Biosensors and Bioelectronics 62 (2014) 79–83

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

Ultrasensitive electrochemical biosensor based on graphite oxide, Prussian blue, and PTC-NH_2 for the detection of α2,6-sialylated glycans in human serum

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ARTICLE INFO

Article history:
Received 7 March 2014
Received in revised form
11 June 2014
Accepted 12 June 2014
Available online 20 June 2014

Keywords:
α2,6-Sialylated glycans
Graphite oxide
Prussian blue
PTC-NH_2
Gold nanoparticles

ABSTRACT

α2,6-Sialylated glycans are crucial molecular targets for cancer diagnosis and clinical research. In this work, a novel ultrasensitive electrochemical biosensor was fabricated based on a graphite oxide (GO), Prussian blue (PB), and PTC-NH_2 (an ammonolysis product of 3,4,9,10-perylenetetracarboxylic dianhydride) nanocomposite for the selective detection of α2,6-sialylated glycans. To increase the sensitivity of the electrochemical biosensor, gold nanoparticles (GNPs) were immobilized on a GO–PB–PTC-NH_2 modified glassy carbon electrode (GCE). *Sambucus nigra* agglutinins (SNAs), which specifically bind with α2,6-sialylated glycans, were covalently immobilized on GNPs for the sensitive detection of α2,6-sialylated glycans in serum. This proposed method can be applied to human serum, and it worked well over a broad linear range (0.1 pg mL^{-1}–500 ng mL^{-1}) with detection limits of 0.03 pg mL^{-1}. Moreover, recovery of the spiked samples ranged from 100.2% to 105.0%, suggesting that this excellent electrochemical biosensor can be used for the practical detection of α2,6-sialylated glycans.

1. Introduction

Sialic acids (Sia), also called N-acetylneuraminic acid (Neu5Ac) which have a nine-carbon backbone, are typically found as terminal monosaccharides attached to cell surface glycoconjugates (Kim et al., 2011). A variety of linkages to the 2-position of the underlying sugar chain and various substitutions at the 4, 5, 7, 8, and 9 positions combine to generate a wide diversity of structures (Angata and Varki, 2002; Schauer, 2000; Varki and Varki, 2007). The α2,6-linkage of sialic acids to N-acetyllactosamine structures (Galβ1–4GlcNAc) is a Golgi-mediated process facilitated by the enzyme β-galactoside α2,6-sialyltransferase (ST6Gal-I) (Harduin-Lepers et al., 2001; Park et al., 2012). Variants of α2,6-sialylation, the outermost monosaccharide unit on the glycan chains of glycolipids and glycoproteins, can have a wide range of biological and pathogenical consequences, such as in the development and progression of some cancers (Chen and Varki, 2010; Park et al., 2012; Pousset et al., 1997). When carcinoma cells die, α2,6-sialylated glycans are formed and released into the bloodstream, which results in increased concentrations of α2,6-sialylated glycans in the serum. High level of α2,6-sialylation in the serum has been detected in a number of tumors (Dall'Olio and Trere, 1993; Pousset et al., 1997). Therefore, α2,6-sialylated glycans are crucial molecular targets for cancer diagnosis and are one of the most extensively used clinical cancer biomarkers (Dall'Olio and Trere, 1993). It is desirable to develop a simple, sensitive, and rapid method for the determination of α2,6-sialylated glycans with high selectivity.

The remarkable chemical diversity of α2,6-sialylated glycans is generated by multiple enzymatic mechanisms. Therefore, it is difficult to detect and quantify glycans present in serum. Recombinant soluble forms of α2,6-sialylated glycans can be used for this purpose. A recombinant Neu5Acα(2-6)Gal β MP Glycoside, because of its similar structure (Fig. S1 in Supplementary information), can specifically probe for α2,6-linkages of sialic acids to N-acetyllactosamine structures in glycoconjugates, which can also be detected by specific lectins. Regardless of the nature of their natural ligands, some Sia-binding lectins have proven to be powerful tools for detecting Sia-specific glycoconjugates. For example, wheat germ agglutinin and Limax flavus agglutinin have been used to detect sialylated glycoconjugates, whereas combinations of *Sambucus nigra*, *Polyporus squamosus*, and *Maackia amurensis* agglutinins can distinguish among different types of Sia-linkages on terminal

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http://dx.doi.org/10.1016/j.bios.2014.06.031
0956-5663/

N-acetyllactosamines. *S. nigra* agglutinins (SNAs) are ideal for detecting α2,6-sialylated glycans (Shibuya et al., 1987).

