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Anionic extracellular polymeric substances extracted from seawater-adapted aerobic granular sludge

Le Min Chen[1] · Paula Beck[1] · Jitske van Ede[1] · Mario Pronk[1,2] · Mark C.M. van Loosdrecht[1] · Yuemei Lin[1]

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Abstract

Anionic polymers, such as heparin, have been widely applied in the chemical and medical fields, particularly for binding proteins (e.g., fibroblast growth factor 2 (FGF-2) and histones). However, the current animal-based production of heparin brings great risks, including resource shortages and product contamination. Recently, anionic compounds, nonulosonic acids (NulOs), and sulfated glycoconjugates were discovered in the extracellular polymeric substances (EPS) of aerobic granular sludge (AGS). Given the prevalence of anionic polymers, in marine biofilms, it was hypothesized that the EPS from AGS grown under seawater condition could serve as a raw material for producing the alternatives to heparin. This study aimed to isolate and enrich the anionic fractions of EPS and evaluate their potential application in the chemical and medical fields. The AGS was grown in a lab-scale reactor fed with acetate, under the seawater condition (35 g/L sea salt). The EPS was extracted with an alkaline solution at 80 °C and fractionated by size exclusion chromatography. Its protein binding capacity was evaluated by native gel electrophoresis. It was found that the two highest molecular weight fractions (438– > 14,320 kDa) were enriched with NulO and sulfate-containing glycoconjugates. The enriched fractions can strongly bind the two histones involved in sepsis and a model protein used for purification by heparin-column. These findings demonstrated possibilities for the application of the extracted EPS and open up a novel strategy for resource recovery.

Key points

- *High MW EPS from seawater-adapted AGS are dominant with sulfated groups and NulOs*
- *Fifty-eight percent of the EPS is high MW of 68–14,320 kDa*
- *EPS and its fractions can bind histones and fibroblast growth factor 2*

Keywords EPS · Nonulosonic acids · Sulfated glycoconjugates · Protein binding · Granular sludge

Introduction

Heparin is the most negatively charged natural polymer, belonging to the family of glycosaminoglycans. Due to the strong anionic charge on its molecular chain, it can bind specific proteins and growth factors and regulate their diffusion (Varki et al. 2017a). Currently, animal-derived heparin is used as a pharmaceutical drug (e.g., sepsis treatment drug and anticoagulant agent), as the component of the endothelial cell culture media to help the binding of growth factors (e.g., Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)), and as packing material for columns in affinity chromatography for proteins purification (Wildhagen et al. 2014; Nicolin Bolten et al. 2018). As heparin is currently derived from animal sources, significant resource shortages and product contaminations may happen, resulting in an unpredictable supply (Nicolin Bolten et al. 2018; McCarthy et al. 2020). It is important to search for sustainable and reliable alternatives for heparin. It has been reported that some algae and bacteria living in the marine environment produce sulfated glycoconjugates (Colliec-Jouault et al. 2012). Sulfated polysaccharides extracted from marine organisms are used as gelling agents in the food and pharmaceutical industries because of their distinct properties. Moreover, due to their bioactive properties (e.g.,

✉ Le Min Chen
L.M.Chen@tudelft.nl

[1] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629, HZ, Delft, The Netherlands

[2] Royal HaskoningDHV, Laan 1914 35, Amersfoort 3800, AL, The Netherlands

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anticancer, anti-inflammatory, and anticoagulant), sulfated polysaccharides from marine organisms have become a source of interest for the development of heparin alternatives and new materials (Colliec-Jouault et al. 2012; Delbarre-Ladrat et al. 2014; Xue et al. 2019; Lee and Ho 2022).

Biopolymers carrying anionic charges have been found in biofilms such as aerobic granular sludge (AGS) as well (de Graaff et al. 2019; Felz et al. 2020; Chen et al. 2023). The AGS technology is increasingly used as an efficient wastewater treatment technology (Pronk et al. 2015; Nancharaiah and Sarvajith 2019). This biotechnological process is based on a microbial community forming granules by a self-produced biofilm matrix using extracellular polymeric substances (EPS). The EPS matrix consists of numerous macromolecular compounds, such as (glyco)lipids, (glyco) proteins, and polysaccharides (Felz et al. 2016; Seviour et al. 2019). Negatively charged glycoconjugates are shown to play an important role in the formation and stabilization of the complex EPS structure, through bridging with multivalent cations (Flemming and Wingender 2010).

One type of strongly negatively charged component in the EPS is nonulosonic acids (NulOs). NulOs are 9-carbon acidic monosaccharides usually reported as part of the glycoconjugates in eukaryotes or pathogenic bacteria (Varki et al. 2017b). Within the NulOs family, neuraminic acid (NeuAc) and its derivatives are well studied, especially in relation to the interaction between pathogens and human cells. Other forms of NulOs such as pseudaminic acid and legionaminic acid are found on lipopolysaccharides produced by pathogenic bacteria (Angata and Varki 2002). Only recently, it has been shown that the NulOs are widespread in non-pathogenic microbial aggregates, such as seawater-adapted AGS, enriched cultures of "*Candidatus* Accumulibacter phosphatis" and anammox granular sludge (de Graaff et al. 2019; Kleikamp et al. 2020; Boleij et al. 2020; Tomás-Martínez et al. 2021). These polymers enriched with NulOs were shown to have interaction with positively charged histones, suggesting a source for sepsis treatment drugs (Tomás-Martínez et al. 2022). Sulfated glycoconjugates are another type of strongly negatively charged compounds that were recently reported in the EPS of various granular sludge systems including AGS (Bourven et al. 2015; Felz et al. 2020; Boleij et al. 2020). Sulfated glycoconjugates, especially sulfated glycosaminoglycans in the extracellular matrix of animals are well-studied (Bedini et al. 2019).

Based on recent research, the EPS of AGS contains anionic glycoconjugates with NulOs and sulfated groups. Thus, it may have great potential to be a sustainable resource for the production of biomaterials for various biotechnological applications. With the AGS system, it is possible to use microbial communities instead of pure cultures and to adjust the operational conditions of the reactor to stimulate the production of the EPS. Therefore, the AGS system may become a new platform to produce biopolymers and provide novel strategies for resource recovery.

