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Effect of Interfacial Properties on Impedimetric Biosensing of the Sialylation Process with a Biantennary N-Glycan-Based Monolayer

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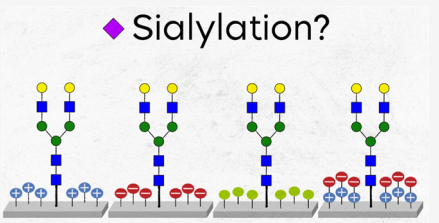
ABSTRACT: Sensing enzymatic sialylation provides new tools for the evaluation of pathological events and pathogen invasion. Enzymatic sialylation is usually monitored via fluorescence or metabolic labeling, which requires relatively large amounts of the glycan substrate with limited availability. Using a label-free biosensor requires smaller quantities of substrates because the interactions induce measurable changes to an interface, which can be translated into a signal. The downside of label-free biosensors is that they are very sensitive to changes at the interface, and the properties of the surface layer can play a major role. Electrochemical impedance spectroscopy was used here to follow the enzymatic sialylation of a biantennary N-glycan acceptor in mixed monolayers. The surfaces contained either neutral, positively or negatively charged, or zwitterionic functional groups. The systems were characterized by contact potential difference, ellipsometry, and contact angle analyses. We found that the characteristics of the mixed monolayer have a profound effect on the biosensing of the enzymatic sialylation. Positively charged layers were found to adsorb the enzyme under the reaction conditions. Negatively charged and zwitterionic surfaces were nonresponsive to enzymatic sialylation. Only the neutral mixed monolayers provided signals that were related directly to enzymatic sialylation. This work demonstrates the importance of appropriate interface properties for monitoring enzymatic sialylation processes.



INTRODUCTION

Sialic acid (neuraminic acid) is a monosaccharide terminating the sequences of many N-glycans, O-glycans, and gangliosides.[1−3] Sialic acid residues are thus involved in cell recognition, communication, and adhesion. Sialylated glycans are targets for viral infections.[3−7] Sialylation is an enzymatic process in which a sialyltransferase (ST) attaches sialic acid to glycans.[1,4] Overexpression or lack of ST activity is a biomarker for cancer or infection.[5,7] Therefore, it is important to monitor this enzymatic activity.

Many techniques were developed to monitor sialic acid.[8] The presence of sialic acid in glycans can be detected by the interaction with lectins or antibodies specific for sialic acid to produce a measurable signal.[9,10] In situ evaluation of sialylation-related enzymatic processes can be performed by following a fluorescent, metabolic, or electrochemically active tag attached to sialic acid.[5,11] The labeling of sialic acid for biosensing applications is not trivial and requires large quantities of the functional glycan to provide a measurable signal. Label-free analytical methods for enzymatic biosensing can be used as an alternative to the techniques mentioned above.[12]

Electrochemical impedance spectroscope (EIS),[13−15] field-effect transistors (FET),[15−17] quartz crystal microbalance (QCM),[18−20] surface plasmon resonance (SPR),[21,22] and bio-layer interferometry (BLI)[23,24] are used for label-free biosensing and rely on the modified interfaces.[15,17−19,25] While SPR, QCM, and BLI rely on the accumulation of the material on a surface,[19,20] EIS and FET are sensitive to changes in the properties of the sensory layer.[13,15,16] The signals measured using FET and EIS are related to changes in charge, conformation, dipole, organization, and other properties of the surface layer that results from a specific interaction.[13,26−28]

Sensing of analytes such as small molecules, metal ions, pathogens, proteins, and others is directly related to recognition events at the sensory layer.[17] Sensing enzymatic reactions differ substantially from the common detection of an analyte because these processes are correlated with the ability of the enzyme to reach the substrate on the surface of the sensor, perform the reaction, and subsequently detach from the interface.[29−31]

In a previous study, we established the monitoring of enzymatic sialylation and desialylation reactions of complex N-glycans using EIS.[13] Electrochemical analysis enabled us to monitor the change in impedance as a response to the introduction or the removal of sialic acid from the N-glycan. In that study, the N-glycan was anchored to a glassy carbon

