, B., et al. 2018). Mucin glycosylation changes (Linden, S., Mahdavi, J., et al. 2008, Linden, S.K., Sutton, P., et al. 2008, Venkatakrishnan, V., Quintana-Hayashi, M.P., et al. 2017) are important, can affect host-pathogen interactions and can serve as biomarkers and diagnostic tools (Benktander, J., Sundh, H., et al. 2021, Linden, S., Mahdavi, J., et al. 2008, McGuckin, M.A., Linden, S.K., et al. 2011). Sulfomucins detection in early diagnosis plays an important role in determining patient treatment (Mishra, R.K., Ahmad, A., et al. 2020), e.g. in determining the stage of gastrointestinal pathologies (Filipe, M.I. 1969, Filipe, M.I. 1972, Rugge, M., Fassan, M., et al. 2011). However, in addition to human diagnostics, studies often involve animals and tissues where the sulfation and sialylation levels are unknown. Different protocols for staining of sulfomucins are used, including Alcian Blue (AB) pH 1.0 staining (Cohen, M.C., Rúa, E.C., et al. 2000) and colloidal iron/high iron diamine stains (HID) (Spicer, S.S. 1965). These stains have the potential to negatively impact staining specificity by non-specific staining of sialic acids (Reid, P.E., Owen, D.A., et al. 1989). Other methods like Alcian yellow (AY) pH1.0 or the laborious, and often incomplete, digestion of sialic acids before AB2.5 staining are also error prone (Padra, J.T., Sundh, H., et al. 2014, Scillitani, G., Mentino,
D., et al. 2012, Sorvari, T.E. and Nanto, V. 1971). Due to ease of use, AB pH 1.0 has gained popularity compared to the more traditional iron stains (Cohen, M.C., Rúa, E.C., et al. 2000, Samar, M.E., Avila, R.E., et al. 1999). Upon trying one of the widely used AB pH1.0 protocols on Atlantic salmon histology sections, we noticed a large discrepancy between the histological staining of the mucin producing goblet cells and results obtained by MS.

The main objectives of this study were therefore to compare the results obtained by several AB staining protocols and identify the protocol(s) that best reflects the sulfation abundance results obtained by mass spectrometry, in the presence of different sialic acid levels.
Results

Alcian blue staining of PVDF membrane-blotted mucins is a good indicator of sialylation and sulfation levels but has positive error in the lower range of detection

First, we tested the AB2.5 and AB1.0 protocols for specificity on mucins with variable sialylation and sulfation dotted onto PVDF membranes. Both the AB2.5 and AB1.0 staining methods correlated well with the total sialylation and sulfation versus the sulfation level, determined by MS, but yielded unspecific weak staining in negative samples (Detailed description in the Supplementary file and Fig S1A-C).

Rinsing tissue sections with 0.1 M HCl/MetOh 9:1 (pH 1.0) followed by 0.5 M NaCl/HCl (pH 1.0) reduces false positive sulfate staining

Mucosal tissue sections (n=7) were stained using four different AB pH 1.0 staining protocols (Supplementary Table II) to visualize sulfation and with a standard PAS/AB pH 2.5 staining protocol to visualize sialic acids, sulfates and neutral mucins (Fig. 1A). Figure 1A shows representative images of tissues stained with each of the protocols. With all four AB pH 1.0 protocols, there was a clearly stronger staining of porcine tissues (55% and 50% relative abundance of sulfation as determined by MS) than that of the human (0% and 0.3% sulfation) and fish tissues (sulfation not-detected).