Expanding on the recent findings of anionic glycoconjugates in AGS, the aim of the current research was to evaluate the anionic properties of the EPS from seawater-adapted AGS. We are specifically focusing on NulOs and sulfated glycoconjugates and exploring the potential application in the medical field (e.g., raw material for sepsis treatment drugs) and chemical field (e.g., column material for protein purification). The extracted EPS was fractionated by size exclusion chromatography to obtain fractions containing different apparent molecular weight. The EPS and the derived fractions were characterized for the presence of anionic compounds by measuring the content of NulOs and sulfated glycoconjugates. Finally, the potential applications of the extracted EPS were evaluated by using a protein binding assay.

Material and methods

Reactor operation and biomass microbial community analysis

Reactor operation

Seawater-adapted aerobic granular sludge was cultivated in a 2.8 L bubble column with an internal diameter of 6.25 cm as a sequencing batch reactor (SBR) adapted from de Graaff et al. (2019). The reactor was inoculated with sludge collected from a lab-scale reactor with glycerol as the carbon source under freshwater condition (Elahinik et al. 2022). The room temperature was controlled at 20 °C, and the pH was controlled at pH 7.3 ± 0.1 by dosing 1.0 M NaOH or 1.0 M HCl. The DO was controlled by a mixture of nitrogen gas and air at 0% and 80% saturation during the anaerobic and aerobic phases, respectively.

Reactor cycles consisted of 5 min settling, 5 min effluent withdrawal, 5 min N_2 sparging, 5 min of feeding, 50 min N_2 gas sparging (anaerobic phase), and 110 min of aeration (aerobic phase). Artificial seawater (Instant Ocean® Sea Salt, Instant Ocean) was gradually introduced by incrementally increasing the seawater concentration with 10 g/L Instant Ocean® Sea Salt each week while maintaining complete anaerobic acetate removal. After the final concentration of 35 g/L Instant Ocean® Sea Salt was reached, the average sludge retention time (SRT) was kept at 14 days. Granules were sampled for EPS extraction and microbial community analysis after 12 days of exposure to 35 g/L Instant Ocean® Sea Salt.

The feed of 1.5 L per cycle consisted of 1.2 L artificial seawater (final concentration 35 g/L Instant Ocean® Sea Salt), 150 mL of medium A, and 150 mL of medium B.

Medium A was composed of 8506 mg/L of sodium acetate trihydrate. Medium B contained 2200 mg/L of NH_4Cl, 340 mg/L of K_2HPO_4, 270 mg/L of KH_2PO_4, 70 mg/L of allylthiourea to inhibit nitrification and 10 mL/L of trace elements solution similar to Vishniac and Santer (1957). The trace element solution contained 4.99 g/L $FeSO_4.7H_2O$, 2.2 g/L $Zn.SO_4.7H_2O$, 7.33 g/L $CaCl_2.2H_2O$, 4.32 g/L $MnSO_4.H_2O$, 2.18 g/L $Na_2MoO_4.2H_2O$, 1.57 g/L $CuSO_4.5H_2O$, 1.61 g/L $CoCl_2.6H_2O$, and 50 g/L EDTA. The combination of these feed streams led to influent concentrations of 400 mg/L COD, 57.6 mg/L NH_4-N, and 12.2 mg/L PO_4-P. To monitor the performance of the reactor, samples were taken at certain interval and filtered through a 0.22 µm PVDF filter. Acetate concentration was measured through high-performance liquid chromatography on the Vanquish HPLC system (Thermo Scientific, Waltham, USA) at 50 °C (0.75 mL/min) with 1.5 mM phosphoric acid as eluent and Aminex HPC-87H (Bio-Rad, California, USA) as a column. Phosphate and ammonia concentrations were measured by using a Thermo Fisher Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, USA). Visualization of the granules was performed by using a stereo zoom microscope (M205 FA, Leica Microsystems, Germany). The images of the granules were captured, processed, and exported in ".jpg" format with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany).

The organic and ash fractions of the biomass were determined according to the standard methods (APHA 1995), after washing the granules three times with five times of its volume with demi-water. For EPS extraction and characterization, the washed granules were lyophilized immediately and stored at room temperature.

Microbial community analysis by fluorescent in situ hybridization (FISH)

The granules were collected from the reactor at the end of the aerobic phase. The handling, fixation, and staining of FISH samples were performed as described in Bassin et al. (2011). The PAOmix combination (PAO462, PAO651, and PAO846) was used for visualizing polyphosphate accumulating organisms (PAO) (Crocetti et al. 2000). The GAOmix (GAOQ431 and GAOQ989) were used for visualizing glycogen accumulating organisms (GAO) (Crocetti et al. 2000). Probes targeting the clade I and clade II of Accumulibacter (Acc444-I and Acc444-II) were used for visualizing the clades of Accumulibacter (Flowers et al. 2009). EUBmix (EUB338, EUB338-II, and EUB338- III) were used for staining all bacteria (Amann et al. 1990; Daims et al. 1999). Images were taken with a Zeiss Axio Imager M2 microscope equipped with the fluorescent light source X-Cite Xylis 720L. The image acquisition was performed with the Zeiss Axiocam 705 mono camera. The images were processed and exported in ".tif" format with the Zeiss microscopy software (ZEN version 3.3).

EPS extraction from aerobic granular sludge and characterization

EPS extraction

Lyophilized granules were extracted in 0.1 M NaOH (1% VS w/v) for 30 min at 80 °C while stirring at 400 rpm. The solution was cooled down and centrifuged at 4000 × *g* for 20 min at 4 °C. The supernatant was collected and subsequently dialyzed against demi-water overnight in dialysis tubing with a molecular weight cut-off of 3.5 kDa MWCO (Snakeskin™, ThermoFisher Scientific, Landsmeer). The dialyzed EPS solution was lyophilized and stored at room temperature until further analysis.

EPS fractionation by size-exclusion chromatography

Forty milligrams lyophilized EPS was solubilized in 8 ml of running buffer containing 0.15 M NaCl and 0.05 M glycine-NaOH (pH 10) with gentle stirring overnight. The pH was adjusted to 10 using NaOH. All solutions were filtered through a 0.45 µm membrane filter before application to the column to allow the samples to remain dissolved as much as possible.