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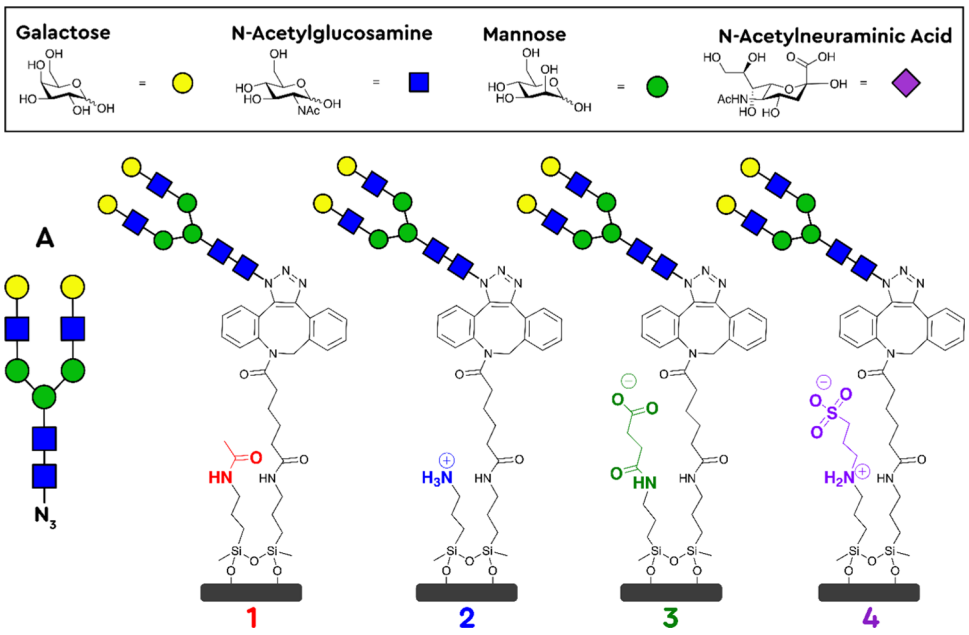

Figure 1. Monosaccharide code for the complex biantennary N-glycan (box). Differently modified mixed surfaces used in this study and the sialylation substrate glycan **A**; see also Schemes S1−S4 for the detailed structure.

electrode (GCE) using a copper-free strain-promoted click-reaction and the remaining amines on the surface were acetylated providing surface **1** (Figure 1).[13] We observed that a lower enzyme concentration led to faster sialylation of the mixed monolayer **1**.[13] This finding in addition to previous observations on enzyme surface interactions led to the assumption that the structure of the mixed interface might affect the enzymatic reaction on the surface.[25,32,33] In this work, the effect of different enzyme concentrations on N-glycan sialylation was evaluated on four differently charged mixed monolayers. The neutral surface (**1**) was compared to a positively charged surface (**2**), a negatively charged interface (**3**), and an antifouling zwitterionic surface (**4**).[34,35] The effect of the different interfaces on the detection of the enzymatic reaction was studied in detail (Figure 1). The mixed monolayers were characterized by variable angle spectroscopic ellipsometry (VASE), contact angle (CA) analysis, and Kelvin probe derived contact potential difference (CPD) analysis. The enzymatic electrochemical biosensing capabilities were optimized for enzyme concentration and net surface charge of different mixed monolayers.

EXPERIMENTAL SECTION

Materials. All reagents such as cytidine-5′-monophospho-N-acetylneuraminic acid sodium salt (CMP-NANA), buffers, salts, and redox-active couple were obtained from Sigma-Aldrich (Merck) unless stated otherwise. Triple distilled water (TDW) from Milli-Q Water (18.3 MΩ/cm, Millipore Milli-Q system, Bedford, MA) was used in the experiments. Dibenzocyclooctyne-N-hydroxysuccinimidyl ester (DBCO) was obtained from Lumiprobe Ltd. Biantennary N-glycan azide **A** (see Figure 1) was obtained as described.[36]

