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Research Article

Fabrication of pH-Adjusted Boronic Acid−Aptamer Conjugate for Electrochemical Analysis of Conjugated *N*-Glycolylneuraminic Acid

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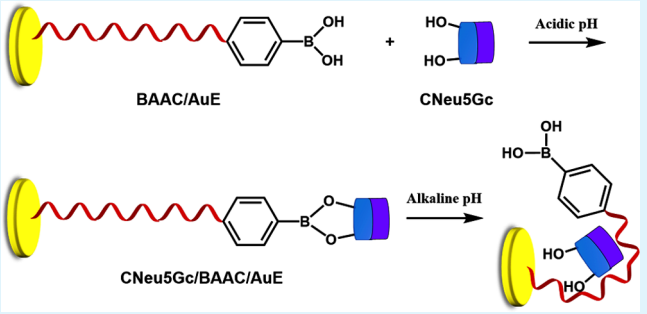
ABSTRACT: In this work, the boronic acid−aptamer conjugate (BAAC) is elaborately designed and explored as a recognition unit. The admirable properties of the pH-dependent boronic acid ester are integrated with the specific capturing capability of the modified aptamer; thus, BAAC can efficiently and selectively bind with the target by adjusting the pH values. An electrochemical biosensor based on pH-adjusted BAAC has been further developed for the analysis of CNeu5Gc, an important biomarker of different kinds of cancer. The boronic acid moiety in BAAC can react with CNeu5Gc to form a BAAC−CNeu5Gc complex under acidic conditions, followed by the release of CNeu5Gc from the complex and subsequent capture by the aptamer moiety with the adjustment of the pH value to alkalinity. With simplicity, high specificity, and efficiency, the biosensor exhibits a wide linear range from 2.816 to 3603.960 ng/mL with a low detection limit of 1.224 ng/mL and can be applied to analyze CNeu5Gc in animal food samples. Besides, this work can also provide a kind of modified aptamer, i.e., the chemical capturing group-modified aptamer, to give a new viewpoint for the exploration of other functionalized aptamers.



KEYWORDS: *boronic acid−aptamer conjugate, conjugated N-glycolylneuraminic acid, electrochemical biosensor, pH adjustment, N-glycolylneuraminic acid*

INTRODUCTION

As well known, aptamers are single-stranded DNA or RNA molecules that were screened from a library of random oligonucleotide sequence via "systematic evolution of ligands by exponential enrichment".[1−4] Due to their unique three-dimensional structures and folding complexity,[5] aptamers can bind selectively and specifically to targets such as protein, amino acid, antibiotic, peptide, small chemical, virus, cell, and metal ions,[6−12] through van der Waals forces, hydrogen bonding, hydrophobic forces, or other mechanism.[2,13,14] Moreover, aptamers can be chemically modified to perform a variety of functions. According to the roles of chemical groups, the modified aptamers are mainly divided into the following categories: (1) A linkage group-functionalized aptamer, for example, sulfhydryl-modified aptamers used to anchor the gold interface and the related materials,[15] and carboxyl-modified aptamers covalently bound to amino groups exposed on the surface.[16,17] (2) A report group-functionalized aptamer, for instance, fluorescent dye-modified aptamers serving for the fluorescence signal output and electroactive probe-labeled aptamers contributing to the electrochemical signal output.[18−20] (3) A capture group-modified aptamer, for example, the glycoprotein aptamer with the incorporation of the boronic acid moiety.[21] Considering the accuracy of double recognition and capturing, it is of great significance to explore the chemical capturing group-modified aptamer.

As a kind of chemical capturing group, the boronic acid group can reversibly bind with the diol-containing substance to form borate ester. The stability of borate ester depends on the pH value, the structure of the boric acid derivative, and the bound molecule.[22−24] Boric acid is an electron-deficient Lewis acid, in which B atom adopts sp^2 hybridization with a planar triangular configuration. For the anionic boric acid, B atom adopts sp^3 hybridization with a tetrahedral structure.[25−27] Generally, anionic boric acid facilitates its binding to a diol-containing substance due to the reason that neutral boronic ester is easily hydrolyzed.[22,25,28,29] For instance, neutral sugars, including glucose and galactose, can coordinate with the boronic acid group at alkaline pH values.[23] However, as an acidic saccharic derivative, *N*-acetylneuraminic acid can combine with boronic acid at acidic pH values owing to the occurrence of the trigonal-formed complex stabilized by the binding between an amide group of *N*-acetylneuraminic acid at

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the C-5 position with the boron atom.[24] The dependency of borate ester on the pH value endows it with controllable and adjustable characteristics.

As one of the most important molecules in the living organism, sialic acid exists in the form of *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc).[30,31] Neu5Gc is effectively absent from normal human tissues, owing to an irreversible mutation in the gene encoding cytidine monophosphate-Neu5Ac hydroxylase.[32] However, high levels of Neu5Gc can be observed in ovarian cancer, breast cancer, colon cancer, lung cancer, and prostate cancer.[33,34] Dietary absorption is the only possible way for exogenous Neu5Gc to enter human tissues.[35] Different from free Neu5Gc, which can be excreted in the urine, conjugated Neu5Gc (CNeu5Gc) mainly in the form of glycoconjugates can be metabolically incorporated into tissues, resulting in the occurrence of various cancers. Moreover, epidemiological studies have concluded that the consumption of animal food is associated with an increased risk of cancer.[31] Hence, the detection of CNeu5Gc is of great significance.