Size exclusion chromatography (SEC) was performed using a Hiload 16/600 Superose 6 prepacked column (Cytiva Lifesciences, Marlborough, MA) fitted on a Bio-Rad system containing a UV detector. Superose 6 column has a high fractionation range (fractionation range Mr ~5–5000 kDa (globular proteins) with an exclusion limit of Mr ~40,000 kDa (globular proteins), calibration of the column, upon which the elution volume was determined, was done using a Cytiva HMW marker set (Cytiva Lifesciences, Marlborough, MA). This set consists of ovalbumin (44 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). The molecular weight of the fractions of the sample was determined based on the calibration line. Molecular weights higher than the standards were calculated by linear extrapolation of the calibration line.

EPS fractions were collected based on the chromatogram (280 nm) of the elution. They were subsequently dialyzed against demi water with a 3.5 kDa MWCO dialysis bag, frozen at −80 °C, and lyophilized. The lyophilized samples were stored at room temperature until further analysis.

EPS and fractions characterization

Native agarose gel staining with Coomassie Blue and Alcian Blue Native agarose gel electrophoresis was run on a submerged horizontal platform, with the wells positioned in the

center of the gel. Lyophilized EPS samples were resolubilized in 50 mM Tris at 2.5 mg EPS/mL concentration for 1 h at 30 °C. Next, 10 µL of the sample was loaded in the wells on a 0.8% agarose gel in 500 mM Tris/HCl, 160 mM boric acid, 1 M urea, and pH 8.5. Electrophoresis was performed with a running buffer (90 mM Tris/HCl, 90 mM boric acid, pH 8.5) at 80 V for 90 min. Proteins carrying a net negative charge migrate toward the anode, whereas proteins carrying a positive charge migrate toward the cathode. To determine if high molecular weight proteins could pass the gel, a high molecular weight marker was used as a ladder (HMW – SDS Calibration kit, Cytiva, Marlborough, MA). The ladder was negatively charged due to the presence of sodium dodecyl sulfate (SDS). The proteins in the EPS were revealed using Coomassie Blue staining (SimplyBlue™ Safestain, Invitrogen, Waltham, MA) according to the manufacturer's instruction and destained overnight. To visualize glycoconjugates containing the carboxyl groups R-COO^- and the sulfated groups R-OSO^{3-}, staining with Alcian Blue was performed at pH 2.5 and pH 1.0, respectively, as described by Boleij et al. (2020). Other negatively charged groups, e.g., phosphate groups, are hardly stained since they are unable to bind as strongly as carboxyl and sulfate groups with Alcian Blue under low salt conditions done in this study (Scott and Dorling 1965). The gel pictures were taken on a ChemiDoc MP imager (Bio-Rad, Hercules, CA).

Functional group analysis by Fourier-transform infrared spectroscopy Functional group analysis was performed by Fourier-transform infrared (FTIR) spectroscopy on a Spectrum 100 spectrometer (PerkinElmer, Shelton, CT). The spectra of the lyophilized samples were recorded at room temperature over a wavenumber range of 600–4000 cm^{-1} with 16 accumulations and 4 cm^{-1} resolution.

Sulfated glycoconjugates assay Detection and quantification of sulfated glycoconjugates were performed with the Blyscan sulfated glycosaminoglycan (sulfated GAGs) assay (Biocolor, Carrickfergus, UK), according to the manufacturer's instructions. Samples (2–5 mg) were digested with 1 mL of papain protein digestion solution at 65 °C for 3 h at 300 rpm (Sigma-Aldrich, Zwijndrecht, Netherlands). The supernatant was recovered after centrifugation at 10.000 × *g* for 10 min. Fifty microliter of sample was then added to 1 mL of DMMB dye reagent. Sulfated GAGs positive components bind and precipitate with the dye, which are subsequently isolated and resolubilized. The concentration of sulfated GAGs was measured with a multimode plate reader at 656 nm (TECAN Infinite M200 PRO, Switzerland) as chondroitin sulfate equivalents. Lastly, the distribution of N-linked and O-linked sulfate in the samples was measured by performing nitrous acid cleavage as by the manufacturer's instructions prior to sulfated GAGs quantification.

Nonulosonic acid analysis with mass spectroscopy The NulOs measurement was done according to the approach described by Kleikamp et al. (2020) with small modifications. Lyophilized EPS fractions were hydrolyzed by 2 M acetic acid for 2 h at 80 °C and dried with a Speed Vac concentrator. The released NulOs were labelled using a final concentration of 7 mM DMB (1,2-diamino-4,5-methylenedioxybenzene dihydrochloride) for 2.5 h at 50 °C.

The LC-MS analysis was performed on a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific, Bleiswijk, Netherlands) coupled to an Acquity M-Class Ultra Performance Liquid Chromatograph (Waters, Milford, MA). The chromatographic separations were performed with a 1.0 × 100 mm C18 1.7 µm column (Acquity UPLC® BEH) at a constant flow rate of 40 µL/min. Solvent A consisted of 0.1% formic acid in MS-grade H_2O and solvent B of 0.1% formic acid in MS-grade acetonitrile. A linear gradient from 2.5 to 35% B was applied over 10 min, followed by a linear gradient up to 65% B over another 2.5 min. Each sample was analyzed in duplicate followed by two blanks.

Electrospray ionization was performed in positive ionization mode, and MS1 analysis was executed at a 35K resolution, an AGC target of 2.0E5, and a maximum injection time of 50 ms. Continuous fragmentation of small mass segments was performed in 5.0 Da steps from 340 to 530 Da (isolation window of 5.5 m/z). Fragmentation was performed using a normalized collision energy of 26. For MS2 analysis a 17.5K resolution, an AGC target of 2.0E5 and a maximum IT of 50 ms were used. The number of micro scans was set to 1. Raw data were analyzed using XCalibur 4.1 (Thermo Fisher Scientific, Germany) and MATLAB R2022a (MathWorks). The mass spectrometer was calibrated using the Pierce™ LTQ ESI positive ion calibration solution (Thermo Fisher Scientific, Germany).

Semi-quantitative estimation of the relative amounts of each type of NulOs was done by using the peak area of spiked 10 pmol of KDN as a reference signal. The integrated peak areas in the mass spectrometry chromatograms were calculated for each type of NulOs in each EPS fraction. The peak area was used as a parameter proportional to the amount of NulOs. The relative amount of each type of NulOs in each EPS fraction was presented as a ratio to the peak area of 1 µg of KDN for comparison.