Preparation of the Modified Glassy Carbon Electrode. The DBCO-modified surface of GCE using 3-aminopropyltriethoxysilane (APTES) was prepared according to the previously reported protocol.[14] DBCO-modified electrodes were incubated in 0.1 mM solution of N-glycan azide **A** in 50 mM phosphate buffer (PB) for 16 h at 25 °C to give **2**. DBCO-modified electrodes were incubated with 1 mg/mL 4-(dimethylamino)pyridine (DMAP) in 2:3 acetic anhydride/acetonitrile (ACN) solution for 1 h at 25 °C followed by washing with ACN, EtOH, and TDW. These electrodes were incubated in 0.1 mM solution of **A** in a 50 mM PB for 16 h at 25 °C to give **1**. DBCO-modified electrodes were incubated with 1 mg/mL DMAP and 5 mg/mL succinic anhydride in 5% *N*,*N*-diisopropylethylamine (DIPEA)/ACN solution for 1 h at 25 °C followed by washing with ACN, EtOH, and TDW. These electrodes were incubated in 0.1 mM solution **A** in 50 mM PB for 16 h at 25 °C to give **3**. DBCO-modified electrodes were incubated with 5 mg/mL 1,3-propane sultone in ACN for 1 h at 25 °C followed by washing with ACN, EtOH, and TDW. These electrodes were incubated in 0.1 mM solution **A** in 50 mM PB for 16 h at 25 °C to give **4**.

Electrochemical Measurements. EIS analyses were performed under single sine AC excitation at a potential of 0.21 V with 10 mV amplitude in the frequency range from 100 kHz to 0.1 Hz using a three-electrode standard electrochemical cell with a BioLogic SAS SP-300 potentiostat. The system contains the following three electrodes: (a) Ag/AgCl (in 3 M KCl) as the reference electrode, (b) Pt rod as the counter electrode, and (c) GCE with a diameter of 3 mm as the working electrode. The measurements were performed in a solution of 5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$, 100 mM KCl, and 50 mM PB. The results were fitted to the equivalent circuit of $R_S[(R_{CT}W)\|Q]$, where R_S is the resistance of the solution, R_{CT} is the charge-transfer resistance of the layer, Q is the constant phase element, and W is the Warburg diffusion element. The normalized R_{CT} is obtained by calculating the ratio between R_{CT} after the enzymatic reaction, R_{CT} ($t = x$); and of the glycan surface before exposure to ST, R_{CT} ($t = 0$).

Preparation of the Stock Solution. Stock solutions of α-2,6-sialyltransferase (ST) from *Photobacterium damsela* (E.C. 2.4.99.1) and CMP-NANA were prepared according to the previously reported procedure.[13]

Enzymatic Reaction Conditions. Stock samples of ST and CMP-NANA were dissolved in 200 μL of 50 mM PB (pH 7.4). Each stock solution (20 μL) was added to 1760 μL of 50 mM PB giving a final volume of 1.8 mL (3 mU ST/mL). For lower enzyme concentrations the reaction stock was diluted with 50 mM PB. Each modified electrode was drop-cast with 50 μL of the reaction stock for different durations ranging from 5 to 120 min. After the reaction, the electrodes were washed with PB. Acetate buffer (50 mM) was used for the experiment at pH 5 and 1 M KCl in PB at pH 7.4 was used for the experiment with high ionic strength.

Silicon Wafer Modifications. Silicon wafers (⟨100⟩ n-type, resistivity <0.005 Ω·cm, Si-Mat) modified with DBCO were prepared

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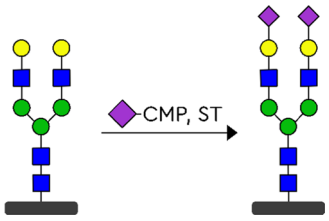
by a previously reported procedure.[14] The preparations of **1**, **2**, **3**, and **4** on Si substrates from DBCO-modified Si wafers were performed by the same protocol as that used for GCE.

Characterization of Modified Wafers. Surface characterizations of modified silicon substrates were performed by VASE, CA, and CPD analysis. VASE measurements were performed with an α-SE ellipsometer (J.A. Woollam Co.) at a silicon Brewster angle of 75° and the results were fitted with the Cauchy model for the organic layer. CA measurements were performed with TDW using an Attension Theta Lite goniometer (Biolin Scientific). CPD measurements were performed with Kelvin probe S, operated by a Kelvin control 07 unit (DeltaPhi Besocke, Julich, Germany), with vibrating gold grid reference electrode in a homebuilt Faraday cage under an argon atmosphere, and the signal was recorded with a Keithley 2450 source meter unit.