In view of capturing precision of the aptamer and the dependence of boronic recognition on the pH value, a boronic acid-modified aptamer has been designed and prepared in this work. On the one hand, the boronic acid-modified aptamer will contain two capturing units, i.e., boronic acid and the aptamer, which are beneficial for high capturing efficiency. On the other hand, it has controllable properties adjusted by the pH values. Therefore, pH-adjusted double recognition can be achieved with the aid of the boronic acid−aptamer conjugate. Taking CNeu5Gc as an example, the double recognition functionality of the boronic acid−aptamer conjugate (BAAC) has been validated, and the corresponding electrochemical biosensor has been established in this work.

EXPERIMENTAL SECTION

Materials and Reagents. The aptamer, reported by Gong et al.,[2,38] and other DNA strands were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). *para*-Sulfonatocalix[4]arene (pSC_4) was purchased from TCI Development Co., Ltd. (Shanghai, China). *N*-Glycolylneuraminic acid (Neu5Gc), *N*-acetylneuraminic acid (Neu5Ac), 4-mercaptophenyl boronic acid (MPBA), $NaBH_4$ (98%), $AgNO_3$ (99%), *N*-hydroxysuccinimide (NHS), 3-aminophenylboronic acid (APBA), 4-carboxybenzeneboronic acid (CPBA), mercaptohexanol (MCH), tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), ovalbumin (OVA), hemoglobin (HGB), α-D-fructose (FRU), α-D-glucose (GLC), potassium chloride, chitosan (CHI), 4,5-methylenedioxy-1,2-phenylenediamine dihydrochloride (DMB), potassium hexacyanoferrate(III), and potassium hexacyanoferrate(IV) were obtained from Sigma-Aldrich (Shanghai, China). Carboxyl magnetic beads (CMB) and immunomagnetic beads (IMB) were purchased from Invitrogen (Shanghai, China). Bovine serum albumin (BSA), 1-(3-dimethlaminopropyl)-3-ethyl-carbodimide hydrochloride (EDC), phosphate-buffered saline (PBS), and ethyl alcohol were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Glacial acetic acid, sodium thiosulfate, sodium sulfite, and 2-mercaptoethanol were obtained from Aladdin (Shanghai, China). Raw milk, chicken meat, pork meat, beef meat, and raw eggs were purchased from the local agricultural market. A commercial ELISA assay kit was purchased from Shanghai Zhen Ke Biological Technology Co., Ltd. (Shanghai, China). All other reagents are of analytical grade. The deionized water, with a resistance value of 18 MΩ cm, was purified through a Millipore Milli-Q water purification system (Barnstead).

High-Performance Liquid Chromatography (HPLC) for Functionality of Boronic Acid−Aptamer Conjugate. Neu5Gc (4 mM) and Neu5Ac (4 mM) were dissolved in buffer at different pH values (pH = 3, 4, 5, 6, 7, 8, 9, and 10). For the preparation of APBA-modified CMB (APBA/CMB), 1 mg/mL CMB was activated by adding EDC (0.22 M) and NHS (0.22 M) for 30 min. After washing repeatedly, 4 mM APBA in anhydrous alcohol was further added to give APBA/CMB after 3 h. For the preparation of aptamer-modified immunomagnetic beads (aptamer/IMB), the biotin-modified aptamer (1.2 μM) was added into IMB (1 mg/mL) and incubated at 30 °C for 3 h to give aptamer/IMB after magnetic separation. Subsequently, 400 μL Neu5Gc samples (4 mM) or Neu5Ac samples (4 mM) in buffer with different pH values were separately mixed with APBA/CMB or aptamer/IMB at 37 °C for 1 h. After magnetic separation, the obtained supernatants (90 μL) with different pH values were mixed with 10 μL DMB derivative solution at 50 °C for 150 min. The products were analyzed through HPLC with methanol−acetonitrile−ultrapure water (7:8:85) as the mobile phase, 0.9 mL/min of flowing rate, 5 μL of injection volume, 30 °C of column temperature, 373 nm of the excitation wavelength, and 448 nm of emission wavelength. The average peak area was calculated for three tests.

For the synthesis of BAAC-modified immunomagnetic beads (BAAC/IMB), IMB (1 mg/mL) was mixed with BAAC (2 μM) at 30 °C for 3 h. Then, by adjustment of the pH values from 3 to 10 assisted by magnetic separation, 400 μL Neu5Gc samples (4 mM) or Neu5Ac samples (4 mM) in buffer were separately mixed with BAAC/IMB at 37 °C for 1 h. After chemical derivatization, each supernatant obtained was analyzed using HPLC.