EPS binding potential with cationic proteins

To study the binding potential of EPS and its fractions with cationic proteins, the interactions between EPS and three known cationic proteins, histone H2A, H2B, and fibroblast growth factor 2 (FGF-2) were evaluated according to Zlatina et al. (2017) with modifications. Solutions of EPS

were prepared in 50 mM Tris at a concentration of 2.5 mg/mL. Five micrograms of a cationic protein was incubated with 10 µg from the EPS solution to a final concentration of 50 mM Tris at 30 °C, 300 rpm for 1 h. One microliter of glycerol was added to the sample, vortexed, and spun down briefly. The entire sample was loaded on a horizontal 0.8% agarose gel (500 mM Tris/HCl, 160 mM boric acid, 1 M urea, pH 8.5), with the wells positioned in the middle of the gel. The electrophoresis was performed at 80 V for 90 min with a running buffer (90 mM Tris/HCl, 90 mM boric acid, pH 8.5). Staining was performed with Coomassie Blue G-250 (SimplyBlue™ Safestain, Invitrogen, Waltham, MA) according to the manufacturer's instruction. In parallel, as a comparison to the EPS, the solutions of heparin (from porcine intestinal mucosa, Sigma-Aldrich) and cationic protein-heparin samples with the same concentrations as described above were prepared accordingly.

Results

Reactor operation and EPS extraction

After stable granulation and complete acetate and phosphate removal were achieved (Fig. 1), the EPS was extracted from the granules. The reactor operation and behavior were similar to previously reported AGS experiments (de Graaff et al. 2019). The yield of the EPS extracted was 640 ± 42 mg VS EPS / g VS granules. The microbial community consisted, according to FISH staining, mainly phosphate accumulating organisms (PAO), specifically "*Candidatus* Accumulibacter phosphatis" sp. were the dominant microorganisms present, together with small amounts of glycogen accumulating organisms (GAO) (Fig. 2).

EPS fractionation and characterization

EPS fractionation and functional groups

The extracted EPS was fractionated by size exclusion chromatography to obtain fractions containing different apparent molecular weight (aMW). Five fractions with aMW ranging from < 12 kDa to more than 14,320 kDa were collected (Table 1). There was no clear difference in the amount of mass among these five fractions. Specifically, fractions F1 and F2 were collected at the beginning of the chromatogram (Supplementary Information Figure 1); both had a high aMW and low absorbance at 280 nm; their mass in total contributed to almost 40% of the extracted EPS. Judging from the chromatogram, F1 and F2 might be proteins which are heavily glycosylated.

To investigate the differences among the obtained fractions, FT-IR spectroscopy was used to analyze the functional groups. The spectrum of each fraction is shown in Fig. 3. The relative intensity ratio between the typical band of carbohydrates (C-O-C stretching at ~1030 cm^{-1}) and the typical band of proteins (N-H bending at ~1630 cm^{-1}) was much higher in the spectrum of F1 and F2 than in the spectrum of F3 and F4, suggesting that the glycoconjugate content is decreasing while the protein content is increasing as the molecular weight decreases from F1 to F4 (Tomás-Martínez et al. 2022). The spectrum of F5 was significantly different from the other fractions, the N-H bending of proteins downshift to ~1620 cm^{-1}, while the band of carbohydrates split into two bands at 1068 cm^{-1} and 978 cm^{-1}, indicating the appearance of furan ring in the glycoconjugates (Zou et al. 2019).

To further characterize the EPS fractions on the possible types of acidic glycoconjugates, native agarose gel stained with Coomassie Blue (proteins), Alcian Blue at pH 2.5

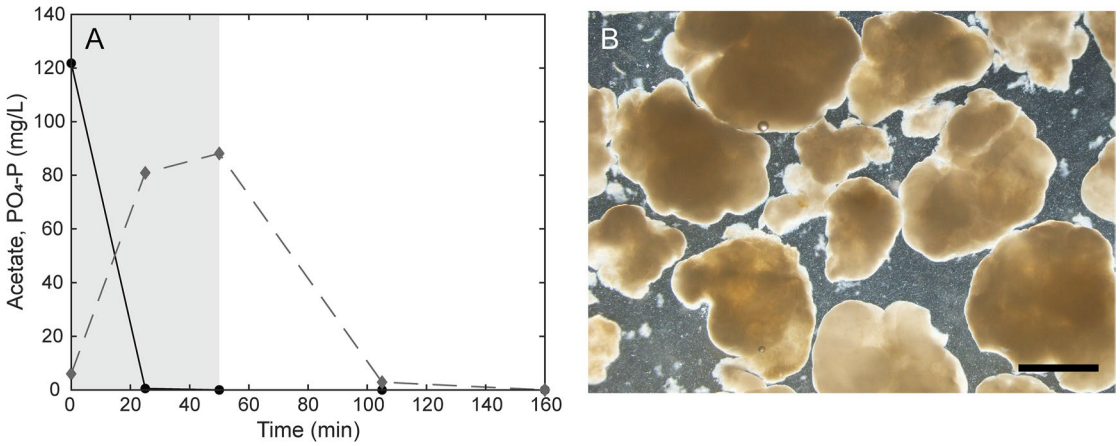

Fig. 1 Profile of acetate (●) and phosphate (♦) in the seawater-adapted AGS reactor (**A**). The shaded area indicates the anaerobic phase. Granules grown at 35 g/L Instant Ocean® Sea Salt with a scale bar representing 1 mm visualized through stereo imaging (**B**)