RESULTS AND DISCUSSION

In our previous report, EIS measurements on glycan-modified GCE **1** (Figure 1 and Scheme S2) showed that a decrease in the ST concentration resulted in a time-depended increase in R_{CT}.[13] This encouraged us to evaluate the concentration-dependent response rates for the enzyme. Sialylation reactions were performed with **1** using ST concentrations of 0.03, 0.09, and 0.9 mU/mL in the presence of CMP-NANA (see Scheme 1). EIS response of each electrode during the sialylation reactions was measured after 5, 10, 20, 40, 60, and 120 min (Figure 2).

Scheme 1. ST Enzymatic Sialylation of the Biantennary N-Glycan Monolayer on Solid Surfaces (1)



Subsequently, the time-dependent response of electrode **1** was recorded for the enzymatic process with ST concentrations ranging from 0.03 to 3.0 mU/mL (Figure S1). For all concentrations tested there was a clear time-dependent progression resulting from the enzymatic transfer of sialic acid onto the glycan presented on electrode **1**. It was evident that the concentration of ST significantly affects the reaction rate on the surface. To visualize the effect, the normalized R_{CT} was plotted against log (t) for each concentration of ST tested (Figure 3a). Linear regression provided the rates showing a nonlinear correlation to the concentration of the enzyme (Figure 3b).

The rate of sialylation increased proportionately when the enzyme concentration was varied from 0.03 to 0.3 mU/mL (Figure 3b). This is in line with enzymatic behavior in solution where a higher concentration of an enzyme results in an increase in the reaction rate.[25] Taking into account the linear behavior in the concentrations range of 0.03−0.3 mU/mL (Figure S2), implies quantitative ST detection at this range.

The effect of the CMP-NANA concentration, ranging from 0.01 to 100 μg/mL, on the enzymatic reaction was evaluated with the optimal enzyme concentration of 0.3 mU/mL (Figures S3 and S4). The sialylation process showed a typical co-factor dependency below 10 μg/mL and saturation above this concentration. This study proved that in the above experiments the co-factor concentration is not a limiting factor.

Surprisingly, the reaction rates dropped to about half on increasing the concentration of the enzyme above 0.3 mU/mL. The decrease in enzyme activity at higher concentrations might be correlated with electrostatic repulsion at the interface.[25] Enzymatic reactions on a modified electrode can deviate from reactions in bulk solution and may involve surface effects, which are not readily deciphered. To address the potential influence of the properties of the monolayer on the enzymatic reaction we considered the application of specifically modified surfaces.

Enzymes acting on immobilized substrates are affected by the surface layer density, its molecular constituents, and many other interfacial properties like charge, hydrophobicity, hydration level, and more.[25,32,33] The sensing ability of a label-free biosensor might be affected by changing the layer properties either by chemical modifications of the substrate of interest or by adjusting other components in the mixed monolayer. We realized that the properties of N-glycan-modified GCE **1** can be changed readily by modifying the remaining free amino groups after functionalizing the surface of the electrode with APTES and strained alkyne DBCO. Such modifications can change the surface dipole, charge, and hydrophobicity.[37] The properties of the interface can affect the enzymatic reaction due to the interactions between the surface and the enzyme.[25] Therefore, a mixed monolayer can be designed to adjust the electrostatics of the interface to change the sensing of enzymatic reactions.

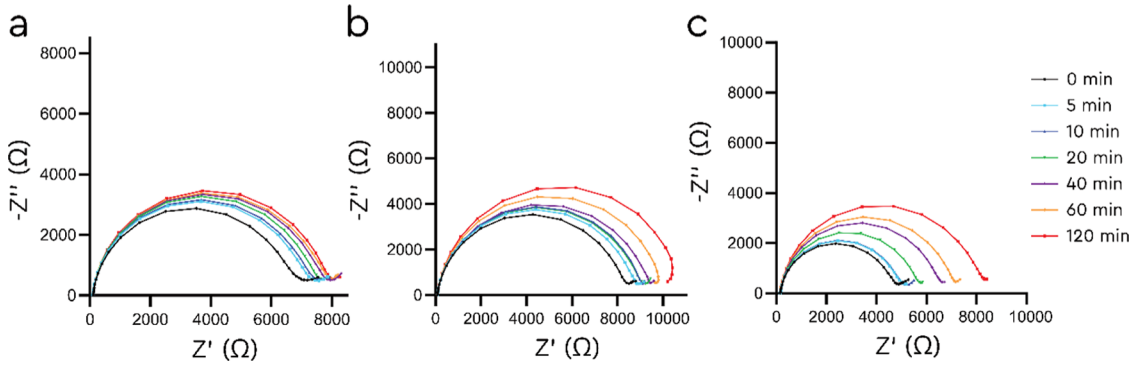


Figure 2. Time-dependent Nyquist plots of the enzyme reaction on **1** with ST concentrations of (a) 0.03 mU/mL, (b) 0.09 mU/mL, and (c) 0.9 mU/mL.