Many analytical methods such as gas chromatography–mass spectrometry (Bratosin et al., 2007; Bugla-Ploskonska et al., 2010; Kim et al., 2006), high-performance liquid chromatography–mass spectrometry (Harvey, 2011), capillary electrophoresis (Liu et al., 2001; Rustandi et al., 2013), and nuclear magnetic resonance spectroscopy (Hobb et al., 2010; Jones, 2005) have been used to detect sialylated glycans. However, no means exist for the specific detection of α2,6-sialylated glycans. Therefore, novel methods capable of simple, rapid, and sensitive detection of α2,6-sialylated glycans are required. Nanocomposites have stimulated intense research in recent years due to their potential applications for bioassays (Kaushik et al., 2013; Li et al., 2010; Sun et al., 2011), especially in biosensor fabrication (Silva Fde et al., 2013). Graphene oxide (GO), for example, has been widely utilized in the development of various biosensors (Kakran et al., 2011; Wang et al., 2011; Wei et al., 2008). Owing to its large absorption cross section, GO has been regarded as an excellent candidate for fabricating electrochemical biosensors (Feng et al., 2011; Wang et al., 2009). PTC-NH_2, which is a well-known matrix for biological substances and nanomaterial absorption, could lower the background current signal because of its amino-functionalized interface and unique electrochemical properties (Han et al., 2013a). Prussian blue (PB) possesses characteristics of an ideal mediator such as a redox potential, good stability, enhanced electron transfer rate, and high surface activity, which has been applied in the development of several recent biosensors (Han et al., 2013b; Li et al., 2013). Therefore, nanocomposites containing GO, PB, and PTC-NH_2 play a crucial role in promoting sensitivity and stability in biosensors. Herein, we used these materials to prepare a novel nanocomposite, denoted GO–PB–PTC-NH_2, for the detection of α2,6-sialylated glycans.

To increase the sensitivity of our electrochemical biosensor, gold nano-particles (GNPs) were chosen for their large surface area and favorable micro-environment for promoting biological activity in proteins (Li et al., 2011). Lectins, natural decipherers and translators for sugar, can distinguish between free sugars and glycans attached to biomolecules. The sialic-acid-binding lectin SNA is routinely used for detecting glycans containing α2,6-Neu5Ac (Cheng et al., 2009, 2008; Ding et al., 2008, 2009). Thus, the determination of α2,6-sialylated glycans relies on specific recognition by SNA.

In this paper, we describe a novel electrochemical biosensor based on a GO–PB–PTC-NH_2 nanocomposite for the highly sensitive detection of α2,6-sialylated glycans. Sensitivity was achieved with enormous loadings of SNA on the GNPs. The absorption of α2,6-sialylated glycans relies on specific recognition by the surface-confined SNA. The characteristic feature of our electrochemical biosensor is its simple surface structure. The preparation, characterization, optimal conditions, and preliminary analysis of serum samples were investigated for the determination of α2,6-sialylated glycans. The proposed method was simple and achieved highly sensitive analysis of α2,6-sialylated glycans. Our biosensor could be further used as a promising platform to monitor the alteration of α2,6-sialylated glycans in serum.

2. Materials and methods

2.1. Reagents and chemicals

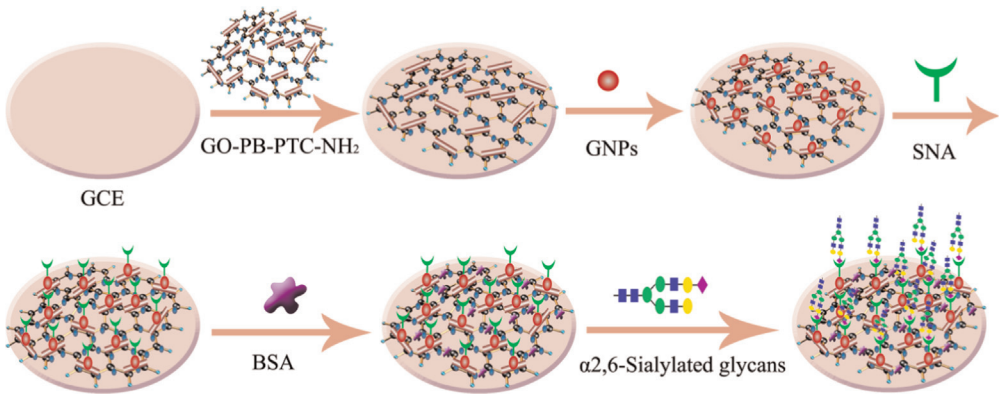
$HAuCl_4 \cdot 3H_2O$ was purchased from Sigma-Aldrich (St. Louis, USA, www.sigmaaldrich.com). Neu5Acα(2-6)Gal β MP Glycoside was purchased from Tokyo Chemical Industry (Japan, www.TCI-chemicals.com, Lot. SGPSH-HF, > 98%). *S. nigra* agglutinin was purchased from Gentaur (Kampenhout, Belgium, www.gentaur.com). 3,4,9,10-Perylenetetracarboxylic dianhydride (PTCDA) was obtained from Liaoning Liangang Pigment and Chemicals Co., Ltd. (China). Ascorbic acid (AA), dopamine (DA), L-cysteine and glucose were purchased from Aladdin. Graphene oxide was obtained from Shanghai Boson Technology Co., Ltd. (China). Other chemicals employed were of analytical reagent grade and used without further purification. All solutions were prepared in ultrapure water.