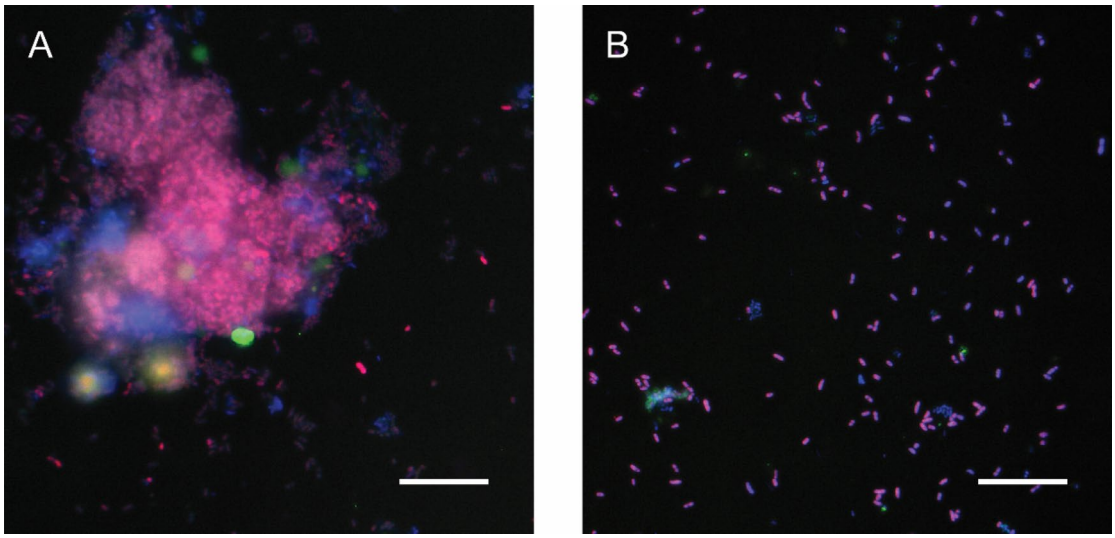

Fig. 2 Fluorescent in situ hybridization of seawater-adapted AGS. **A** Eubacteria (blue), GAO (green), PAO (red). **B** Eubacteria (blue), Acc I (red), and Acc II (green). Overlapping probes of blue and red colors result to a magenta color. Overlapping green and blue colors result in a turquoise color

Table 1 Fractionation yields of the total eluted EPS through size exclusion chromatography and the corresponding apparent MW (aMW). The calibration is based on globular proteins and thus the molecular weight range is here expressed in aMW. The fractions are collected based on the eluted peaks at 280 nm of the chromatogram (Supplementary Information Figure 1). The fractions marked with an asterisk (*) are based on extrapolation of the calibration line

Fraction no.	aMW range (kDa)	Weight percentage of EPS (%)
F1	2815– > 14,320*	20
F2	439–2815	19
F3	68–439	19
F4	12–68	26
F5	< 12*	16

(strongly acidic R-COO^- and R-OSO^{3-}) and 1.0 (R-OSO^{3-}) was performed (Fig. 4). Firstly, Coomassie Blue staining showed that the EPS migrated toward the anode as a light smear (Fig. 4A), indicating an overall negative charge on the extracted EPS. A large proportion of F1 and F2 was retained within the well toward the anode, implying that both F1 and F2 are negatively charged. Probably, due to their high molecular weight and/or the overall low negative charge density, it was difficult for these two fractions to migrate further in the gel. In comparison, both F3 and F4 migrated toward the anode as an intense smear without being retained in the well. Likely, due to their lower molecular weight, the negative charge density is sufficient for the molecules to migrate. Secondly, both F1 and F2 in the well were stained with Alcian Blue at both pH 2.5 and

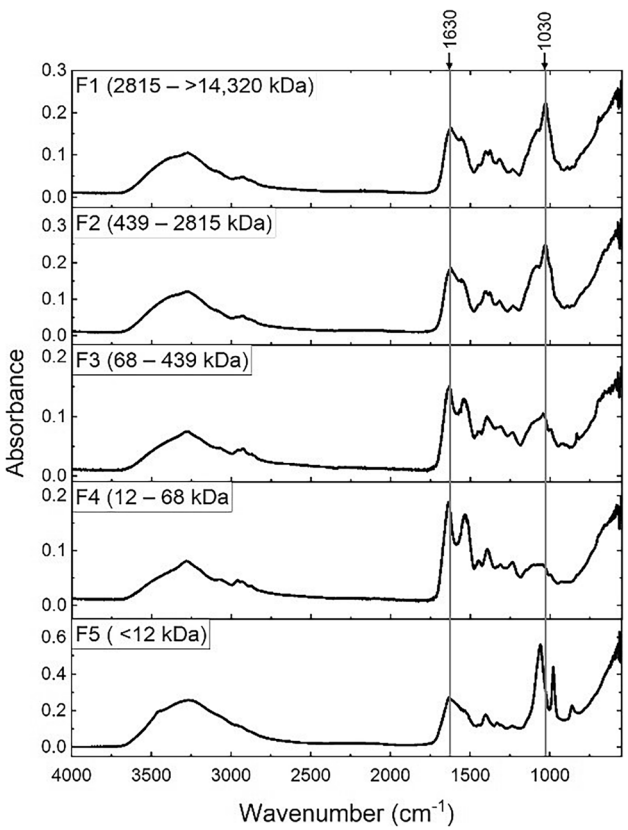

Fig. 3 FT-IR spectrum of fractionated EPS. The gray lines indicate the wavenumbers 1630 cm^{-1} (-NH bending) and 1030 cm^{-1} (C-O-C stretching)

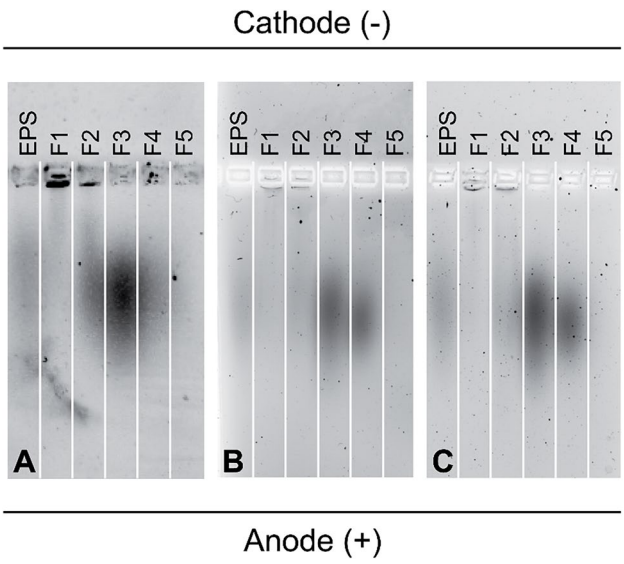

Fig. 4 Native gel electrophoresis on agarose stained with Coomassie Blue (**A**), Alcian Blue pH 2.5 (**B**), and Alcian Blue pH 1.0 (**C**) with 10 µg of unfractionated EPS (EPS), and the different molecular weight fractions F1 (2815– > 14,320 kDa), F2 (439–2815 kDa), F3 (68–439 kDa), F4 (12–68 kDa), and F5 (< 12 kDa)