Electrochemical Analysis for Functionality of Boronic Acid−Aptamer Conjugate. The gold electrodes (3.0 mm diameter) were consecutively polished with sandpaper (3000 and 5000 mesh) and alumina powder (1, 0.3, and 0.05 μm), respectively, followed by ultrasonically cleaning by ethanol and distilled water. Subsequently, the gold electrodes were immersed with piranha solution (98% H_2SO_4/30% H_2O_2 = 3:1) for 5 min and then sonicated with distilled water for 3 min. Then, the electrode was activated by cyclic voltammetry in 0.5 M H_2SO_4 in the range from 0 to 1.6 V and 100 mV/s of the scanning rate. The activated electrodes were incubated with 2 μM MPBA, aptamer, or BAAC in PBS buffer containing 10 mM TCEP (pH 6) at 4 °C overnight, followed by soaking in 1 mM MCH for 1 h to give an MPBA-modified gold electrode (MPBA/AuE), aptamer-modified gold electrode (aptamer/AuE), and BAAC-modified gold electrode (BAAC/AuE), respectively. Subsequently, MPBA/AuE was separately immersed in 100 μL CNeu5Gc or CNeu5Ac solution (pH 6) for 1 h. Meanwhile, aptamer/AuE was separately soaked in 100 μL of CNeu5Gc or CNeu5Ac solution with pH 9 for 1 h. Moreover, BAAC/AuE was first immersed in 100 μL CNeu5Gc or CNeu5Ac solution (pH 6) for 30 min. After thorough washing, BAAC/AuE was consecutively soaked in 100 μL buffer (pH 9) for 30 min. Finally, all modified electrodes were incubated with pSC_4−AgNPs at 4 °C for 1 h. The modified electrodes were detected using linear sweep voltammetry.

Quantitative Analysis of Conjugated *N*-Glycolylneuraminic Acid. BAAC/AuE was immersed in 100 μL conjugated Neu5Gc (CNeu5Gc) in PBS buffer (pH 6) at 37 °C for 1 h. After being rinsed thoroughly with 10 mM PBS (pH 6) and distilled water, the modified electrodes were placed in Tris-HCl buffer (pH 9) at 37 °C for 1 h, followed by incubation with pSC_4−AgNPs for 1 h at 4 °C, to give pSC_4−AgNPs/CNeu5Gc/BAAC/AuE for the measurement of linear sweep voltammetry.

Investigation for Specificity and Recovery of the Established Method. The specificity of the method has been evaluated using the interfering substances, including neutral saccharides (GLC and FRU), acidic saccharides (Neu5Ac and Neu5Gc), conjugated acidic saccharide (CNeu5Ac), saccharic derivative (CHI), protein (BSA), and glycoproteins (OVA and HGB), instead of CNeu5Gc. Different samples in PBS buffer (pH 6) were used to immerse BAAC/AuE at 37 °C for 1 h. After washing, the modified electrodes were placed in Tris-HCl buffer (pH 9) at 37 °C for 1 h, followed by incubation with pSC_4−AgNPs for 1 h at 4 °C. The obtained pSC_4−AgNPs/CNeu5Gc/BAAC/AuE was measured by linear sweep voltammetry.

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Different real samples, including raw milk, chicken meat, pork meat, beef meat, and raw chicken egg, have been prepared so as to evaluate the applicability of the established method. Fresh raw milk was diluted by 10 times with purified water to give the raw milk sample. The meats from chicken, pork, and beef were minced with an electric kitchen blender, and 5 mg samples of the homogenate were weighed, followed by the addition of 20 mL purified water. The mixture was incubated at 100 °C for 10 min and then centrifuged for 10 min at 4 °C, followed by the collection of a clear supernatant. The mixture of egg white and yolk was stirred and diluted by 10 times with purified water to give a raw chick egg sample. Subsequently, 50 μL CNeu5Gc in PBS buffer (pH 6) was added into different animal food samples (50 μL). BAAC/AuE was immersed into the obtained 100 μL mixture for 1 h at 37 °C. After rinsing thoroughly, the modified electrodes were placed in Tris-HCl buffer (pH 9), followed by incubation with pSC$_4$–AgNPs, to give pSC$_4$–AgNPs/CNeu5Gc/BAAC/AuE for electrochemical detection.

Electrochemical Measurement. The cyclic voltammetry (CV) and linear sweep voltammetry (LSV) measurements were carried out on a CHI660C electrochemical workstation (CH Instruments, Shanghai, China) at room temperature. The modified gold electrode was utilized as the working electrode with the saturated calomel electrode (SCE) as the reference electrode and the platinum wire electrode as the counter electrode. CV was measured in the range from −0.4 to 0.6 V at a scanning rate of 100 mV/s with 1 M KCl solution as the electrolyte solution. LSV was performed over the potential range from −0.2 to 0.3 V with 100 mV/s of the scanning rate with 1 M KCl solution as the electrolyte solution. The LSV was performed over the potential range from −0.2 to 0.3 V with 100 mV/s of scanning rate.

RESULTS AND DISCUSSION

Functionality of pH-Adjusted Boronic Acid–Aptamer Conjugate (BAAC). Both Neu5Ac and Neu5Gc are sialic acid derivatives, and Neu5Ac contains the *N*-acetyl group at the C-5 position, while Neu5Gc has the *N*-glycolyl group at the C-5 position. Considering its structure similar to Neu5Gc and its coexistence with Neu5Gc in the animal samples, such as red pork meat, raw milk, beef meat, chicken meat, and mutton, Neu5Ac has been chosen as the seriously interfering substance to evaluate the function of boronic acid–aptamer conjugate (BAAC). The pH value has an obvious effect on the formation of the boronic acid–Neu5Ac complex or boronic acid–Neu5Gc complex. As shown in Figure 1A, along with the increase of pH values, the contents of boronic acid–Neu5Gc complexes decrease, suggesting that the complexes easily produce under acidic conditions. The result can be explained for the conformation, a spatial arrangement formed between the *N*-glycolyl group and the glycerol side chain, favorable for inducing the stable complexation of boronic acid with Neu5Gc, including the B–N/B–O bond.[24] Meanwhile, the increasing pH values result in the reduction of contents of the boronic acid–Neu5Ac complex (Figure 1B), due to the reason that the conformation deriving from the acetamide group at the C-5 position and glycerol side chain is beneficial to stabilize the complex.[24] Different from neutral saccharides (glucose, mannose, and galactose), which can react with boronic acid to form the stabilized ester under alkalic conditions,[23,24] the two complexes exhibit the unusual pH dependency. Therefore, boronic acid can selectively bind acidic saccharide derivatives, such as Neu5Ac and Neu5Gc, under acidic conditions. However, boronic acid cannot differentiate Neu5Gc and Neu5Ac.