2.2. Apparatuses

The electrochemical experiments were performed utilizing an electrochemical workstation (CHI660D) with a conventional three-electrode system (Shanghai Chenhua Apparatus Corporation, China). A glassy carbon electrode (GCE, 4 mm in diameter) was used as the working electrode, with a saturated calomel electrode (SCE) as the reference electrode, and platinum as the counter-electrode which is a platinum wire with a diameter of 0.5 mm. Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM) were investigated using Hitachi-7500158 (Hitachi Limited, Japan). UV–vis absorption spectrums were measured on a UV-2450 spectrophotometer (Shimazu, Japan). Fourier transform infrared (FT-IR) spectroscopy was recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Nicolet, USA).

2.3. Preparation of GNPs and the GO–PB–PTC-NH_2 nanocomposite

A detailed method for the preparation of GNPs and the GO–PB–PTC-NH_2 nanocomposite can be found in Supplementary information (S11).



Scheme 1. Schematic for fabrication of the electrochemical biosensor.

2.4. Preparation of test serum samples

Blood samples were obtained from healthy volunteers. After collection, the samples were centrifuged for 5 min at 5000 rpm to obtain the serum, which was stored at −20 °C until assay. An aliquot of serum was spiked with Neu5Acα(2-6)Gal β MP Glycoside dissolved in 0.1 M PBS, transferred into a 2-mL volumetric flask, and diluted with 0.1 M PBS to achieve a final concentration of 1.0 μg mL^{-1}, which was stored at −20 °C until further use.

2.5. Fabrication of the biosensor

The fabrication of the electrochemical biosensor is outlined in Scheme 1. The GCE was carefully polished with an alumina slurry (grain size, 0.3 μm and 0.05 μm) until a mirror finish was obtained, then ultrasonicated and rinsed with ethanol and ultrapure water to remove any alumina residue from the electrode surface. First, 6 μL of the prepared GO–PB–PTC-NH_2 nanocomposite was dropped onto the pretreated GCE and then dried in air. Next, the GO–PB–PTC-NH_2 nanocomposite-modified electrode was added to the GNPs and incubated for 12 h at 4 °C, followed by coating with 4 μL of 1.5 mg mL^{-1} SNA solution. This was followed by incubation with 1 wt% BSA solution for 0.5 h at 4 °C to block non-specific binding sites between the analyte and electrode. Finally, Neu5Acα (2-6)Gal β MP Glycoside at various concentrations was added to the electrode and allowed to react for 2.5 h. PB was used as the redox probe to record the electron transfer generated from $Fe^{2-/3-}$ to the electrode during binding of Neu5Acα(2-6)Gal β MP Glycoside. The current change in response to the biological reaction versus the varying concentrations of Neu5Acα(2-6)Gal β MP Glycoside was used as the analytical signal.

