

Electrochemistry of chitosan amino-glycan and BSA protein mixture under seawater conditions

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Abstract

We investigated the electrochemical properties of the models chitosan oligosaccharide (CHOS), an amino-glycan, and BSA protein, as major components of N-bearing polymeric organic material (N-POM) in seawater. The chronopotentiometric stripping method (CPS) and a hanging mercury drop working electrode (HMDE) were used to detect catalytic peaks (E_p around -1.8 V/ -1.9 V vs. Ag/AgCl (3M KCl)) generated by the catalytic hydrogen evolution reaction (CHER). “Peak H_{OS}” of CHOS was not observed under seawater conditions (pH 8.2). Since both analytes measured separately produced catalytic peaks in CPS after their adsorptive transfer (AdT) from seawater into slightly acidic and neutral buffers (pH-s 5.3-7.1), we investigated measurement conditions that might allow selective AdT detection of CHOS in the CHOS+BSA seawater mixture. Only one catalytic peak was observed in the AdT CPS of mixture, which was attributed to the involvement of both compounds in adsorption on HMDE and consequently in CHER. The height and E_p of this peak tended to correspond to the analyte with the higher concentration in the mixture. Regardless of the choice of pH or buffer, the combination of CPS measurement conditions or the rinsing mode by relevant solvents and solutions during AdT, BSA was not efficiently removed from the HMDE and selective detection of CHOS in the mixture was not achieved. These results will be applicable in the electrochemical analysis of amino-glycans in natural seawater samples containing a mixture of N-POM, but also represent an investigation of the basic electrochemical properties of CHOS and BSA.

Keywords: catalytic peak, chitosan oligosaccharide, chronopotentiometry, organic matter, protein, seawater.

1. Introduction

Entrapping about 10^{15} g of carbon, dissolved organic matter (DOM) in ocean represents the largest pool of reduced and thus potentially reactive carbon on Earth [1]. Within DOM, dissolved organic nitrogen (DON) represents an important form of fixed N. DON is directly involved in the biogeochemistry of N, the most abundant biogenic element after C and O. On the other hand, DON serves as a potential source of bioavailable N after microbial transformation and can therefore control marine primary production [2]. The chemical components of DON vary in size, complexity and resistance to degradation and transformation. Structurally, 70–90% of oceanic DON is in the form of amides, the majority of which belong to amino acids and the smaller portion to amino sugars [3]. The structure and associated concentration of certain DON molecules provide information about their biogeochemical function. The oldest analytical methods for quantification of N-containing organic compounds in seawater are based on the specific chemical reactions of added reagent with free primary amino groups and were studied intensively in the 1970s and 1980s [4]. Colorimetric (ninhydrin reaction) and fluorimetric methods (o-phthaldialdehyde or fluorescamine reaction) were the most commonly developed methods for the analysis of total free amines in seawater [5]. The development of separation methods allowed the quantification of specific amine-containing compounds. Marine proteins have been characterized by gel electrophoresis [6], immunochemical assays [7] and HPLC-MS [8]. Since its introduction in 2000 [9], HPLC coupled with pulsed amperometric detection (HPLC-PAD) has been used for the qualitative and quantitative determination of specific amino sugars such as glucosamine, galactosamine, mannosamine and muramic acid in seawater [10, 11]. This method does not require derivatization and its detection limit is (2–10) nmol/L. Recently, we highlighted the electrochemical method of chronopotentiometric stripping (CPS) as a sensitive, rapid, label-free, reagent-free and low-priced method for the detection of protein-like polymers directly in seawater [12, 13]. The CPS analysis is based on the detection of a specific catalytic “peak H” at slightly less negative potential than the hydrogen wave due to the catalytic hydrogen evolution reaction (CHER) at mercury-containing electrodes [14]. However, at that time, amino-bearing polysaccharides such as chitosan (containing glucosamine and *N*-acetyl glucosamine groups) together with short amino-oligosaccharides were not known to produce a similar catalytic peak as proteins and peptides [15, 16]. Specific residues of Arg, Lys, His and Cys in proteins and peptides [17, 18] and free amine groups in amine-containing glycans [16] are mandatory for the occurrence of CHER. CPS experiments with chitosan, first performed in 2014 [15] and then in 2016 [16], were carried out in acetate

(pH 5.2) and McIlvaine buffers (pH 5.6) under weakly acidic conditions. Since the occurrence and characteristics of the catalytic peak depend drastically on the measurement conditions, especially pH and buffer capacity [19], it is of electroanalytical interest to determine experimental conditions for the selective and sensitive CPS detection of amino-glycans (as recently discovered to participate in CHER) in the presence of proteins (well-studied CHER molecules) under seawater conditions. Seawater contains a natural buffer system of carbonate (2.3 mmol/L) and borate (0.41 $\mu\text{mol/L}$) that ensures a slightly alkaline pH around 8.20 [20]. For comparison, while HSA protein provides a peak H [12], sulfated polysaccharides κ -, ι - and λ -carrageenans do not yield a catalytic peak under seawater conditions [21]. In this context, we performed electrochemical studies of chitosan oligosaccharide (CHOS) glycan, BSA protein and their mixtures (CHOS+BSA) at the hanging mercury drop electrode (HMDE) in a model seawater electrolyte. Our aim was to investigate measurement conditions for the selective electrochemical detection of CHOS in a CHOS+BSA mixture under seawater conditions. In addition to the electrochemical analysis of protein-like compounds, this work extends for the first time the CPS analysis to amino-like glycans under seawater conditions. Since protein-like and glycan-like organic molecules are ubiquitous in natural waters and the most abundant N-organic molecules in the dissolved organic fraction (<0.45 μm) in seawater, this work contributes to their electrochemical analysis, which should allow a detailed interpretation of their biogeochemistry in the marine environment.