Results of the microscopy scoring (scale 0-10) were correlated with sulfation levels measured with MS (Fig. 1B-E, n=7). Protocol #3 was found to correlate best with sulfate levels quantified with mass spectrometry (Fig. 1D). The other protocols (#1, #2 and #4) had a weaker correlation due to that highly sialylated samples scored positive in spite of lacking sulfation (Fig. 1B, C and E).
Rinsing tissue sections with MetOh/HCl (pH 1.0) followed by 0.5 M NaCl/HCl (pH 1.0) (‘protocol #3’) yields accurate relative quantification of sulfates in tissues with lower than 24% sulfation

To test protocol #3 further, we stained 17 tissue sections from fish, mice, pig and human with sulfated mucin glycan content ranging from 0% to 55% and variable sialic acid content. The gradient of blue staining matched the MS results (Fig. 2A). Tissues ranging from zero to 23% sulfation were visibly distinguishable from each other based on staining intensity and neither Arctic char intestine (0% sulfation) nor human stomach (0.3% sulfation) were positive (Fig. 2A). The porcine gastric and colon tissues ranging from 23% to 55% sulfation all had stronger blue staining than the 15% sulfated mouse colon tissue (Fig. 2A). However, the 51% and 55% sulfated porcine tissues did not stain stronger than the 23% and 36% sulfated porcine tissues (Fig. 2A).

We found a positive correlation between the histological scoring of protocol #3 and the sulfation levels quantified by Mass spectrometric analysis (Fig2B, r²=0.53, p≤0.001, n=17). The shape of the curve was only linear until ca 25% sulfation score, which is due to the variable staining strengths and a proportionally weaker staining of the porcine tissues with the higher sulfation levels (Fig. 2A). Correlating the MS and AB-stain results excluding the high sulfate porcine samples resulted in a very strong linear correlation (Fig. 2C, r²=0.94, p≤0.0001, n=13). The staining score did not correlate with the sum of the sialylation and sulfation (Fig. 2D; r²=0.03, p=n.s., n=17). This indicates that unspecific Alcian blue staining of sialic acids is not likely with this protocol.
Discussion

Here, we identified that commonly used staining protocols with AB at pH 1.0 used for identifying sulfomucins gives false positive results when sialic acids are present and therefore developed a protocol with more stringent washing steps to avoid this problem. We did not see marked differences in the staining due to high sialic acid levels on mucins dotted to PVDF membranes. This suggests that the washing steps we applied here, 0.1M HCl followed by further destaining with methanol three times, prevented the false positive staining of sialomucins. Despite the thorough washing in methanol that dissolves unbound Alcian blue particles (Schenk, E. 1981), there seems to be certain physicochemical properties of the mucins that contribute to the appearance of weak residual stain in the absence of sulfate and sialic acid.

Tissue staining scores of four AB pH 1.0 staining methods differing in the washing steps each resulted in strong positive correlation with the relative abundance of sulfates. However, samples lacking sulfation also bound the blue stain weakly. The most precise method was protocol #3, which did not show a staining level distinguishable from the background staining for the samples with non-detectable sulfate levels using MS. This is in line with the PVDF membrane staining. The similarity between the two methods is the low pH (pH 1.0) and the washing with methanol, which seems to be protective against the undesirable sialomucin staining.

In samples with sulfation levels between 0% and 55%, the correlation between histological scoring of tissues stained with protocol #3 and MS detection is strong but not linear. In the range of 0% to ca. 24% relative abundance of sulfates the staining intensity follows the sulfation levels closely but reaches a plateau above 24%. This means that protocol #3 is a useful tool in the relative quantification of sulfomucins in all samples with low to moderate sulfomucin content but only as a qualitative detection method in samples with high sulfation. In this context
we also want to highlight that sulfates ionizes efficiently and therefore are most likely somewhat overestimated in the MS data. The 24% should therefore not be used as an absolute value but as an indication for what type of samples that are suitable to perform semi-quantification on. The lack of correlation between the summarized relative abundance of sulfation and sialylation on these mucin samples and the histological scores of sulfomucins suggest that the effect of sialic acids is negligible with protocol #3.