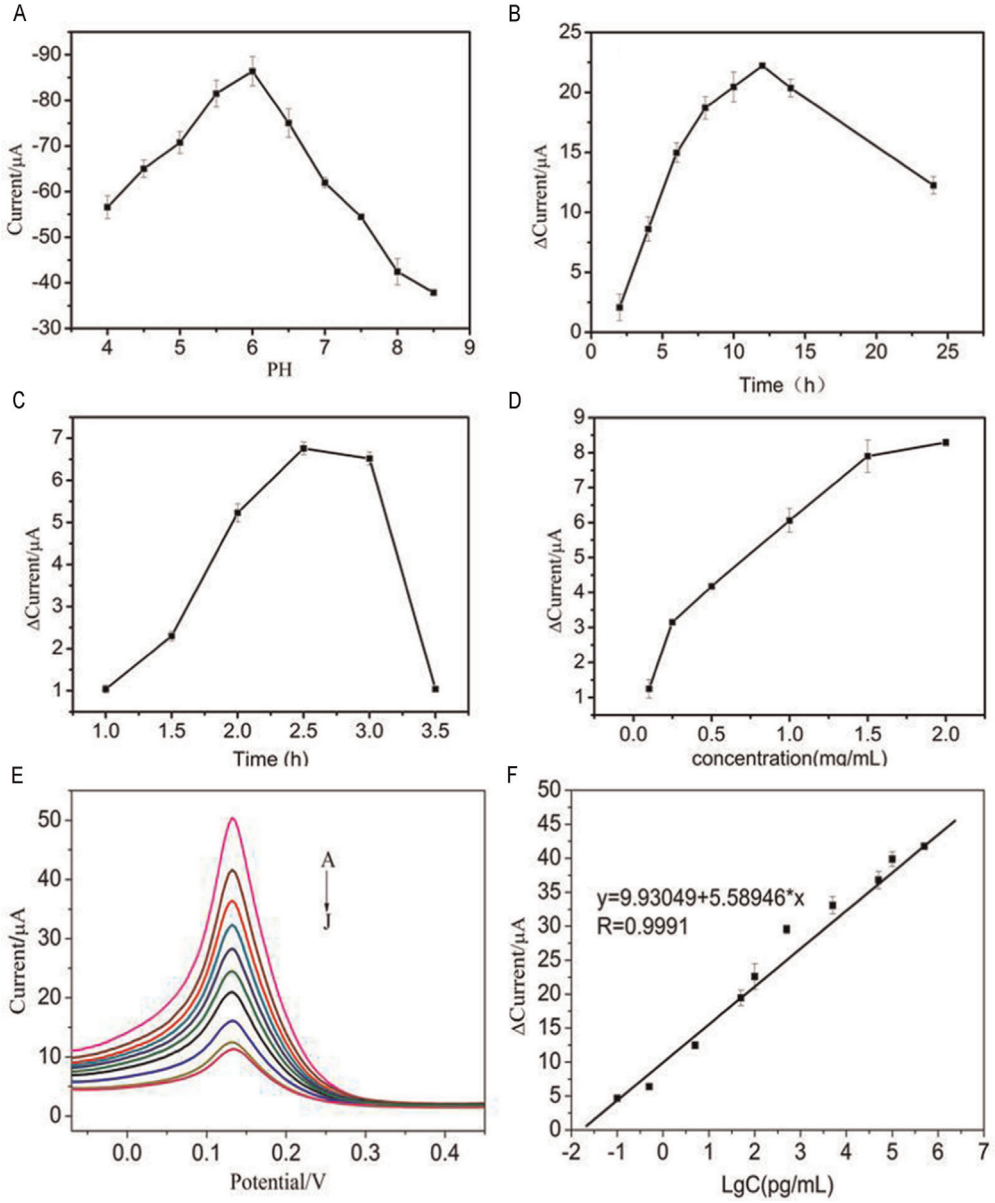


Fig. 1. Effects on the current brought about by (A) pH of detection solution, (B) incubation time in GNPs, (C) incubation time of the receptor/ligand, and (D) concentration of SNA. Mean values and standard deviations were obtained from at least three independent experiments. (E) Typical DPVs of an electrochemical assay with GO–PB–PTC-NH_2 films in PBS (pH=6.0) with increasing Neu5Acα(2-6)Gal β MP Glycoside concentrations of 0.1 pg mL^{-1}, 0.5 pg mL^{-1}, 5 pg mL^{-1}, 50 pg mL^{-1}, 100 pg mL^{-1}, 500 pg mL^{-1}, 5 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, and 500 ng mL^{-1}, along with (*F*) the resulting calibration curve.

3. Results and discussion

3.1. Morphology and structural characterization of the as-prepared materials

Morphology and structural characterization of the as-prepared materials using TEM, SEM, UV–vis, and FT-IR spectrometry are available in Supplementary information (S12).