2. Materials and methods

2.1. Instrumentation and methods

Electrochemical experiments were performed using a 663VA Stand multimode three-electrode system (Metrohm, Herisau, Switzerland) connected to a potentiostat/galvanostat ($\mu\text{Autolab}$ type III) and an Interface for Mercury Electrode (IME) module (Autolab, Utrecht, The Netherlands). A hanging mercury drop electrode (HMDE, 0.40 mm^2 surface area) served as the working electrode and a glassy carbon (GC) rod as the auxiliary electrode. All potentials were defined with respect to the Ag/AgCl (3 M KCl) reference electrode. All measurements were guided PC by *GPES 4.9* software.

CPS setup included 60 s (t_a) long accumulation at $E_a = -0.1$ V (unless otherwise specified) followed by constant current ($I_{\text{str}} = -5$ to -160 μA) stripping, under air with an equilibration time of 1 s and a maximum time of measurement of 3 s. Square-wave voltammetry (SWV) was used to investigate the nature of the electrochemical process of CHOS in 0.05 mol/L

phosphate buffer (pH 7.05), under the following measurement conditions: 300 s deaeration by introducing nitrogen gas (99.999 %), $E_a = -0.1$ V, $t_a = 60$ s, step potential $E_s = 4$ mV, and amplitude $a = 25$ mV. The adsorption/desorption of CHOS and BSA on HMDE was studied by phase-sensitive alternating current voltammetry (ACV) with *phase-out* mode (phase angle 90°) in seawater without prior oxygen removal, with $E_a = -0.6$ V, $t_a = 60$ s, frequency $f = 77.35$ s⁻¹, $E_s = 10$ mV, $a = 10$ mV. CPS, SWV and ACV accumulation steps were accompanied by stirring with a Teflon stirrer at 1500 rpm. Each measurement was repeated twice and the mean value was taken for further processing.

The *ex situ* adsorptive transfer (AdT) technique involves the accumulation of the analyte (CHOS, BSA, or their mixture) on the HMDE surface in seawater ($E_a = -0.1$ V, $t_a = 60$ s) and the transfer of the modified HMDE (with the surface-attached molecules) into the blank background electrolyte (buffer). There, the CPS was performed at constant I_{str} or AC voltammogram was recorded in the potential range from -0.1 V to -1.9 V (AdT ACV).

The stability of the adsorbed CHOS layer on the HMDE surface was determined after AdT of CHOS-modified HMDE to the 0.05 M phosphate buffer pH 7.05, with CPS detection after a certain time t_b during which the buffer was stirred (1500 rpm) without applied potential.

Rinsing of the BSA-modified HMDE (after accumulation of 20 $\mu\text{g/mL}$ BSA from seawater, $E_a = -0.1$ V, $t_a = 60$ s) was performed during transfer with specific solvents and solutions in two ways: 1) by immersing the BSA-modified electrode in the solvent or solution for 60 s, without stirring, and 2) by immersing the BSA-modified electrode in the solvent or solution three consecutive times. The BSA-modified electrode was then rinsed with Milli-Q water (so as not to contaminate the stripping background electrolyte) and immersed in phosphate buffer pH 7.05, where CPS ($I_{str} = -40$ μA) was performed.

A bench pH meter with glass Ag/AgCl electrode (Hanna Instruments, Croatia) was used to control the pH of the prepared buffers and the measured solutions after calibration with Hanna pH 4.01 and 9.01 buffer standards.

2.2. Chemicals and solutions

Chitosan oligosaccharide lactate (CHOS, $(\text{C}_{12}\text{H}_{24}\text{N}_2\text{O}_9)_n$, M_w 4 000-6 000 Da (average 5 000 Da), degree of deacetylation > 90 %, water soluble) and bovine serum albumin (BSA, M_w 66 463 Da, lyophilized) were purchased from Sigma-Aldrich, Steinheim, Germany. Appropriate Milli-Q water dilutions of 2 mg/mL CHOS and 5 mg/mL BSA stock solutions were used.

The "organic matter-free" seawater was a background electrolyte in which the accumulation

step took place. It was prepared by removing all organic molecules from the seawater previously collected in the Adriatic Sea. The seawater was first filtered through a filter with a pore size of 0.7 μm (GF/F, Whatman) to remove particulates, then the filtrate was exposed to UV radiation (150 W UV lamp) for 24 hours to destroy the dissolved organic fraction, and then it was left in an activated charcoal mixture for the next 24 hours, which was finally filtered off.