We further investigate the capture of the aptamer on Neu5Gc and Neu5Ac, and the corresponding results have been exhibited in Figure 1C,D. The pH values have an impact on

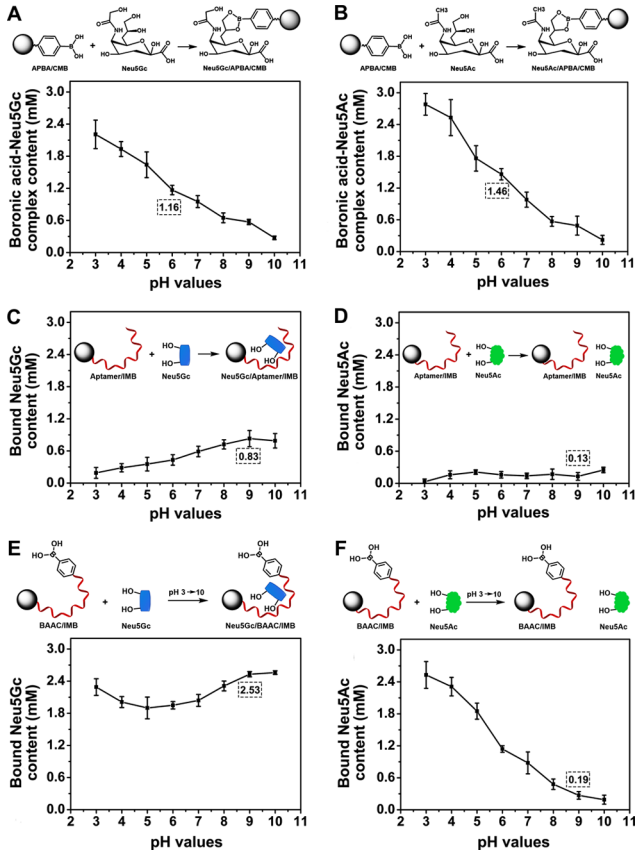

Figure 1. (A) Reaction between boronic acid derivatives and Neu5Gc (up) and the influence of pH values on the formation of the boronic acid–Neu5Gc complex (down). (B) Reaction between boronic acid derivatives and Neu5Ac (up) and the influence of pH values on the formation of the boronic acid–Neu5Ac complex (down). (C) Influence of pH values on the binding of Neu5Gc with the aptamer. Inset: Schematic illustration for the binding of Neu5Gc with the aptamer. (D) Influence of pH values on the binding of Neu5Ac with the aptamer. Inset: schematic illustration for no interaction between Neu5Ac and the aptamer. (E) Schematic illustration for the binding of Neu5Gc with BAAC by the adjustment of pH values from 3 to 10 (up). Influence of adjusted pH values on the binding of Neu5Gc with BAAC (down). (F) Schematic illustration for the interaction between Neu5Ac and BAAC by the adjustment of pH values from 3 to 10 (up). Influences of adjusted pH values on the binding of Neu5Ac with BAAC (down).

the binding between the aptamer and Neu5Gc. Along with the increase of pH values, the amount of bound Neu5Gc gradually increases and reaches a plateau at pH 9 (Figure 1C). Inversely, the negligible binding of the aptamer with Neu5Ac can be found (Figure 1D), as a result of specific capture of the aptamer on Neu5Gc. Moreover, the aptamer can also effectively capture Neu5Gc from the mixture of Neu5Gc and Neu5Ac at pH 9 (Figure S8 in the Supporting Information).

In view of the stability of boronic acid–Neu5Gc complex under acidic conditions and favorable binding of the aptamer with Neu5Gc at alkalic pH, we design and synthesize the boronic acid–aptamer complex, which can doubly separate Neu5Gc through adjusting pH values. The aptamer covalently linked with CPBA through an amide bond to form BAAC, which can be characterized by FT-IR (Figure S1 in the Supporting Information), and the function of BAAC has been investigated by HPLC. As given in Figure 1E, the amounts of Neu5Gc captured by BAAC reduce and then increase with the

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adjustment of pH values from 3 to 10. Moreover, the amount of bound Neu5Gc (2.53 mM) is three times higher than that obtained in the presence of only aptamer (0.83 mM) at pH 9. It can be explained for the reason that the aptamer moiety of BAAC can capture Neu5Gc. Moreover, this can also be ascribed to the binding of Neu5Gc through the formation of boronic acid ester at acidic pH values and its further release from the boronic acid−Neu5Gc complex and the following highly efficient capture by the aptamer at alkalic pH values as a result of proximity effect. Almost the same changes of contents can be observed for bound Neu5Ac in the presence of boronic acid or BAAC (Figure 1B,F), suggesting that the aptamer cannot bind with Neu5Ac. These results well signify that BAAC maintains the specific recognition capability of Neu5Gc in the presence of Neu5Ac and can capture Neu5Gc with high efficiency. Considering the requirement for the detection of conjugated Neu5Gc (CNeu5Gc) in the real sample, we have chosen pH 6 for the formation of the boronic acid−CNeu5Gc complex and pH 9 for binding of the aptamer moiety with CNeu5Gc using BAAC as the recognition unit.