Contrary to AB pH1.0 kit suppliers instructions, we have shown that as low as 5% relative abundance of sulfation is detectable with this protocol as it stains distinctively stronger than tissues having no sulfomucins. Moreover, rinsing with 0.1M HCl or blotting with paper alone is not enough to avoid false positive staining as we have seen strong staining with some of the non-sulfated samples with protocol #1 and #2. Five out of ten reviewed commercially available kits suggested washing with 3% acetic acid or running tap water, which is alarming since it has been long established that the pH of the rinsing solution should be kept low and water should be avoided (Lev, R. and Spicer, S.S. 1964).

In conclusion, we bring attention to that widely used AB pH 1.0 staining kits and protocols lack vital, or provide false, information for accurate assessment of sulfomucins. As a substitute protocol we recommend using Alcian blue pH 1.0 staining followed by three rinses of MetOH/HCl (9:1 v/v; pH 1.0) and three rinses of 0.5 M NaCl/HCl (pH 1.0). This method safely circumvents false positive sialomucin staining, enabling reliable use of the Alcian blue pH 1.0 staining method in histopathological applications.

**Materials and Methods**

The materials and methods used in this study can be found among the supplementary files.
Data Availability Statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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Figure 1. PAS/AB2.5 and four varieties of AB1.0 staining to visualize sulfation in animal tissues and correlation with relative abundance of sulfate. (A) Tissues with decreasing levels of sulfation (quantified by MS), from top to bottom, were stained. PAS/AB2.5 shows neutral mucins with magenta color and acidic mucins with blue color. The black in the Atlantic salmon skin samples is an artifact present in skin with this stain, but goblet cells can be observed in areas without black. 100× magnification. (B-E) Sulfate levels of the seven tissues studied correlated positively with all four protocols. Protocol #3 (D) shows the strongest association between the parameters ($r^2=0.99$, $p \leq 0.0001$, $n=7$) followed by protocol #1 (B) ($r^2=0.96$, $p \leq 0.01$, $n=7$), protocol #4 (E) ($r^2=0.77$, $p \leq 0.01$, $n=7$) and protocol #2 (C) ($r^2=0.77$, $p \leq 0.01$, $n=7$). Samples having high relative abundance of sialic acids (black dots) cause elevated staining levels with protocol #1, #2 and #4, but not with #3. Sialic acid abundance is marked with black dots over 40%, grey dots between 10 and 39% and white dots at or below 9%. Statistics: Pearson’s correlation test ($r^2$, $n=7$).
Figure 2. Relationship between MS quantification vs. staining with ‘protocol #3’ for sulfates. (A) Eight representative tissues with increasing levels of sulfation (quantified by MS) from top to bottom were stained with ‘protocol #3’. Arctic char: There is weak staining in the non-mucosal region and no apparent staining in the mucosal region. Human stomach: There is no visible staining of the tissue. Murine colons: There is moderate staining in both the 5% sulfated and the 15% sulfated mucus in the goblet cells and the in the luminal space. Pig tissues: All pig tissues stained strong for sulfates. Out of the four tissues, the stomach samples (36% and 51% sulfated) show slightly stronger staining than the colon sections (23% and 55% sulfated). 100x magnification. (B) Correlation analysis showed positive correlation between the two quantification methods ($r^2=0.73$, p≤0.001, n=17), however the linear relationship disappeared at sulfation values above 24%. (C) There is a strong correlation between histological staining and MS quantification for samples with low to moderate sulfation (under 25% abundance; $r^2=0.97$, p≤0.0001, n=13). On graph (B) and (C) the samples with high sialic acid content (black dots) have histological scores in line with the samples with low sialic acid content (white dots). (D) The staining scores of ‘protocol #3’ did not correlate with the total abundance of sialic acids and sulfates in the samples ($r^2=0.03$, p=n.s., n=17). Sialic acid abundance is marked with black dots over 40%, grey dots between 10 and 39% and white dots at or below 9%. Statistics: Pearson’s correlation test ($r^2$).