Phosphate and acetate buffers were the stripping electrolytes. Phosphate buffers (0.05 mol/L, pH 6.06, 7.05 and 8.06, unless otherwise indicated) were prepared by mixing a specific volume ratio of solutions prepared by dissolving $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (ACS reagent, 98.0-100.0 %, Sigma-Aldrich, Steinheim, Germany) and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (Merck-Millipore, Darmstadt, Germany) in Milli-Q water. Acetate buffer (pH 5.28, 0.05 mol/L) was prepared by mixing a specific volume ratio of acetate acid and a solution prepared by dissolving solid CH_3COONa (both from Kemika, Zagreb, Croatia) in Milli-Q water.

The following solvents and solutions were used for rinsing the modified HMDE: ethanol (Kemika, Zagreb), 2-propanol (99,9 %, Riedel-de Haen, Germany), KOH (2 mol/L) by dissolving solid KOH (Kemika, Zagreb), KCl (2 mol/L) by dissolving solid KCl (Riedel-de Haen, Germany), ammonium acetate (0,25 mol/L, Sigma-Aldrich, Steinheim, Germany), acetone (Kemika, Zagreb), HNO_3 (10 %, p.a., Kemika, Zagreb), urea (4 mol/L, by dissolving the solid chemical (Sigma-Aldrich, Steinheim, Germany), $\text{Pb}(\text{NO}_3)_2$ (10 $\mu\text{mol/L}$, Sigma-Aldrich, Steinheim, Germany), and SDS (10 mmol/L, Sigma-Aldrich, Steinheim, Germany).

3. Results and discussion

3.1. Electrochemistry of CHOS on Hg electrode

CPS analysis of CHOS in seawater (pH 8.20) did not reveal a peak in the chronopotentiogram either at high concentration (100 $\mu\text{g/mL}$), low reduction stripping current ($-3 \mu\text{A}$) or long accumulation (300 s) (Fig. 1A). The adsorptive transfer (AdT) CPS of 50 $\mu\text{g/mL}$ CHOS from seawater into 0.05 M phosphate (pH 7.05, 6.06) or acetate buffer (pH 5.28) showed a well-defined peak with E_p between -1.80 and -1.90 V (Fig. 1A). One reason for this is the ratio of protonated and deprotonated amino groups, which is determined by their intrinsic $\text{p}K_a$ and the pH of the stripping medium. The highest protonation would be at $\text{pH} \ll \text{p}K_a$, although it is not advisable to measure at $\text{pH} < 4$ because of a high hydrogen evolution peak that narrows the working electrode window. Since $\text{p}K_a = 6.3$ for $-\text{NH}_2$ groups in CHOS [22], they would be protonated to a very low percentage at the pH of 8.2 in seawater, which means that they

would hardly undergo CHER. On the other hand, the chitosan oligosaccharide lactate we used produced a peak in phosphate buffer pH 7.05, probably because it carries protonated $-\text{NH}_3^+$ groups.

The peak height in AdT CPS did not change significantly even 20 min (t_b) after transfer to buffers, demonstrating solid adsorption of CHOS on the HMDE surface during and after transfer. CHOS, as a relatively long oligosaccharide, adsorbed strongly on the HMDE, which is very similar to the adsorption of polysaccharides [21, 23], while relatively short oligosaccharides adsorb very weakly [16]. Moreover, 10 consecutive independent AdT CPS measurements of 50 $\mu\text{g/mL}$ CHOS ($E_a -0.1$ V, t_a 60 s) gave very good reproducibility, as evidenced by a low relative standard deviation (% RSD = (SD/mean) x100): RSD of ± 7 % was calculated for phosphate buffer pH 7.05 and acetate buffer pH 5.28 ($I_{\text{str}} -20$ μA), and ± 8 % in phosphate buffer pH 6.06 ($I_{\text{str}} -30$ μA).

Using ACV, SWV and CPS we have confirmed the electrochemical catalytic hydrogen evolution reaction (CHER) on adsorbed CHOS at neutral pH. We have recently shown that CHER of catalytically active molecules such as proteins and polysaccharides is usually accompanied by strong adsorption, giving a tensammetric minimum (T_{min}) at very negative potentials in ACV (*phase-out* mode) [24]. The ACV of CHOS did not give T_{min} in seawater, but a well-defined T_{min} was detected in AdT ACV in phosphate buffer pH 7.05 (Fig. 1B), in agreement with the results of CPS. SWV dependence of peak height on frequency (10-220 s^{-1}) in 0.05 mol/L phosphate buffer pH 7.05 revealed a maximum peak height at 100 s^{-1} and an exponential decrease in I/f vs. f (Fig. 1C), which are features of CHER proceeding on organic molecules adsorbed on the mercury electrode surface [25]. Both the forward and the backward current components were in the negative current range, suggesting an irreversible electrode reaction [26] as CHER. We also investigated the AdT CPS effect of pH and stripping (phosphate) buffer capacity. The peak height gradually increased as we increased the concentration of phosphate buffer (pH 7.05) in the range of 0.01-0.1 mol/L (Fig. S1). The buffer is indirectly involved in CHER the step of regeneration of CHOS catalyst [19]. When the pH was changed from 6.00-8.00 of 0.05 mol/L phosphate buffer, the peak height increased at lower pH values (Fig. S2), indicating a low protonation rate constant of the catalyst at higher pH and a high one at lower pH [19]. The effects of buffer concentration and pH on the CHOS peak are consistent with the behavior of the catalytic peak of proteins, peptides [27] and polysaccharides [15]. Our experiments showed that the amino groups of glycan are catalytically inactive in seawater at pH 8.2, giving no peak H in CPS and no T_{min} in ACV, but allowing CHER not only in slightly acidic, as it is known [16], but also in neutral buffer

solution at $\text{pH} > \text{p}K_a$. Moreover, the peak recorded in phosphate buffers pH 6.06 and 7.05 is higher than the peak obtained in acetate buffer pH 5.28 (Fig. 1A), which in terms of analytical sensitivity gives preference to phosphate buffers to be used as stripping buffer. In the following, the CPS catalytic peak of CHOS will be referred to as “peak H_{OS} ”.