The capturing capabilities of boronic acid, aptamer, and BAAC on CNeu5Gc and CNeu5Ac have been investigated, and the corresponding results have been exhibited in Figure 2.

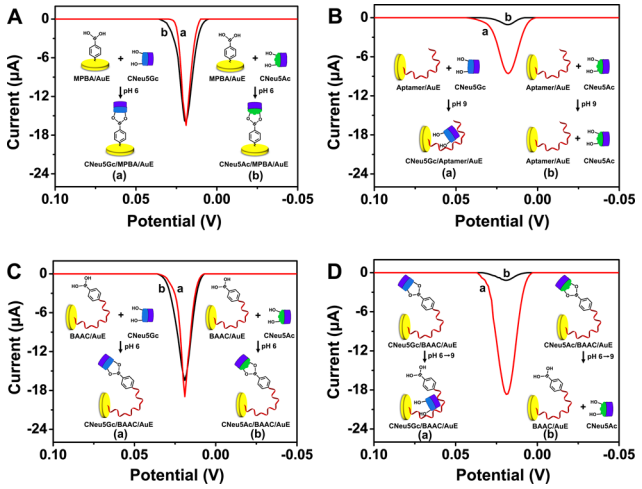

Figure 2. (A) Linear sweep voltammograms obtained for the boronic acid-modified gold electrode (MPBA/AuE) at pH 6. Inset: schematic illustration for the binding of CNeu5Gc and CNeu5Ac onto MPBA/AuE at pH 6. (B) Linear sweep voltammograms obtained for the aptamer-modified gold electrode (aptamer/AuE) at pH 9. Inset: schematic illustration for the binding of CNeu5Gc and CNeu5Ac onto the aptamer/AuE at pH 9. (C) Linear sweep voltammograms obtained for the BAAC-modified gold electrode (BAAC/AuE) at pH 6. Inset: Schematic illustration for the binding of CNeu5Gc and CNeu5Ac onto the BAAC/AuE at pH 6. (D) Linear sweep voltammograms obtained for BAAC/AuE with the adjustment of pH values from 6 to 9. Inset: schematic illustration for the binding of CNeu5Gc and CNeu5Ac onto the BAAC/AuE with the adjustment of pH values from 6 to 9.

Both CNeu5Gc and CNeu5Ac can covalently bind onto the surface of the boronic acid-modified electrode at pH 6 (Figure 2A). It can be attributed to the formation of stable boronic acid esters under acidic conditions. In contrast, only obvious peak current can be found for CNeu5Gc with the aptamer as the recognition unit at pH 9 (Figure 2B). It suggests the high specificity of the aptamer to capture CNeu5Gc under alkalic conditions. Similar to those for MPBA/AuE, almost the same peak current values can be given for CNeu5Ac with the BAAC-modified electrode at pH 6 (Figure 2C). However, for CNeu5Gc, the peak current value (18.94 μA) increases in comparison with that obtained with aptamer/AuE (8.45 μA). It can be explained for the simultaneous binding of the boronic acid moiety and the aptamer moiety with CNeu5Gc. With the adjustment of the pH value to 9, the negligible peak current can be obtained for CNeu5Ac (Figure 2D), due to the dissociation of boronic acid−CNeu5Ac complex and the resultant release of free CNeu5Ac into solution. Inversely, the apparent peak can be observed for CNeu5Gc (Figure 2D), and the current value (18.68 μA) is nearly two times higher than that with aptamer/AuE (8.45 μA). For one thing, a small quantity of the bound CNeu5Gc by the aptamer moiety of BAAC at pH 6, can remain unchanged under alkalic conditions. For another thing, the numerous free CNeu5Gc released from the boronic acid−CNeu5Gc complex at pH 9 can be efficiently captured by the aptamer moiety of BAAC as a result of the proximity effect. Therefore, compared with the aptamer, BAAC, with two capturing moieties, exhibits good advantage for CNeu5Gc recognition so as to serve for the construction of biosensor.

Mechanism Investigation of the Established Electrochemical Biosensor for Conjugated *N*-Glycolylneuraminic Acid. CNeu5Gc has been successfully synthesized (Figure S3 in the Supporting Information), and the mechanism for its analysis using BAAC as the recognition unit has been illustrated in Figure 3A. BAAC can be immobilized onto the surface of the gold electrode through the Au−S bond. Under acidic conditions, a large quantity of CNeu5Gc can bind with the boronic acid moiety of BAAC to form the stable boronic acid−CNeu5Gc complex through the ester bond. With the