3.2. Electrochemical characterization of the stepwise-modified electrode

CV is a convenient tool for monitoring the characteristics of the modified electrode surface. The CVs of the fabricated biosensor during stepwise modification, which were conducted in PBS (pH=6.0) at a scan rate of 50 mV s^{-1}, are shown in Fig. S5(A). A well-defined oxidation and reduction peak for $Fe^{2-/3-}$ was observed at the GO–PB–PTC-NH_2 modified electrode (curve a). Curve b shows that the peak current of the GNPs-modified electrode increased markedly, indicating that GNPs increase the biosensor's detection sensitivity (Li et al., 2011). When SNA molecules were adsorbed onto the modified electrode, the current decreased sharply (curve c). After blocking with BSA (curve d) and modification with 5 ng mL^{-1} Neu5Acα(2-6)Gal β MP Glycoside (curve e), a decrease in peak current was observed, indicating that SNA had specifically binded with Neu5Acα(2-6)Gal β MP Glycoside and facilitated the adsorption of analytes onto the surface of the modified electrode. As shown in Fig. S5(B), the detailed electron transfer behavior of GO–PB–PTC-NH_2/Au/SNA/BSA/Neu5Acα(2-6) Gal β MP Glycoside/GCE was also investigated by electrochemical impedance spectroscopy (EIS) in the presence of 5 mM potassium ferricyanide. The results are in agreement with the conclusions obtained from CV.

Typical CVs of the GO–PB–PTC-NH_2 modified electrode at different scan rates ranging from 10 to 600 mV s^{-1} are presented in Fig. S6. Both oxidation and reduction peak currents increased linearly and were proportional to the square root of the different scan rates (Fig. S7), indicating that the reaction was a surface-controlled process, which is in agreement with the previous results (Hui et al., 2014).

3.3. Optimization of the experimental conditions

Elements that influence the performance of the electrochemical biosensor have been previously discussed. As the buffer's pH significantly impacts the stability of PB and the activity of SNA-Neu5Acα(2-6)Gal β MP Glycoside, we investigated the effect of pH on performance of the biosensor. The peak current of the modified electrode initially increases then decreases when the pH of PBS ranged from 4.0 to 8.5, with the highest peak current at pH=6.0, as shown in Fig. 1A. The previous study proved that PBS at pH=6.0 is the most suitable pH for stabilizing PB (Jiang et al., 2010). Therefore, in the present study, phosphate buffer (pH=6.0) is chosen as the optimal buffer solution.

The incubation time of GO–PB–PTC-NH_2/GCE in GNPs was also investigated. As shown in Fig. 1B, the change in current increased sharply from 2 h to 12 h and reached a maximum at 12 h. Further increase in incubation time seemed to sharply decrease the current change, possibly because the excessive amount of GNPs decreased the surface area of the electrode and deterred electron transfer (Zhang et al., 2013). Therefore, the optimized incubation time in GNPs is 12 h.

To optimize the reaction time, the assembly time of analytes on GO–PB–PTC-NH_2/Au/SNA/BSA/GCE was investigated by the change in response current (Fig. 1C). The experimental data indicated that assembly time has some influence on the response signal, which reveals that the quantity of adsorbed SNA relies on the reaction time. The highest response was observed at 2.5 h; gradually increasing the reaction time led to a sharp decrease in signal, which implies that excessive SNA molecules were immobilized on non-specific binding sites and deterred electron transfer. In this work, an incubation time of 2.5 h is recommended.

The effect of SNA concentration (0.1–2.0 mg mL^{-1}) on the bioreaction efficiency was investigated (Fig. 1D). Our experimental data revealed that the amperometric response increased with increased SNA concentration. When the concentration of SNA was increased to 2.0 mg mL^{-1}, the amperometric response increased slightly compared with that of the 1.5 mg mL^{-1} sample, possibly because only adsorbed SNA in a suitable orientation participates in the reaction and a higher concentration may have no significant effect on the response signal. Therefore, 1.5 mg mL^{-1} SNA was chosen in the present experiment.

3.4. Analysis and detection

The calibration plot for the detection of Neu5Acα(2-6)Gal β MP Glycoside was determined under the optimal experimental conditions. Fig. 1E presents the DPV analysis and detection of Neu5Acα (2-6)Gal β MP Glycoside with the prepared electrochemical biosensors in 0.1 M PBS (pH=6.0) where the analyte concentration varied from 0.1 pg mL^{-1} to 500 ng mL^{-1}, which were dissolved and quantified in human serum containing 0.1 M PBS as in Section 2.4. The amperometric signal decreased with increasing analyte concentration. As seen in Fig. 1F, the electrochemical biosensor worked well over a broad linear range (0.1 pg mL^{-1}–500 ng mL^{-1}) with a correlation coefficient of R=0.9991. The limit of detection offered by this electrochemical biosensor was 0.03 pg mL^{-1}. The developed electrochemical biosensor clearly provides a model for clinical research and diagnostic applications.