3.1.1. AdT CPS dependence on E_a , I_{str} and CHOS concentration

The AdT CPS dependence of the peak H_{OS} height of 50 $\mu\text{g/mL}$ CHOS on the accumulation potential ($E_a = -0.1 \text{ V}$ to -1.2 V , $t_a = 60 \text{ s}$) was tested in phosphate buffer pH 7.05 ($I_{\text{str}} = -20 \mu\text{A}$) and pH 6.06 ($I_{\text{str}} = -30 \mu\text{A}$) and in acetate buffer pH 5.28 ($I_{\text{str}} = -20 \mu\text{A}$). Measurements in phosphate buffer pH 8.06 were not performed because the peak was very small even at very low stripping currents (such as $-5 \mu\text{A}$, Fig. S3, A). In all buffers, the change of E_a did not significantly affect either the height of the peak H_{OS} or the E_p (not shown), e.g. the peak height in phosphate buffer pH 7.05 was $(dE/dt)^{-1} = (7.9 \pm 0.13) \text{ s V}^{-1}$ in the measured range of E_a -s.

The intensity of the stripping current affects the rate of potential changes and controls the amount of time the adsorbed molecules are exposed to the electric field [28]. By applying more negative stripping currents ($I_{\text{str}} -10$ to $-160 \mu\text{A}$) in the phosphate buffers pH 7.05 and 6.06 and in the acetate buffer pH 5.28, the peak H_{OS} height of 50 $\mu\text{g/mL}$ CHOS decreased exponentially (Fig. S3, A). Adsorbed CHOS was exposed to the effects of the electric field for a shorter time near the electrode surface and therefore produced smaller peaks. At less negative currents, the opposite was true: CHOS near the surface was exposed to the electric field for a longer time, resulting with higher peaks. In phosphate buffer pH 8.06, peak H_{OS} was not observed at currents more negative than $-25 \mu\text{A}$. The E_p of peak H_{OS} shifted toward more negative potentials at all pH values at more negative I_{str} (Fig. S3, B), which also confirmed that CHER favored less negative I_{str} . For each pH, a different optimal current intensity was most appropriate for further measurements: $-20 \mu\text{A}$ was selected for pH-s 7.05 and 5.28 and $-30 \mu\text{A}$ for pH 6.06.

Increasing the CHOS concentration (0.2-100 $\mu\text{g/mL}$, $E_a = -0.1 \text{ V}$, $t_a = 60 \text{ s}$, Fig. 2) in seawater linearly increased the AdT CPS peak height in phosphate buffer pH 7.05 ($I_{\text{str}} = -20 \mu\text{A}$) up to 10 $\mu\text{g/mL}$ ($(dE/dt)^{-1} = (0.86 \pm 0.06) \times [\text{CHOS}]$, $R^2 = 0.97$, inset in Fig. 2A). The peak levelled off at higher concentrations. This result is consistent with the behavior of CHER peaks [19]. The E_p shifted to less negative potentials with increasing CHOS concentration (Fig. 2B), suggesting that CHER is favored when more catalytic groups are

adsorbed on the electrode surface. A detection limit of 1.44 $\mu\text{g/mL}$ was calculated.

3.2. Electrochemistry of BSA on HMDE in seawater

Examining BSA as a model for protein-like material in seawater, we detected peak H *in situ* in seawater along with T_{min} in ACV for 20 $\mu\text{g/mL}$ BSA (Fig. 3), both of which indicate the involvement of BSA in CHER at around -1.80 V under seawater conditions. BSA is involved in CHER under seawater conditions because the intrinsic pK_a -s of the catalytic side-chain groups are higher than the pH of seawater, which was a stripping electrolyte. The pK_a values for Cys is 8.2, Arg is 12.5, Lys is 10.5, and His is 6.0 [29], indicating that Cys, Arg, and Lys were highly protonated and carried labile protons under seawater conditions. The CPS peak H of 20 $\mu\text{g/mL}$ BSA in seawater was not significantly changed by changing E_a from -0.1 V to -1.4 V ($t_a = 60$ s, $I_{\text{str}} = -10$ μA ; $(dE/dt)^{-1} = (11.6 \pm 0.16)$ s V^{-1}), and the peak position remained the same ($E_p - 1.78$ V). By changing I_{str} from -5 μA to -40 μA , peak H height decreased exponentially and finally disappeared at I_{str} more negative than -40 μA , while E_p shifted significantly toward negative potentials, from -1.72 to -1.95 V, indicating a more energetically demanding electrochemical CHER (Fig. S4).