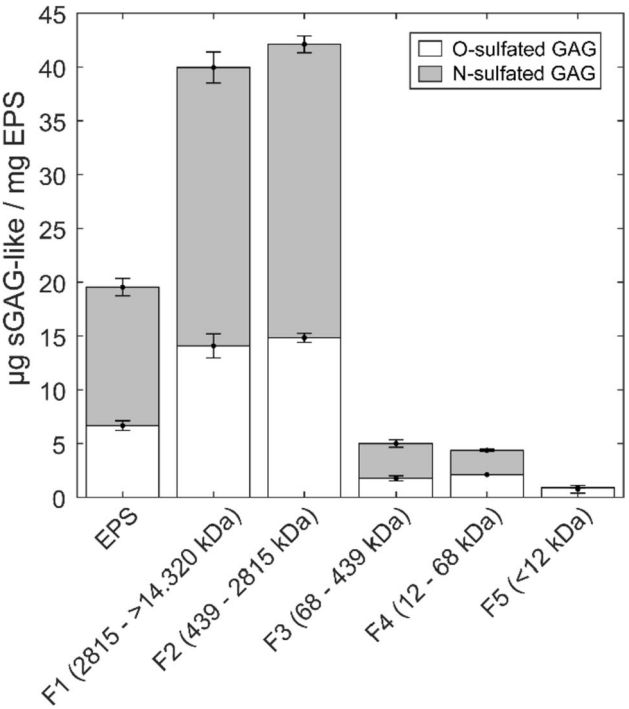

Fig. 5 The content of sulfated glycosaminoglycans in the EPS and its fractions (µg sGAG-like/mg EPS) with chondroitin-4-sulfate as reference. White: O-linked sulfated GAGs; Gray: N-linked sulfated GAGs.

1.0, indicating the presence of glycoconjugates containing both R-COO^- and R-OSO^{3-} groups. In comparison, both F3 and F4 were stained as a smear with Alcian Blue at both pH 2.5 and 1.0, implying the glycoconjugates which contained R-COO^- and R-OSO^{3-} groups have a range of chain length (Fig. 3C and Fig. 4B). In contrast, F5 did not react with any of the dyes. Probably because the agarose gel has large pore size (Stellwagen 2009), F5, which has the molecular weight of < 12 kDa, could not be retained in the gel.

Distribution of sulfated glycoconjugates and NulOs in the EPS fractions

Sulfated glycoconjugates and the carboxylic groups of NulOs confer negative charges to the EPS. To confirm the presence and the types of sulfated glycoconjugates and NulOs, the extracted EPS and the fractions were analyzed by DMMB assay for sulfated glycoconjugates and mass spectrometry for NulOs, respectively.

Quantification of sulfated glycoconjugates by DMMB assay has been reported to be more sensitive and reliable than Alcian Blue (De Jong et al. 1994; Zheng and Levenston 2015). As shown in Fig. 5, the amount of sulfated glycoconjugates (chondroitin-4-sulfate equivalent) in F1 and F2 was increased by 2-fold in comparison to that of the unfractionated EPS. The amount in F3 and F4 was much lower, and F5 showed negligible amounts of sulfated glycoconjugates. These results correspond well to the Alcian Blue staining for R-OSO^{3-} (pH 1.0). Apparently, the fractionation with SEC allowed to obtain fractions which are highly enriched with sulfated glycoconjugates. F1 and F2, which were the two fractions with the highest molecular weights, contained the majority of the total sulfated glycoconjugates.

In addition, it was possible to differentiate the two types of sulfated glycoconjugates, N-sulfation and O-sulfation, by performing nitrous acid pretreatment. N-sulfated glycoconjugates were found to be between 52 and 66% of the total sulfated glycoconjugates in the EPS and the fractions. This suggests that the N-sulfated glycoconjugates are relatively predominant.

To identify the types of nonulosonic acids (NulOs) and estimate their relative amounts in the EPS and its fractions, derivatization with DMB and mass spectrometry was used (Fig. 6) (Tomás-Martínez et al. 2022). The analysis showed that the EPS and all fractions contain predominantly double acetylated NulO, likely the bacterial NulOs pseudaminic or legionaminic acid ($PseAc_2$ or $LegAc_2$), which cannot be distinguished as they have the same molecular mass. However, other types of double acetylated NulOs could not be ruled out. Trace amounts of NeuAc were detected in the EPS across F1-F4, while a higher amount was detected in F5. Thus, NulOs were found in all fractions, with F1–F3 containing a higher amount of NulOs than the original EPS and the other fractions.

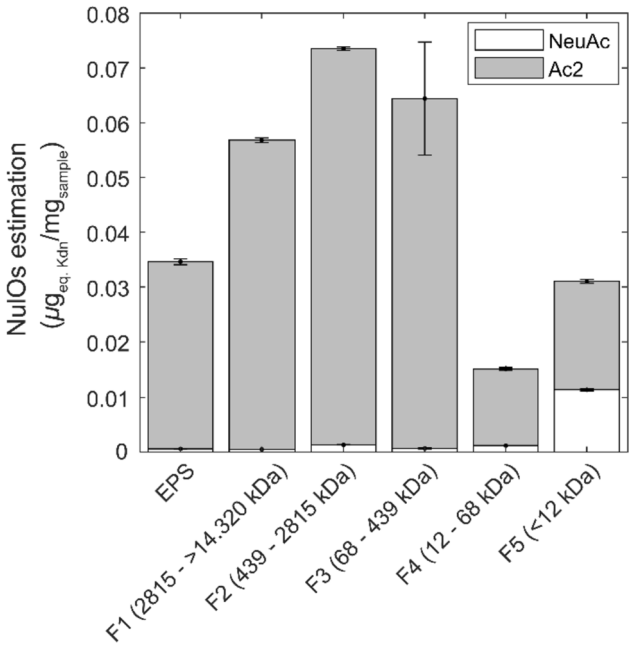

Fig. 6 Relative quantification of nonulosonic acids in the extracted EPS and each fraction (F1-F5) based on the relative area signal of a spiked standard of Kdn. Correlating the spiked signal of KDN with the signal of the detected nonulosonic acids species gives a relative estimation of the EPS and the fractions. The different nonulosonic acids found are NeuAc (white) and double acetylated variant (Ac2), PseAc2, or LegAc2 (gray)

Histone and FGF binding assay

Negatively charged polymers, such as heparin or polysialic acids, can be used to bind cationic proteins due to their high concentration of anionic groups. These polymers have been used for binding histones as the treatment of sepsis and as a column material for protein purification (Ke et al. 1992; Seeger and Rinas 1996; Nicolin Bolten et al. 2018). To explore the potential of the extracted EPS and the fractions for these applications, histones (H2A and H2B, which are the most abundant histones causing sepsis (Urban et al. 2009)) and the model protein FGF-2 binding tests were performed (Nicolin Bolten et al. 2018). These three cationic proteins were incubated with EPS and its fractions and their migration characteristics before and after incubation were compared (Fig. 7). Cationic proteins migrate toward the cathode due to their positive charge. Upon interacting with the EPS, which is anionic, the charge will be neutralized resulting in a reduction of the migration toward the cathode.