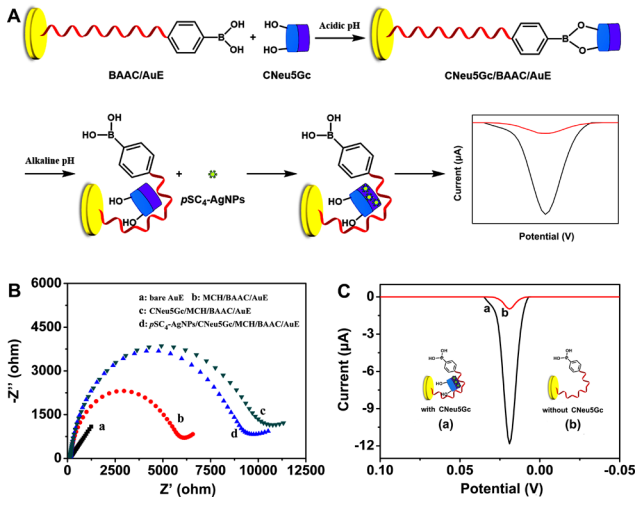

Figure 3. (A) Schematic illustration for the mechanism of CNeu5Gc analysis using BAAC. (B) Complex plane plot for the electrochemical impedance measurements of the gold electrode at different modification stages: (a) bare AuE, (b) MCH/BAAC/AuE, (c) CNeu5Gc/MCH/BAAC/AuE, and (d) pSC_4−AgNPs/CNeu5Gc/MCH/BAAC/AuE. Electrolyte: phosphate buffer (5 mM, pH 7.0). Electrochemical species: 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl. Biasing potential: 0.224 V. Amplitude: 10 mV. Frequency range: 0.01 Hz to 10 kHz. (C) Linear sweep voltammograms obtained in the presence of CNeu5Gc (curve *a*) and in the absence of CNeu5Gc (curve *b*). Inset: schematic illustration for the final modified electrode with CNeu5Gc (image a) and without CNeu5Gc (image b).

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adjustment of pH values from 6 to 9, CNeu5Gc can be dissociated from the complex and subsequently be quickly captured by the aptamer moiety of BAAC as a result of the proximity effect. With the addition of synthesized pSC_4–AgNPs (Figure S4 in the Supporting Formation), in which pSC_4 can recognize and bind to aromatic amino acid residues of the protein part of CNeu5Gc, and AgNPs can give a sensitive electrochemical signal,[36] an obvious peak current can be observed. In contrast, a negligible peak current can be found in the absence of CNeu5Gc.

The modification process of the electrode surface has been characterized by electrochemical impedance spectroscopy, and the corresponding results have been exhibited in Figure 3B. Almost no semicircle can be found for the bare AuE (curve *a*), suggesting that there is no resistance for electron transfer. After consecutively modifying with BAAC and MCH, a large semicircle can be tested (curve *b*). On the one hand, it can be explained for insulating the properties of two substances. On the other hand, it can be attributed to the repulsive interaction between the negatively polarized nucleic acid strand and the negatively charged probe $[Fe(CN)_6]^{3-/4-}$. Along with the further addition of CNeu5Gc, the semicircle becomes larger (curve *c*), due to its nonconductive properties. However, the resistance value reduces slightly with further modification of the electrochemical probe pSC_4–AgNPs (curve *d*), resulting from the beneficial electron-transfer ability of AgNPs. These results well confirm that the electrode surface has been successfully modified.

As depicted in Figure 3C, nearly no redox peak can be observed in the absence of CNeu5Gc (curve *b*), as a result of no occurrence of pSC_4–AgNPs on the surface of the BAAC-modified electrode. In contrast, an evident redox peak can be detected in the presence of CNeu5Gc (curve *a*), suggesting the existence of abundant pSC_4–AgNPs on the BAAC/AuE surface. pSC_4–AgNPs can bind with aromatic amino acid residues exposed on the surface of CNeu5Gc captured by the aptamer moiety of BAAC at alkaline pH values. Therefore, the amount of electrochemical signal probe (pSC_4–AgNPs) is related to the concentration of CNeu5Gc, and the electrochemical biosensor can be developed with BAAC as the recognition unit.

Quantitative Analysis of Conjugated *N*-Glycolylneuraminic Acid Using pH-Adjusted Boronic Acid–Aptamer Conjugate as the Recognition Unit. In view of its advantages, including high sensitivity, high resolution, and strong antireduction ability, linear sweep voltammetry has been used to analyze CNeu5Gc with different concentrations, and the corresponding results are shown in Figure 4A. As the concentrations of CNeu5Gc increase from 0 to 3603.960 ng/mL, the peak current values gradually increase. The boronic acid section in BAAC can react with the Neu5Gc residue in the structure of CNeu5Gc to form the stable complex under acidic conditions. With the change of the pH value to 9 in the reaction system, CNeu5Gc will become free and dissociate from the complex. The releasing CNeu5Gc can be quickly captured by the aptamer moiety in BAAC, resulting in its specific immobilization on the surface of the modified electrode, while pSC_4–AgNPs can bind with aromatic amino acid residues on the surface of CNeu5Gc through the host–guest interaction. As shown in Figure S5, the linear fitting equation of $C_{AgNPs} = -0.01434 + 0.0291 \log C_{CNeu5Gc}$ can be given with a correlation coefficient of 0.98431. Therefore, the increase of CNeu5Gc concentrations can lead to an increased

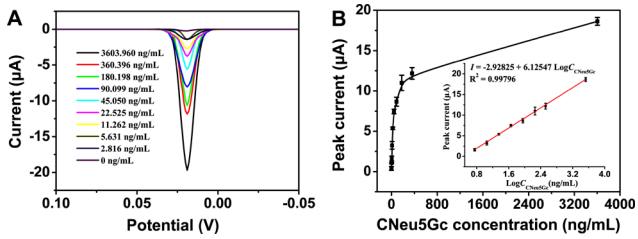

Figure 4. (A) Linear sweep voltammograms for the detection of CNeu5Gc with different concentrations (0, 2.816, 5.631, 11.262, 22.525, 45.050, 90.099, 180.198, 360.396, and 3603.960 ng/mL). (B) Peak current values versus different CNeu5Gc concentrations. Inset: the linear relationship between peak current values and logarithmic values of CNeu5Gc concentrations.

number of pSC_4–AgNPs on the surface of the modified electrode, and the corresponding increase of peak current values.