We observed peak H of BSA in AdT CPS in all phosphate (pH-s 6.06, 7.05, 8.06) and acetate (pH 5.28) buffers used in our work (Fig. S4). The CHER of BSA as a model protein in phosphate buffer at neutral (physiological) pH has been very well elucidated for more than two decades [30]. Accordingly, we obtained similar peak H with respect to the CHER after AdT of BSA from seawater into phosphate buffer and the CHER of BSA measured directly in phosphate buffer (not shown), indicating the importance of the involvement of the stripping electrolyte in CHER. This will be relevant in the context of selective AdT CPS detection of CHOS in the CHOS-BSA mixture, where BSA may be an interferent, which will be explored in Section 3.4.

3.3. CHOS affects CPS of BSA in seawater

We studied a BSA-CHOS mixture under seawater conditions to determine whether catalytically inactive CHOS molecules could affect the peak H of BSA in their mixture. The effect of CHOS on CHER of BSA in seawater was investigated by gradually increasing the concentration of CHOS (0.2-100 $\mu\text{g/mL}$) in seawater containing 20 $\mu\text{g/mL}$ of BSA (Fig. 3A). We observed a very sharp decrease in CPS peak H height when the concentration of CHOS increased up to 20 $\mu\text{g/mL}$ CHOS, whereas at >20 $\mu\text{g/mL}$ the decrease was gradual. Since

only adsorbed molecules on the electrode surface participate in CHER, we investigated the adsorption/desorption of CHOS, BSA and their mixture in detail using ACV (*phase-out* mode). 20 $\mu\text{g/mL}$ BSA alone in seawater was strongly adsorbed on the electrode throughout the measurement range from -0.1 to -1.8 V (Fig. 3B). The addition of CHOS affected the BSA adsorption in a negative manner. Specific features of BSA viewed in AC voltammograms, a reorientation peak around -0.6 V (due to reduction of Hg-S bonding [31]) and T_{min} at -1.8 V (due to adsorption associated with CHER [24]), were less pronounced at higher CHOS concentrations (Fig. 3B). T_{min} of BSA disappeared completely at 20 $\mu\text{g/mL}$ CHOS (Fig. 3B). According to the adsorption isotherm (for analytes measured alone) showing a concentration dependence of electrode surface coverage, ≥ 10 $\mu\text{g/mL}$ BSA ensured complete coverage of the electrode surface, whereas CHOS confirmed complete surface coverage for ≥ 20 $\mu\text{g/mL}$ CHOS (Fig. S5). In the mixture, the most intense effect of CHOS on BSA peak H was visible after addition of a CHOS concentration that would normally cover the entire electrode surface (≥ 20 $\mu\text{g/mL}$ CHOS). The height of the peak H of BSA decreased probably due to the progressive displacement of BSA from the electrode by surface-active CHOS. Moreover, the E_p shifted from -1.80 V to -1.90 V with increasing CHOS concentration, indicating a more energetically demanding CHER of BSA when its adsorption was disturbed. The formation of a BSA-CHOS complex via electrostatic interactions [32], which could mask the catalytic groups of BSA, would be less likely in seawater due to its high ionic strength (0.7 mol/L), which induces the dissociation of such a complex [33]. In addition, we hypothesize that other natural surface-active organic substances would also reduce the height of the peak H, especially catalytically inactive ones under seawater conditions, but this remains to be investigated.

3.4. BSA affects AdT CPS of CHOS

We examined the effect of (0.2-100) $\mu\text{g/mL}$ BSA on CHER of 50 $\mu\text{g/mL}$ CHOS adsorbed in seawater and stripped in 0.05 M phosphate buffer pH 7.05 (Fig. 4). Regardless of the BSA concentration, we observed only one catalytic peak in the CHOS-BSA mixture (Fig. 4A, B). The height of this peak did not change significantly up to 8 $\mu\text{g/mL}$ of added BSA, but increased at >8 $\mu\text{g/mL}$ of BSA, while at 50-100 $\mu\text{g/mL}$ of BSA the peak actually doubled its height and levelled off with a height around 4.5 s V^{-1} . The same concentration range of BSA alone ((0.2-100) $\mu\text{g/mL}$) measured in AdT CPS produced a higher peak H with a maximum height around 15 s V^{-1} at ≥ 10 $\mu\text{g/mL}$ BSA (Fig. 4A) when complete surface coverage was

achieved. The peak obtained in the mixture was higher than the peak of CHOS alone and smaller than the peak of BSA alone. In the mixture, the E_p shifted from -1.91 V to -1.80 V at higher BSA concentration, which E_p is more similar to the peak H of BSA alone. It is evident that with increasing BSA concentration, more BSA molecules were adsorbed and involved both with CHOS molecules in CHER. The adsorption characteristics may contribute to the understanding of CHER in the CHOS-BSA mixture. T_{min} recorded in AdT ACV (adsorption in CHOS-BSA seawater mixture, stripping in 0.05 M phosphate buffer pH 7.05) accompanied the catalytic peak: it decreased, had a minimum at $8 \mu\text{g/mL}$, increased at $>8 \mu\text{g/mL}$, and leveled off at $60\text{-}80 \mu\text{g/mL}$ BSA (Fig. 4C, D). T_{min} shifted from -1.82 to -1.74 V and at $80 \mu\text{g/mL}$ BSA its shape resembled the T_{min} of BSA alone. The reorientation peak at -0.6 V increased due to the higher BSA concentration added in the mixture and the ability of more BSA to form Hg-S bonds (not shown). Regardless of the BSA concentration, only one T_{min} occurred for the CHOS+BSA mixture. Since both analytes are surface active and catalytically active on Hg electrode, their concentration ratio would be crucial for involvement in CHER, i.e., the analyte occupying the larger electrode surface would dominate in CHER. Considering natural seawater samples as a mixture of biologically relevant organic molecules (exuded by phytoplankton or released by cell lysis in seawater), the AdT CPS peak detected in the samples would be a result of the participation of all catalytically active molecules anchoring on the electrode. To detect N-glycans in such a mixture, we investigated measurement conditions that would allow their selective detection.