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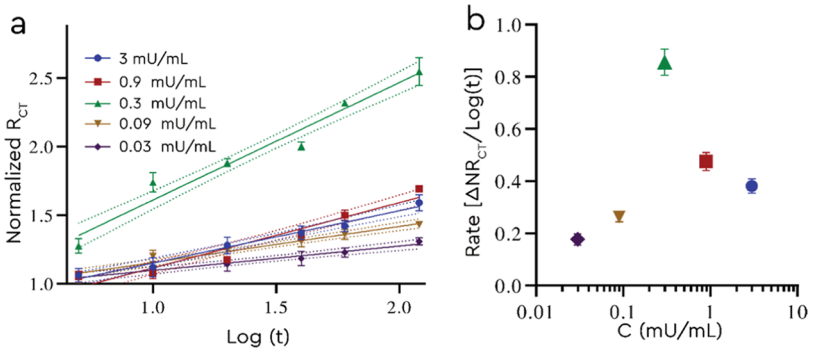

Figure 3. (a) Normalized R_{CT} as a function of log (t (min)) recorded for the sialylation of **1** using different concentrations of ST. Error bars are based on the standard deviation for 3 independent electrodes. (b) Rates calculated from normalized R_{CT} as a function of the concentration of ST. Error bars based on regression fit.

To evaluate the effect of interfacial properties on the sialylation process, three alternative electrode layouts (**2**, **3**, and **4**, Figure 1) were prepared. While retaining the connectivity for the N-glycan substrate the properties of the mixed monolayer were varied. The primary amine of APTES was used to introduce charges at the interface.

Mixed monolayer **2**, displaying free amino groups, was prepared by skipping the acetylation step in the preparation of **1**, which should result in a positively charged interface (Figure 1 and Scheme S1).[38] The carboxy-modified mixed monolayer **3** was obtained by reacting the amino groups of **2** with succinic anhydride providing a negatively charged mixed monolayer (Figure 1 and Scheme S3). The zwitterionic-modified mixed monolayer **4** was obtained by reacting **1** with 1,3-propane sultone. **1**, **2**, **3**, and **4** were characterized by VASE, CPD via the Kelvin probe, and CA measurements on silicon wafers that were functionalized according to the same procedure as that used for the preparation of GCE **1**, **2**, **3**, and **4** (Table 1).

Table 1. VASE, CA, and CPD Characterization Results for the Different Substrates[a]

mixed monolayer on silicon	thickness (Å)	contact angle (deg)	V_{CPD} (V)
1	13 ± 2	66 ± 2	−0.92 ± 0.01
2	12 ± 1	60 ± 1	−0.99 ± 0.01
3	15 ± 2	61 ± 1	−0.88 ± 0.01
4	16 ± 2	46 ± 1	−0.90 ± 0.01

[a]Errors are based on three repeats.

VASE measurements did not show significant differences in modified layer thickness proving that different modifications on the surface did not result in additional layers. Wettability studies by CA measurements showed that **1** was slightly more hydrophobic than **2** and **3**, which is in line with the neutral moieties of **1**, whereas the zwitterionic monolayer **4** was significantly more hydrophilic. V_{CPD} values of the four modified silicon substrates were also measured. Since V_{CPD} is affected by the dipoles formed in the interface; hence, it can be related to the orientation and net-charge of the interface.[37]

The net positive charge on **2** showed a decrease of V_{CPD} compared to the neutral surface **1** while the net negative charge of **3** led to an increase of V_{CPD}. The V_{CPD} value of **4** ranges between the negatively charged **3** and the neutrally charged **1**. Since the glycan part was identical for all wafers, the combined V_{CPD}, CA, and VASE results showed that the modification of the APTES amino group has significant effects on the surface properties. Time-dependent EIS measurements for the enzymatic sialylation of GCE **2**, **3**, and **4** were performed at ST concentrations of 0.03, 0.3, and 3 mU/mL in the presence of CMP-NANA (Figures S5−S8). Although the response rate for **2** increased with the concentration of ST (Figure 4a), only a very small increase in R_{CT} was recorded for **3** regardless of the enzyme concentration or the incubation time (Figure 4a).