The peak current values have been further used to quantitatively analyze CNeu5Gc, and the corresponding results are given in Figure 4B. The current values linearly increase with logarithmic values of increasing CNeu5Gc concentrations from 2.816 to 3603.960 ng/mL, and the linear range is wider than previous reports.[37,38] The linear fitting equation of $I = -2.92825 + 6.12547 \log C_{CNeu5Gc}$ can be given with a correlation coefficient of 0.99796, where I is the peak current value and $C_{CNeu5Gc}$ is the CNeu5Gc concentration. The detection limit has been estimated to be 1.224 ng/mL (S/N = 3), which is lower than the values in the previous reports.[38] Moreover, based on three independent electrochemical measurements, the RSD value has been calculated to be 3.64% using the slopes of three regression equations with CNeu5Gc concentrations from 2.816 to 3603.960 ng/mL. It signifies good reproducibility and precision of the developed electrochemical biosensor. Meanwhile, an adsorption model of $q = 18.407 \times 0.028\ c^{0.835}/(1 + 0.028\ c^{0.835})$ with a correlation coefficient of 0.98418, where q is the adsorption capacity and c is the CNeu5Gc concentration, 18.407 is the maximum adsorption capacity, and 0.028 is the adsorption equilibrium constant, can be obtained for the binding between CNeu5Gc and BAAC. Besides, the mathematical relationship between peak current values and different CNeu5Gc concentrations in the mixture of CNeu5Gc and Neu5Ac or in the mixture of CNeu5Gc and CNeu5Ac has also been established (Figure S9A,B in the Supporting Information).

Investigation for Specificity of the Developed Electrochemical Biosensor Using pH-Adjusted Boronic Acid–Aptamer Conjugate as the Recognition Unit. To investigate the specificity of the established method, different samples, including the neutral saccharides (GLC and FRU), acidic saccharides (Neu5Ac and Neu5Gc), conjugated acidic saccharide (CNeu5Ac), saccharic derivative (CHI), protein (BSA), and glycoproteins (OVA and HGB), have been used instead of CNeu5Gc. As exhibited in Figure 5A,B, there is no distinct peak with the separate addition of the other substances, except for CNeu5Gc. Meanwhile, there is no significant change of peak current values for the mixtures in comparison with that of only CNeu5Gc (Figure 5C,D). It has been well confirmed that neutral saccharides such as GLC and FRU can coordinate with boronic acid under alkaline conditions,[23,39] so only a small quantity of these saccharides can bind with the boronic acid moiety in BAAC at pH 6. Though a large number of acidic saccharides such as Neu5Ac

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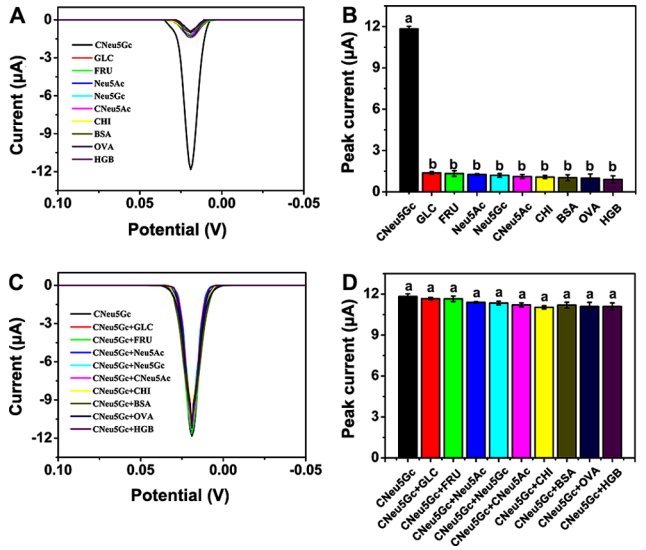

Figure 5. (A) Linear sweep voltammograms for the detection of different substances, including CNeu5Gc, GLC, FRU, Neu5Ac, Neu5Gc, CNeu5Ac, CHI, BSA, OVA, and HGB. (B) Peak current values versus different substances. (C) Linear sweep voltammograms for CNeu5Gc and its mixtures with GLC, FRU, Neu5Ac, Neu5Gc, CNeu5Ac, CHI, BSA, OVA, and HGB. (D) Peak current values versus CNeu5Gc and its mixtures. CNeu5Gc concentration is 360.396 ng/mL and other substance concentrations are 3603.960 ng/mL. Columns with different alphabets are significantly different.

and its corresponding complex CNeu5Ac can react with the boronic acid moiety of BAAC at pH 6, these substances will be released into the solution when the pH values of the reaction system are adjusted to 9. Similar to CNeu5Gc, free Neu5Gc can react with the boronic acid moiety under acidic conditions and further transfer to bind with the aptamer moiety under alkaline conditions. However, free Neu5Gc has no capability to capture the signal probe pSC_4–AgNPs due to the lack of aromatic amino acid in its structure. The saccharic derivative (CHI), protein (BSA), and glycoproteins (OVA and HGB) cannot be recognized by BAAC. These results well signify the specific recognition capability of pH-adjusted BAAC on CNeu5Gc and the high specificity of the established electrochemical method based on pH-adjusted BAAC.