3.5. Testing conditions for selective AdT CPS of CHOS in seawater

The goal was to explore measurement conditions that: largely attenuate the effect of $20 \mu\text{g/mL}$ BSA on peak H_{OS} of $50 \mu\text{g/mL}$ CHOS when both are adsorbed on the electrode in seawater, selectively eliminate BSA from the electrode when both analytes are adsorbed, or allow detection of both analytes via two separate peaks. We considered the effect of different I_{str} intensity, varying E_b , and rinsing of the BSA-modified HMDE during transfer on the AdT CPS E_p and peak height of the CHOS+BSA mixture.

3.5.1. Effect of I_{str} in phosphate (pH 7.05) vs. acetate (pH 5.28) buffer

A comparison of the heights of peak H of BSA and peak H_{OS} of CHOS measured separately in phosphate buffer pH 7.05 and acetate buffer pH 5.28 shows a large influence of the stripping buffer composition on CHER. As evidence, we can compare the catalytic peak

heights at $I_{\text{str}} -20 \mu\text{A}$, which for CHOS alone was 8.2 s/V in phosphate, while 4.8 s/V in acetate buffer and for BSA alone was 48.8 s/V in phosphate and 12.4 s/V in acetate buffer (Fig. 5). The CHER of both BSA and CHOS measured separately was more pronounced in phosphate buffer, which was probably due to the interplay of $\text{p}K_{\text{a}}$ and pH of the stripping buffer used. In phosphate buffer with $\text{p}K_{\text{a}2}$ 7.2 for the pair $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$, a higher concentration ratio of $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$ at pH 7.05 probably allowed a more efficient reprotonation of the catalyst [34]. In acetate buffer with $\text{p}K_{\text{a}}$ 4.8 for $\text{CH}_3\text{COOH}/\text{CH}_3\text{COO}^-$, there was a higher concentration of unprotonated acetate ions than protonated acetic acid at a working pH of 5.28. In addition to the interplay of $\text{p}K_{\text{a}}$ and pH, the effects of the electric field on the electrode surface were crucial for the very high peak H in phosphate buffer at $-I_{\text{str}} < 55 \mu\text{A}$, when the structural transition of BSA from the native to the denatured form was induced, exposing more catalytic groups of denatured BSA to CHER. There was no similar change in the structure of CHOS by changing I_{str} . However, in terms of sensitivity, phosphate buffer would be a better choice as a stripping electrolyte when BSA or CHOS is studied alone. Regardless of the application of different I_{str} between -15 and $-100 \mu\text{A}$, we obtained only one AdT CPS peak of similar height in both buffers/pH-s for CHOS+BSA seawater mixture. The peak height decreased with more negative I_{str} , and the E_{p} shifted to more negative potentials, similar to the results obtained for the analytes measured separately. For all applied I_{str} , the peak of mixture observed in phosphate buffer pH 7.05 was higher than the peak H_{OS} but smaller than the peak H (Fig. 5A, B). It could be that CHOS, when adsorbed on the electrode together with BSA, protects BSA from the electric field effects, so that the structural transition from the native to the denatured form, evidenced by a sharp increase in peak height, does not occur until $-I_{\text{str}} < 20 \mu\text{A}$. It seems that the interplay of $\text{p}K_{\text{a}}$ and pH of the buffers used was not decisive in the CHOS-BSA mixture, but other factors such as their possible interactions on the electrode [33], actually could lead to a decrease in the height of the peak H of BSA in phosphate buffer, which appeared as a peak with a similar height as in acetate buffer. In acetate buffer pH 5.28, the peak height values for $-I_{\text{str}} > 40 \mu\text{A}$ were more similar to peak H of BSA alone, while the peak for $-I_{\text{str}} < 35 \mu\text{A}$ was higher than the peak of CHOS or BSA alone (Fig. 5C, D), indicating an additive effect of both analytes, BSA in its native form and CHOS. However, these results did not show selectivity for CHOS detection in the mixture with BSA, but may be a guide for further studies. The greatest difference between the heights of the catalytic peaks was seen at less negative I_{str} values when the molecules were exposed to the effects of the electric field for a longer time; therefore, lower $-I_{\text{str}}$ such as $-20 \mu\text{A}$ should be used. In the context of selecting a suitable

buffer for the analysis of the N-POM mixture in marine samples, acetate buffer would be a better choice as peak of the mixture was higher in acetate buffer. In the analysis of natural seawater samples, the peak detected in acetate buffer at $-20 \mu\text{A}$ (after AdT from any natural seawater sample) would indicate an additive contribution of analytes to CHER, so it should be ascribed to all catalytically active molecules in the sample rather than to one component of N-POM.