The response of surface **1** (normalized R_{CT}) was compared to those of **2**, **3**, and **4** after 2 h of incubation with ST and CMP-NANA (Figure 4b). Using an ST concentration of 0.03 mU/mL, only **1** showed a change in R_{CT}, while **2** and **3** showed no increase. At a concentration of 0.3 mU/mL, a

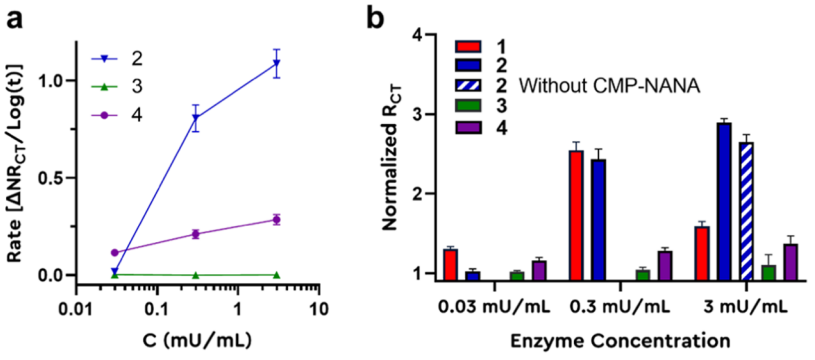

Figure 4. (a) Rates calculated from normalized R_{CT} as a function of the concentration of ST. Error bars based on the regression fit. (b) Impedimetric response to enzymatic sialylation for 2 h with different concentrations of ST on modified electrodes **1**−**4**. Error bars are based on 3 independent electrodes (raw data and results are shown in Figures S1, S6−S10).

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similar R_{CT} was recorded for **1** and **2**, while no change was observed for **3**. The response of **2** to the highest concentration of ST (3 mU/mL) increased further whereas the response of **1** decreased (compared to 0.3 mU/mL). The negatively charged surface **3** remained nonresponsive to the enzymatic reaction at all concentrations tested. The zwitterionic surface **4** showed a low response over all concentrations used.

These results show that surface charges directly influence the enzymatic reactions. In line with previous reports,[32,33] the low response of the negatively charged and zwitterionic mixed monolayers suggests that the negatively charged interface prevented the interaction of the enzyme with the glycan substrate on the surface. Electrode **3** is not suitable as a sensing layer for the evaluation of ST activity since it apparently repels either the negatively charged enzyme surface or the reactant, CMP-NANA. **4** contains a known zwitterionic antifouling submonolayer, which repels the enzyme.[34,35] The glycan-presenting interfaces **3** and **4** are; hence, not suitable for the sensing of enzymatic sialylations.

Surfaces **2** and **1** are potential candidates for responsive layers but differ in their response pattern. **1** shows optimal behavior and **2** a monotonic increase. We assume that the nature of these interfaces determines the type of interaction with the protein. We thus attempted to gain more insights into the difference in the response pattern at variable concentrations of ST. We suspected that the positively charged interface of **2** might introduce electrostatic attraction resulting in nonspecific interactions. To evaluate this, **2** was incubated with ST (3 mU/mL) but without the substrate CMP-NANA, resulting in a similar impedimetric response upon the incubation with CMP-NANA (Figure 4b). By comparison, the analogous incubation of **1** with ST without CMP-NANA showed no response.[13] This implies that the measured response of **2** to ST is a result of nonspecific protein adhesion and not of enzymatic transformation.

We suspected that the nonspecific interaction of the enzyme with the modified surface of **2** occurs via the ammonium head groups of **2**. VASE analysis showed that after incubation of **2** with ST but without CMP-NANA the thickness of the monolayer nearly doubled (from 12 ± 1 to 23 ± 2 Å), whereas after the analogous incubation of **1** the thickness barely changed (from 13 ± 2 to 14 ± 1 Å). This confirmed the presence of an additional layer on the surface of **2** after incubation with the enzyme indicating that the protein adhered to the monolayer. The VASE and EIS analyses complement each other suggesting that ST adhered to positively charged **2** and thus generated a strong response signal, unrelated to an enzymatic transfer of sialic acid.