Conjugated *N*-Glycolylneuraminic Acid Analysis in Real Samples. As shown in Table 1, CNeu5Gc contents in different food samples have been detected using our established method, and the results are almost in accordance with those (none for both chicken meat and raw chicken eggs, and 3.92, 7.89, and 3.82 ng/mL for pork meat, beef meat, and raw milk, respectively) obtained using the commercial ELISA assay kit. To further evaluate the analytical reliability and practical applicability of the electrochemical biosensor, CNeu5Gc with different concentrations have been added into different animal foods and the CNeu5Gc concentrations are determined using our constructed biosensor (Figure S10 and Table 1). The recovery ratio varies between 91 and 110%, and relative errors are basically within 8%. The results indicate that the electrochemical biosensor for the detection of CNeu5Gc with pH-adjusted BAAC has good anti-interference ability and can be applied for the detection of CNeu5Gc in real samples.

Table 1. CNeu5Gc Concentrations Detected by the Established Method and the Comparison with the Given Concentrations in Different Food Samples

samples	CNeu5Gc concentration detected (ng/mL)	standard concentration (ng/mL)	recovery ratio (%)	relative error (%)
raw milk	3.82 ± 0.69	0	none	none
	86.35 ± 0.38	90.10	91.60	4.29
	191.56 ± 0.73	180.20	104.19	6.59
	350.04 ± 0.77	360.40	96.07	6.06
chicken meat	none	0	none	none
	86.99 ± 0.51	90.10	96.55	5.65
	197.59 ± 0.43	180.20	109.65	3.85
	369.65 ± 0.74	360.40	102.57	5.80
pork meat	3.92 ± 0.70	0	none	none
	88.02 ± 0.29	90.10	93.35	3.24
	184.06 ± 0.52	180.20	99.97	4.78
	344.73 ± 0.75	360.40	94.56	5.98
beef meat	7.89 ± 0.40	0	none	none
	91.12 ± 0.32	90.10	92.38	3.56
	177.73 ± 0.80	180.20	94.25	7.33
	344.95 ± 0.65	360.40	93.52	5.14
raw eggs	none	0	none	none
	88.88 ± 0.33	90.10	98.64	3.63
	169.39 ± 0.69	180.20	94.00	6.46
	346.72 ± 0.60	360.40	96.20	4.76

CONCLUSIONS

In this work, the boronic acid–aptamer conjugate (BAAC) has been successfully constructed and its function has been further investigated. Compared to boronic acid and the aptamer, BAAC can capture free Neu5Gc and CNeu5Gc with high specificity and efficiency. Subsequently, BAAC has been utilized as a recognition unit for the establishment of an electrochemical biosensor for analysis of CNeu5Gc. With a wide linear range, low detection limit, and high specificity, the explored electrochemical biosensor has been applied for the analysis of real animal food samples. This work not only provides a new perspective for exploration of recognition molecules but also first develops the electrochemical biosensor to test CNeu5Gc in real samples.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.9b23029.

Characterization of the boronic acid–aptamer conjugate (Figure S1); calibration curve for the measurement of Neu5Ac and Neu5Gc (Figure S2); characterization of conjugated Neu5Gc and Neu5Ac (Figure S3); characterization of pSC_4–AgNPs (Figure S4); linear relationship between CNeu5Gc and pSC_4–AgNPs (Figure S5); fluorescent image for the binding of the aptamer with CNeu5Gc (Figure S6); cyclic voltammograms and electrochemical impedance spectra for the gold electrode at different modification stages (Figure S7); capture of Neu5Gc from the mixture of Neu5Gc and Neu5Ac by the aptamer (Figure S8); linear relationship between the peak current values and logarithmic values of CNeu5Gc concentrations (Figure S9); voltammo-

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grams for the detection of different concentrations of CNeu5Gc in real samples (Figure S10) (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Neu5Gc, *N*-glycolylneuraminic acid
Neu5Ac, *N*-acetylneuraminic
CNeu5Gc, conjugated Neu5Gc
CNeu5Ac, conjugated Neu5Ac
pSC_4, *para*-sulfonatocalix[4]arene
BAAC, boronic acid−aptamer conjugate
CMB, carboxyl magnetic beads
IMB, immunomagnetic beads
MPBA, 4-mercaptophenyl boronic acid
BSA, bovine serum albumin
CPBA, 4-carboxybenzeneboronic acid
APBA, 3-aminophenylboronic acid
OVA, ovalbumin
MCH, mercaptohexanol
NHS, *N*-hydroxysuccinimide
HGB, hemoglobin
FRU, α-D-fructose
GLC, α-D-glucose
EDC, 1-(3-dimethlaminopropyl)-3-ethylcarbodimide hydrochloride
TCEP, tris(2-carboxyethyl)phosphine hydrochloride
CHI, chitosan
PBS, phosphate-buffered saline
HPLC, high-performance liquid chromatography
DMB, 4,5-methylenedioxy-1,2-phenylenediamine dihydrochloride
AuE, gold electrode
CV, cyclic voltammetry
LSV, linear sweep voltammetry

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