3.5.2. Effect of E_b after the transfer

We investigated the effect of E_b on the peak of the CHOS+BSA mixture as well as on the peak of BSA and CHOS measured separately due to possible selective desorption of BSA after AdT. After adsorption of both analytes from seawater to the electrode surface ($E_a = -0.1 \text{ V}$, $t_a = 60 \text{ s}$) and AdT to 0.05 mol/L phosphate buffer pH 7.05, different E_b ranging from -0.1 V to -1.7 V were applied for 1 s before the stripping step ($I_{\text{str}} = -40 \mu\text{A}$) (Fig. 6). When a less negative E_b of -0.1 V to -1.1 V was applied, the BSA peak height was drastically lower, while the CHOS and CHOS+BSA peak heights did not change significantly compared to peaks recorded in AdT CPS without E_b step. At $E_b -1.1 \text{ V}$ to -1.7 V , there was a steep increase in peak heights recorded in all three solutions, probably because a highly negative charge induced denaturation of BSA at the negatively charged electrode [35] and to a possible change in the orientation of the self-assembled CHOS layer [36]. Although applied for 1 s , E_b more negative than -1.7 V efficiently and indiscriminately removed both analytes from the electrode surface, causing an abrupt decrease in peak height. In the CHOS+BSA mixture, BSA denaturation was not as pronounced as in the BSA solution, which may be attributed to the lesser influence of the negative electrode charge on BSA when both CHOS and BSA were adsorbed on the electrode.

3.5.3. Rinsing of BSA-modified HMDE during transfer

Our goal was to find a solvent or solution that could wash adsorbed BSA off the HMDE surface. Table 1 lists the solvents and solutions used and the percent decrease (negative value) or increase (positive value) in peak H height of BSA observed after washing compared to peak H height without washing. Milli-Q water, 2-propanol, acetone and SDS were selected because they efficiently remove the BSA film from the biomaterials [37]. We added to this list ethanol and methanol as organic solvents, HNO_3 as acid, KCl solution due to its high ionic strength, Triton-X-100 as detergent, urea as denaturant and Pb^{2+} as metal

ions binding catalytic residues of amino acids [38]. A maximum decrease in peak H height (-17 %) was obtained after 60 s of rinsing with 0.25 mol/L ammonium acetate solution, probably because this solution was used to wash different proteins off the HPLC column. However, our rinsing experiments were unsuccessful, probably due to mercury-thiolate binding [39], which provides tight binding of BSA to the HMDE surface (the solubility product constant, K_{sp} of HgS is 1×10^{-54} [40]). Rinsing the BSA-modified HMDE with other solvents and solutions resulted in a decrease in the peak H height by up to -12 %. Milli-Q water practically did not change the peak H, which is why it was used to wash the protein-modified electrode in many AdT experiments (to remove nonspecifically adsorbed analytes) [17]. On the other hand, a suitable solvent for the removal of BSA should not remove CHOS from the electrode. When we rinsed the CHOS-modified HMDE with ammonium acetate for 60 s, the peak H_{OS} height was changed by 3 %, implying that it could serve to wash other N-POM components while amino-glycans would remain adsorbed. It should be noted that the HMDE is very sensitive to mechanical stimuli such as physical transfer from one electrolyte to another and can easily fall off, requiring the measurement to be repeated. Prolonged washing or repeated immersion in the solution would therefore not be another option.

4. Conclusions

We investigated measurement conditions for selective CPS detection of amino-glycans under seawater conditions on a mercury electrode. We studied CHOS for the first time in seawater and reported that it does not produce any peak in the chronopotentiogram recorded under seawater conditions. After AdT of CHOS-modified electrode from seawater into acetate or phosphate buffers at weakly acidic and neutral pH-s, a CPS yielded a well-defined peak H_{OS} of CHOS (Fig. 1). Specific SWV and CPS results indicated the involvement of CHOS in the irreversible catalytic (accelerated faradaic) electrochemical process of CHER. BSA yielded peak H both in seawater and in the AdT procedure. With regard to the selective AdT CPS detection of amino-glycans in real natural seawater samples, neither application of different I_{str} or E_b nor rinsing of the protein-modified electrode with any of the solutions or solvents used (Table 1) would probably not result in its selective removal, but all adsorbed catalytically active molecules would contribute to CHER under selected electrochemical conditions. The problem are different N-POM organic components that cannot be detected only in one buffered solution (whether seawater or model buffer) or at one pH, because

different catalytic groups with different dissociation constants or intrinsic dissociation constants (e.g., proteins with many different catalytic groups) participate differently in CHER, depending on the ratio of protonated to deprotonated catalytic groups at the stripping pH. The *in situ* CPS in seawater samples would mainly reflect organic material bearing catalytic groups with $pK_a \geq 8.2$ (seawater pH), such as protein-like compounds, since their catalytic groups in Cys, Arg and Lys are mainly protonated at pH 8.2. The molecules that are catalytically active at pH 8.2 are also involved in CHER at lower pH values (such as BSA in seawater and in acidic and neutral buffers), while the molecules active at lower pH values would not contribute or would contribute to a lesser extent to CHER under seawater conditions (such as CHOS in acidic and neutral buffers and in seawater). In our experiments, catalytically active compounds in the weakly acidic acetate buffer contributed additively to the AdT CPS peak, while in the phosphate buffer interactions during the structural transition of the protein affected peak height. The application of these results to further research on selective detection of amino-glycans or detection of amino-glycans after separation from the pool of N-POM and CPS quantification will be described in another paper.