After finding that monolayer **1** had the best properties for sensing ST activity, we turned to examine the effect of the environmental electrostatics such as solution pH and ionic strength on biocatalysis. Enzymatic reactions on **1** were evaluated for ST (0.3 mU/mL) in 50 mM acetate (pH 5) or in PB (pH 7.4) containing 1 M KCl (Figures S11 and S12). Sialylation under both conditions resulted in a negligible response. Thus, changing the environmental electrostatics affects the capability of the enzyme to carry out the sialylation on the surface of the electrode. Previous work showed that pH and ionic strength can affect the interaction of enzymes with the solid−liquid interface.[32] This is because the electrostatic interactions between the enzyme and the interface play a major role in biocatalysis on the solid−liquid interface.[32] Our observations show that modulating the electrostatics of both, monolayer and solution, affected the enzymatic reaction.

The isoelectric point of the recombinant ST from *P. damsela* (EC 2.4.99.1) is 4.88.[39] This implies that ST is negatively charged at the neutral pH used for the sialylations, which might explain the different response pattern of surfaces **1**−**4**. The high V_{CPD} value of **3** (relative to **1** and **2**, Table 1) suggests that surface **3** is negatively charged.[38] We assume that electrostatic repulsion between the negatively charged surface and the enzyme precludes enzymatic sialylation of the glycan on the surface, and hence, no change in R_{CT} was observed.

CPD analyses suggest that **2** is positively charged. It is conceivable that an interaction between a positively charged surface and the negatively charged ST should result in adhesion or adsorption of the enzyme to the surface due to electrostatic attraction.[40] In practice, this requires the accessibility of acidic residues on the surface of the enzyme. The crystal structure of *P. damsela* (pdb code: 4R83)[35] revealed the presence of an acidic patch and several isolated acidic side chains (Asp and Glu) in the C-terminal region of the enzyme. Thus, the scenario of ionic adsorption of ST onto the complementarily charged surface **2** is further supported by the particular distribution of negatively charged residues on the enzyme and explains the electrochemical response of **2** to ST even without CMP-NANA.

Another factor that may affect the sensing ability of the enzymatic process is the concentration gradient of CMP-NANA. The negatively charged substrate CMP-NANA should be attracted to a positively charged mixed monolayer and repulsed from a negatively charged surface. Although CMP-NANA interacts primarily with the enzyme, local concentration variation of CMP-NANA in proximity to charged glycated surfaces might also alter the reaction kinetics.

In contrast to the charged surfaces **2**, **3**, and **4**, interface **1** is N-acetylated, hence the mixed monolayer is not charged. The neutral surface enables the enzyme to specifically react with the glycan and subsequently detach from the surface. This prevents CMP-NANA from accumulating on or depleting from the surface. Using **1** the EIS signals measured are correlated with the enzymatic addition of sialic acid but not to the adsorption of the protein. The detection of enzymatic activity can be further used to monitor sialylation activity related to cellular function, pathogen infection, immune response, and more.

CONCLUSIONS

In this work, we revealed the intricate interplay between a charge-tunable mixed monolayer containing an N-glycan and a soluble glycan-modifying ST using EIS. The charges deliberately installed in the monolayer of the electrodes strongly affected the interaction of the enzyme with the surface. The negatively charged enzyme was adsorbed by the positively charged interface and repelled by the negatively charged layer. The ability to specifically sense the enzymatic activity was achieved only on a neutral interface. In this work, ST reaction was studied under four different conditions: surface charging, enzyme concentration, co-factor concentration, and pH. Controlling the properties of the interface allows differentiating between enzymatic activity and unspecific attachment. Therefore, in the design of label-free biosensors for enzymatic reactions, the isoelectric point of the enzyme and the physicochemical properties of the substrate monolayer were found to achieve high-sensitivity electrochemical sensing.

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This system can find many biomedical applications to monitor pathogen invasion and as a biomarker for cancer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.langmuir.1c02995.

Mixed monolayer preparation schemes, Nyquist plots, and electrochemical data (PDF)

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Author Contributions

I.A. conceived the idea, performed the experiments, and analyzed the data. A.S. performed surface characterization. Y.S. performed EIS experiments. C.U. conceived the idea, provided the glycan, and supervised the work. M.H. and S.Y. conceived the idea and supervised the work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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