Heparin is the most negatively charged natural polymer. After being incubated with the three proteins, the migration of all of them was completely stopped. Since heparin is a polysaccharide, when it binds with proteins, it may prevent Coomassie Blue staining of the proteins (Zlatina et al. 2017), thus leaving an empty spot on the gel. To compare, the EPS and the fractions demonstrated binding capacity to the three proteins but were not as good as that of heparin. It was observed that after incubation with the EPS, the migration of H2A, H2B, and FGF-2 toward the cathode was indeed reduced, indicating that there was interaction between the EPS and the proteins. The charge neutralization effect of EPS appeared to be stronger for H2B than for H2A, judging from the length of the smear toward the cathode after the incubation. Probably, the binding mechanism is not the same, and/or due to the difference of the cationic property between these two histones. In comparison, the migration of FGF-2 was completely stopped after incubation with the EPS. Likely, since FGF-2 itself already has relatively weak cationic property, binding with the anionic EPS can completely neutralize the charge.

Regarding the fractions, the tendency of their binding capacity to the three cationic proteins is similar. In general,

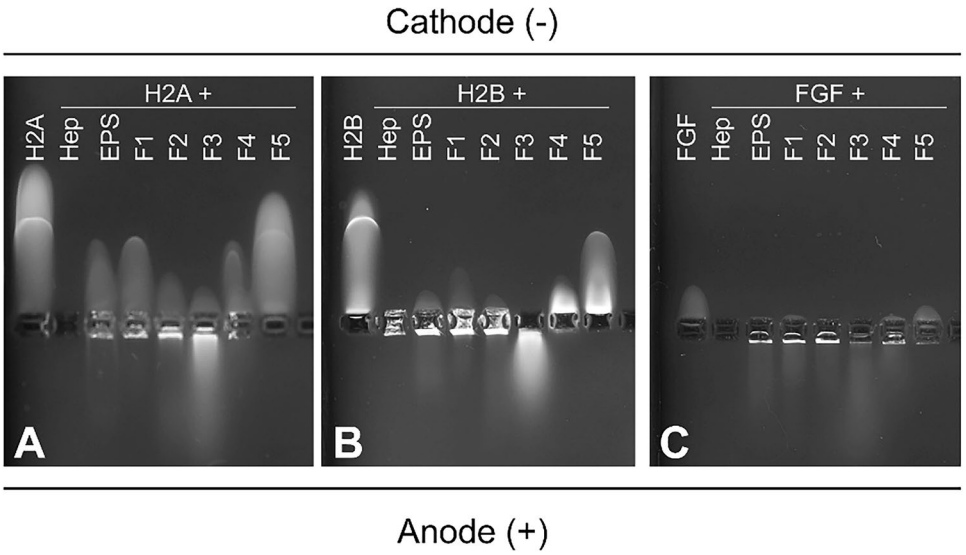

Fig. 7 Protein binding assay. Histone H2A (**A**), histone H2B (**B**), and FGF (**C**) (5 µg) were incubated with heparin (Hep), EPS, and the fractions (10 µg) and stained with Coomassie Blue. F1 (2815– > 14,320 kDa), F2 (439–2815 kDa), F3 (68–439 kDa), F4 (12–68 kDa) and F5 (< 12 kDa)

all five fractions can reduce the migration of the proteins by neutralizing the charge. Their binding capacity was increased from F1 to F3 but decreased from F4 to F5. F1 had roughly the same effect as the EPS, while F2 decreased the migration, and F3 almost stopped the migration of the proteins. It is interesting to see that the two higher aMW fractions (F1 and F2), which have significantly higher amount of sulfated glycoconjugates and the similar amount of NulOs as F3, did not neutralize the charge of the proteins as well as F3. Probably, besides the anionic sulfated glycoconjugates and NulOs in the EPS, other factors such as polymer conformation, molecular weight, and other anionic groups might also be involved in protein-EPS interaction.

Discussion

Sulfate and NulOs containing glycoconjugates are enriched in the high MW fractions of EPS extracted from seawater-adapted AGS

Finding animal-free sources for the production of anionic polymers is of great interest for both medical and chemical applications (Delbarre-Ladrat et al. 2014; Nicolin Bolten et al. 2018). Producing anionic polymer by using bioreactors can be one sustainable alternative. In this study, the anionic polymers were successfully extracted from seawater-adapted aerobic granular sludge and enriched by size exclusion chromatography fractionation. EPS fractions with aMW ranging from 68 to 14,320 kDa were enriched with sulfated glycoconjugates and/or NulOs containing glycoconjugates. They displayed application potential as a raw material in the pharmaceutical and chemical fields.

The presence of anionic polymers with sulfated groups and NulOs has been reported in granular sludge (Bourven et al. 2015; Felz et al. 2020; Boleij et al. 2020; Tomás-Martínez et al. 2022). The amount of sulfated glycoconjugates (chondroitin sulfate equivalent) in anammox EPS was around 6.9%, and in aerobic granular sludge EPS was around 8.7% (Felz et al. 2020; Boleij et al. 2020). In the current research, it was found that the amount of sulfated glycoconjugates was about 2.0% of the extracted EPS, which is relatively lower than what was reported in the literature. However, the molecular weight of the EPS fractions reported here is much higher in comparison to the literature value, i.e., > 439 kDa in this research versus 12 kDa for anammox EPS (Boleij et al. 2020) and 10–50 kDa for freshwater aerobic granular sludge EPS (estimated from the elution time reported in the supplemental material of Felz et al. 2020). Regarding NulOs containing glycoconjugates, the EPS fractions from a "*Ca*. Accumulibacter phosphatis" enriched culture might resemble more closely to what is found in this study (Tomás-Martínez et al. 2022). The high molecular weight fractions (5500– > 15,000 kDa) reported by Tomás-Martínez et al. (2022) contained the majority of NulOs. However, it is unknown if those fractions have sulfated conjugates or not. Although a few factors might cause those differences, such as the microbial communities, the EPS extraction, separation methods, and the operational conditions, it is expected that cultivation in seawater might contribute to the formation of EPS rich in anionic groups such as NulOs and sulfated groups.