Although chitosan has practical applications in a variety of scientific and industrial fields [41], the described study of CHOS in model buffers and under seawater conditions represents a contribution to the understanding of its basic electrochemical properties.

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Figure Captions

Fig. 1. **A)** Chronopotentiogram for 100 $\mu\text{g/mL}$ CHOS measured *in situ*, $E_a = -0.1\text{ V}$, $t_a = 300\text{ s}$, $I_{\text{str}} = -20\ \mu\text{A}$ (black), and 50 $\mu\text{g/mL}$ CHOS measured AdT in 0.05 mol/L phosphate buffer pH 7.05 (red) and 6.06 (blue), and in acetate buffer pH 5.28 (magenta), $E_a = -0.1\text{ V}$, $t_a = 60\text{ s}$, $I_{\text{str}} = -20\ \mu\text{A}$ (pH 5.28 and 7.05), $I_{\text{str}} = -30\ \mu\text{A}$ (pH 6.06). Corresponding baselines are shown in dashed lines. **B)** ACV of 50 $\mu\text{g/mL}$ CHOS in seawater (black) and AdT ACV in 0.05 mol/L phosphate buffer pH 7.05 (red), *in situ* and AdT baselines (---), $E_a = -0.1\text{ V}$, $t_a = 60\text{ s}$, $f = 77.35\text{ s}^{-1}$, $E_s = 10\text{ mV}$, $a = 10\text{ mV}$. **C)** The effect of SWV f on the 50 $\mu\text{g/mL}$ CHOS peak current $-I$ (\bullet) and on $-I/f$ (\circ) in 0.05 mol/L phosphate buffer pH 7.05, $E_a = -0.1\text{ V}$, $t_a = 60\text{ s}$, $E_s = 4\text{ mV}$, $a = 25\text{ mV}$.

Fig. 2. AdT CPS dependence of height (A) and potential (B) of peak H_{OS} on CHOS concentration in 0.05 mol/L phosphate buffer pH 7.05. Inset: linear part of calibration plot. $E_a = -0.1\text{ V}$, $I_{\text{str}} = -20\ \mu\text{A}$, $t_a = 60\text{ s}$.

Fig. 3. Dependence of peak H height in CPS (A) and T_{min} in ACV (B) of 20 $\mu\text{g/mL}$ BSA *in situ* in seawater on added CHOS. Insets: chronopotentiograms (A) and AC voltammograms (B) of BSA with CHOS addition. CPS: $E_a = -0.1\text{ V}$, $t_a = 60\text{ s}$, $I_{\text{str}} = -10\ \mu\text{A}$, ACV: $E_a = -0.1\text{ V}$, $t_a = 60\text{ s}$, $f = 77.35\text{ s}^{-1}$, $E_s = 10\text{ mV}$, and $a = 10\text{ mV}$.

Fig. 4. A) The effect of added BSA concentration in seawater on the height of AdT CPS peak H of BSA alone (\circ) and on the peak height of 50 $\mu\text{g/mL}$ CHOS in CHOS-BSA mixture (\bullet). Stripping electrolyte was 0.05 mol/L phosphate buffer, pH 7.05, $E_a = -0.1\text{ V}$, $t_a = 60\text{ s}$, $I_{\text{str}} = -40\ \mu\text{A}$. The AdT procedure is shown in inset. B) AdT chronopotentiograms of CHOS under the influence of different BSA additions. C) The effect of BSA addition on T_{min} of 50 $\mu\text{g/mL}$ CHOS measured in AdT ACV, $E_a = -0.1\text{ V}$, $t_a = 60\text{ s}$, $f = 77.35\text{ s}^{-1}$, $E_s = 10\text{ mV}$, and $a = 10\text{ mV}$. The scheme of the AdT procedure is shown in A. D) AC voltammograms of CHOS-BSA mixture.

Fig. 5. AdT dependence of peak height of 20 $\mu\text{g/mL}$ BSA + 50 $\mu\text{g/mL}$ CHOS mixture (\blacksquare), 20 $\mu\text{g/mL}$ BSA (\bullet), 50 $\mu\text{g/mL}$ CHOS (\blacktriangle) on I_{str} after transfer in A) 0.05 mol/L phosphate buffer pH 7.05 and C) 0.05 mol/L acetate buffer pH 5.28, $E_a = -0.1\text{ V}$, $t_a = 60\text{ s}$. AdT

chronopotentiograms of BSA+CHOS mixture (—), BSA (—), and CHOS (—) in B) phosphate buffer and D) acetate buffer, $I_{str} = -20 \mu\text{A}$.

Fig. 6. The influence of E_b on the height of the peak H_{OS} of $50 \mu\text{g/mL}$ CHOS (\blacktriangle , red), the peak H of $20 \mu\text{g/mL}$ BSA (\bullet , blue) and the peak of the CHOS+BSA mixture (\blacksquare , black) after AdT CPS in 0.05 M phosphate buffer pH 7.05. Measurement conditions: $I_{str} = -40 \mu\text{A}$, $E_a = -0.1 \text{ V}$, $t_a = 60 \text{ s}$, $t_b = 1 \text{ s}$.