3.5. Selectivity, stability, and reproducibility of the electrochemical biosensor

To evaluate the specificity of the electrochemical biosensor towards the target analyte, we exposed the system to other biological molecules, such as ascorbic acid (AA), dopamine (DA), glucose, and L-cysteine (L-cys). The electrochemical biosensor was incubated in interfering solutions of 500 ng mL^{-1} AA, 500 ng mL^{-1} DA, 500 ng mL^{-1} glucose, 500 ng mL^{-1} L-cysteine, and a mixture of the four interfering analytes and 100 ng mL^{-1} Neu5Acα (2-6)Gal β MP Glycoside. The current change is shown in Fig. S8. The biosensor showed a rather low current response to the interfering analytes at 500 ng mL^{-1} compared with that of the mixture containing 100 ng mL^{-1} of analyte and all four interferences, which implied high specificity of the electrochemical biosensor.

The reproducibility of the electrochemical biosensor was investigated by the variation coefficient of intra- and inter-assays. Using 0.1 pg mL^{-1}, 500 pg mL^{-1}, and 500 ng mL^{-1} Neu5Acα(2-6) Gal β MP Glycoside solutions, the intra- and inter-assays of the electrochemical biosensor were estimated using one biosensor for three repeat assays and three biosensors for one assay, respectively. The relative standard deviations (RSDs) were 2.61%, 1.13%, and 0.10% for intraday repeated assays and 5.09%, 1.80%, and 2.90% for interday repeated assays (Table S1) for 0.1 pg mL^{-1}, 500 pg mL^{-1}, and 500 ng mL^{-1} solutions of Neu5Acα(2-6)Gal β MP Glycoside, respectively. Therefore, the reproducibility of the electrochemical biosensor is satisfactory. The stability was assessed over a 20-day period while the electrochemical biosensor was stored at 4 °C. The peak current of the biosensor retained 93.26% and 86.91% of its initial response after storing for 10 and 20 days, respectively. No obvious change in response was found, which

Table 1
Recovery of serum samples for the electrochemical biosensor using DPV analysis ($n=3$).

Samples	C_{Added}	C_{Found}	Recovery (%)	RSD (%)
1	0.3 pg mL^{-1}	0.315 pg mL^{-1}	105.0	4.31
2	300 pg mL^{-1}	300.61 pg mL^{-1}	100.2	4.95
3	400 ng mL^{-1}	406.4 ng mL^{-1}	101.6	1.05

indicated that the storage stability of the assay system was acceptable.

3.6. Recovery testing

To examine the applicability of the present electrochemical biosensor to biological samples, the determination of Neu5Acα (2-6)Gal β MP Glycoside in human serum was performed using DPV analysis. The developed electrochemical biosensor was applied to human serum samples spiked with 0.3 pg mL^{-1}, 300 pg mL^{-1}, and 400 ng mL^{-1} of Neu5Acα(2-6)Gal β MP Glycoside, and the recoveries of the three Neu5Acα(2-6)Gal β MP Glycoside concentrations were determined. As shown in Table 1, the range of recovery and RSD was 100.2–105.0% and 1.05–4.95%, respectively. Thus, it is clear that the proposed Neu5Acα(2-6)Gal β MP Glycoside electrochemical biosensor has high potential for use with human serum.

4. Conclusions

In summary, we have fabricated, for the first time, a highly conductive electrode modified by graphene oxide, Prussian blue, and PTC-NH_2 for the specific detection of α2,6-sialylated glycans in serum. This proposed method has high sensitivity, can be applied to human serum, and worked well over a broad linear range with a low detection limit, demonstrating the resiliency of this method to endogenous interferences in human serum. The biosensor also has the advantages of low cost, high specificity, and reproducibility, which makes it a promising tool for clinical research and diagnostic applications. However, the proposed biosensor was fabricated with multiple steps, in which some factors may exist and have an effect on the stability of our biosensor. Therefore, it is necessary to further simplify the fabrication process to promote the stability of the biosensor and to be better used for the detection of α2,6-sialylated glycans in serum.

Acknowledgments

This study was supported financially by the National Natural Science Foundation of China (81370403 and 21205146).

Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.06.031.

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