Seawater environments have been shown in stimulating the production of glycoconjugates with anionic properties containing sulfated and carboxylic groups (e.g., NulOs) (Decho and Gutierrez 2017). There are indications that marine bacteria possess NulO production genes (Lewis et al. 2009; McDonald and Boyd 2021). In addition, marine bacteria *Pseudomonas* and *Alteromonas* have been found to produce sulfated polysaccharides for protection and adhesion. The average molecular weight of those polysaccharides is 330 kDa and 1000 kDa, respectively (Raguenes et al. 1996; Matsuda et al. 2003; Colliec-Jouault et al. 2012). Likely, microorganisms produce specific glycoconjugates to adapt to seawater condition. On the other hand, high molecular weight glycoconjugates produced under non-saline condition have been reported as well. For instance, capsular polysialic acid from *E. coli* K-235 can reach up to 1000 kDa (Colley et al. 2014; Rohr and Troy 1980). Whether seawater has an impact on the production of high molecular weight, glycoconjugates containing both NulOs and sulfated groups should be a topic for further investigation.

Binding with cationic proteins reveals potential applications for EPS extracted from seawater-adapted AGS

Anionic polymers have broad applications, e.g., heparin, a highly negatively charged biopolymer extracted from animal mucosa, can be used for medical treatment such as sepsis (Wildhagen et al. 2014) and for chemical applications such as column material for protein separation (Nicolin Bolten et al. 2018). In the current research, the extracted EPS and its fractions displayed binding capacity to histones H2A and H2B, and FGF-2, which are model proteins related to these applications. This indicates that the EPS, especially the higher MW fractions, have great potential to be used as raw material for pharmaceutical and chemical applications.

Among the EPS fractions, F1, F2, and F3 bind the model proteins much stronger than F4 and F5. The most apparent difference is that F1–F3 are both heavily sulfated and contain more NulOs, which suggests that both of these two types of glycoconjugates are crucial for the binding of cationic proteins. Other factors such as molecular weight and charge density influence the binding capacity as well. In fact, it is reported that particular patterns of sulfation on the glycan

chain and the linkage influence the binding strength of heparan sulfate with FGF and histones (Mulloy 2005; Lindahl and Li 2009; Meara et al. 2020). A decrease in molecular weight from a sulfated glycosaminoglycan analog shows increased binding affinity to growth factors (Esposito et al. 2022). The chain length of polysialic acid affects the binding with histone (Zlatina et al. 2017). In addition, other anionic groups such as phosphate may play a role as well. Further research is needed to determine the exact mechanism of protein-EPS interaction in order to manipulate the bioprocess toward producing anionic polymers which have higher binding capacity.

There are limited reports about the interaction between the anionic EPS and cationic proteins. Comparing to a recent study which looked into the interaction between histones and EPS fractions recovered from "*Ca.* Accumulibacter phosphatis" enriched culture, it was found that if the same ratio between the histone and EPS fraction is applied, the binding capacity of the EPS fractions recovered from seawater-adapted aerobic granular sludge, in this study, is higher (judging from the length of the migration smear of histone-EPS complex) (Tomás-Martínez et al. (2022). This might be because the EPS fractions in the current study contain both sulfated and NulO glycoconjugates. Moreover, the binding ability to both histones and FGF-2 protein of the EPS fractions in this research clearly displayed that the EPS recovered from seawater-adapted AGS is not only an attractive raw material in the medical field but also has the potential to be applied as a column material in the chemical field (Nicolin Bolten et al. 2018). It is worth pointing out that, different from animal-based biomolecules, microbial polymers can be produced in strictly controlled environments. This can reduce the variations in the quality of the product and contaminations with generally lower production costs (Sutherland 1998).

Challenges in the characterization of glycoconjugates in EPS

To produce EPS with specific application potential, studies are needed to elucidate their exact molecular structure, which is important to understand their function and identify the regulation pathways. The structural diversity of glycans and the unavailability of well-defined glycoconjugate standards make the in-depth EPS characterization challenging (Schäffer and Messner 2017). In addition, glycan structures cannot be directly translated into a genetic template. External factors such as nutrient availability and environmental conditions can modify the structures as well (Varki et al. 2017a). Glycan structures and modifications (e.g., sulfate and NulOs) may vary depending on the environmental conditions (Poli et al. 2010; Schäffer and Messner 2017). These structural variations can play a critical role in the bioactivity related to their applications. By studying the changes in EPS composition coupled with omics techniques under different environmental conditions, it might be possible to gain a better understanding of the factors regulating their production and chemical structures. This information can ultimately contribute to an improved control of the EPS production in the bioreactor.

To understand which components of the EPS contribute to the protein binding ability, the structure of the EPS components needs to be revealed. For the glycoconjugates, specific labelling methods, such as microarrays, can be employed by targeting functional groups (Seviour et al. 2019). Additionally, a combination of separation methods, such as size exclusion chromatography, coupling with mass spectrometry may enable the structural elucidation of individual components (Zamfir et al. 2011). Finally, when the purified anionic glycoconjugates are obtained, protein-ligand studies are necessary to understand the specificity and affinity, and ultimately, clinical tests are required for evaluating the possible application in the medical field.

In summary, the current study has demonstrated that the EPS extracted from seawater-adapted AGS contain anionic polymers rich in NulOs and sulfated glycoconjugates. The majority of these two glycoconjugates are located at the higher molecular weight EPS fractions. The binding of the EPS and its fractions with cationic histones and FGF-2 protein suggests their potential application as a raw material in pharmaceutical and chemical fields. EPS extracted from seawater-adapted AGS can be a valuable source for the recovery of biomolecules with interesting properties.

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Author contribution LC and YL planned the research based on discussions among PB, MP, and MvL. LC and PB conducted most experiments. JvE performed the mass spectrometry analysis. LC and PB interpreted the data with support of YL, MP, JvE, and MvL. LC and YL drafted and wrote the manuscript with input of MP and MvL. All authors read and approved the manuscript.

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Data Availability The data generated and/or analyzed in the current study are included in this article and its supplementary material.

Declarations

Ethical approval and consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

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