Highly sensitive electrochemical immunosensing for Listeria monocytogenes based on 3,4,9,10-perylene tetracarboxylic acid/graphene ribbons as sensing platform and ferrocene/gold nanoparticles as amplifier

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**Abstract**

As a gram-positive foodborne pathogen, Listeria monocytogenes (LM) can cause many serious diseases to the human health coupled with high mortality rates, thus constructing effective method to detect LM is of great significance. Herein, a novel sandwich-type electrochemical immunosensor was proposed for LM by introducing 3,4,9,10-perylene tetracarboxylic acid/graphene ribbons (PTCA/GNR) nanohybrids as sensing platform and ferrocene/gold nanoparticles (Fc/Au NPs) as signal amplifier. The high conductivity and large surface area of GNR can increase the immobilizing amount of primary antibody (PAb) and enhance the electron transport rate, while Au NPs can carry secondary antibodies (SAb) and Fc derivative (Fc–SH) to form SAb-Au NPs-Fc signal amplifier. Through using Fc molecules as signal probe, its peak current can appear and increase varied from the LM concentrations, hence a highly sensitive sandwich-type immunosensor was constructed: the linear range is wide from 10 to $2 \times 10^4$-CFU mL$^{-1}$ and the limit of detection is low to 6 CFU mL$^{-1}$. Furthermore, the specificity of the immunosensor was also studied and a satisfactory result was obtained.

**Keywords:** Listeria monocytogenes; foodborne pathogen; electrochemical sensing; immunosensor; graphene ribbons; ferrocene.
1. Introduction

Listeria monocytogenes (LM), a gram-positive foodborne pathogen, has posed serious threats to the human health since it can cause many serious diseases such as meningitis, septicemia, abortion and febrile gastroenteritis coupled with the high mortality (~30%) and morbidity rates. In general, the pregnant women, neonates and immunocompromised persons as well as the elderly are susceptible to LM.\textsuperscript{1,2} In addition, LM can easily survive in water, soil, plants, milk, meats, cheese, raw vegetables and muskmelon.\textsuperscript{3,4} Consequently, LM has been confirmed as one of the most life-threatening and hazardous pathogens. In USA, the Centers for Disease Control and Prevention estimated 1000 infections with exceed than 200 deaths each year associated with the listeriosis, and a zero-tolerance policy about the existence of LM in food has been formed; as for Canada, it only allows 100 CFU/g of LM in the food.\textsuperscript{5,6} Therefore, developing sensitive and accurate method for LM detection is needed urgently.

Some conventional technologies including enzyme-linked immunosorbent assay, polymerase chain reaction assay, surface plasmon resonance, quartz crystal microbalance immunosensor and fiber-optic immunosensor have been developed to detect LM.\textsuperscript{3,7-9} It’s no doubt that these methods are very important and can largely fit the demands for detecting LM. However, they usually suffered from some deficiency such as labor-intensive, time-consuming, complex pretreatments or expensive equipments. In contrast, the electrochemical technologies have many unique advantages compared to other methods; specifically, it is simple, sensitive, time-saving, low-cost and easy to operate.\textsuperscript{10} In recent years, some superior electrochemical sensing platforms have been constructed successfully to detect LM.\textsuperscript{11-13} Although these methods have important applications for LM detection, developing new sensing method with higher sensitivity and superiority for LM is still considerable important.

The electrochemical immunosensing coupled with sandwich-type strategies attracted considerable attentions in the past years due to their superiorities in sensitivity and signal amplification ability. As for constructing effective sandwich-type immunosensor, one crucial factor
is to introduce suitable nanomaterial in sensing platform for facilitating electron transfer and increasing the load amount of antibodies. Graphene ribbon (GNR), a new quasi-one-dimensional carbon material after carbon nanotube (CNT) and graphene nanosheet (GNS), possesses large surface area and high electrical conductivity as well as electrochemical stability.

In addition, comparing to general CNT and GNS, GNR has much more edge defects and active sites, which makes GNR easy to be functionalized and has important application in electrochemical sensing. Presently, GNR has been applied successfully widely in various fields such as ion batteries, organic photovoltaics, supercapacitors, device fabrication and optoelectronics, while there are only a few reports by using GNR in electrochemical sensing.

Hence, introducing GNR in sensing platform to construct electrochemical immunosensor for LM is an interesting topic. However, the pure GNR usually tends to agglomerate in solvent owing to its hydrophobic property.

Another key factor for constructing effective sandwich-type immunosensor is to design desirable signalling amplifiers. As we all know, gold nanoparticles (Au NPs) are easy to be obtained and Au NPs have excellent conductivity and electro-catalytic property as well as biocompatibility; meanwhile, ferrocene (FC) and its derivatives have been largely used as signaling probe owing to the highly and stable electrochemical activity. Inspired by these insights, herein GNR was prepared and functionalized with 3,4,9,10-perylene tetracarboxylic acid (PTCA) via π-π stacking, the formed PTCA/GNR was then introduced as sensing platform to immobilize primary antibody (PAb) for capturing LM target. In this process, PTCA can not only improve the dispersibility of GNR in water but also offer the capability to immobilize PAb due to the presence of abundant carboxy groups. While Fc-SH and secondary antibodies (SAb) were assembled on the Au NPs surface to be used as signalling amplifier (SAb-Au NPs-Fc). When the specific immunoreaction of “PAb-LM-SAb” occurs on the sensor surface, which can results the presence of peak current of FC probe, and the corresponding current values are proportional to the concentration of LM. Based on this principle, a highly sensitive and reliable sandwich-type electrochemical immunosensing platform for LM was constructed successfully in this work (Scheme 1). After optimizing the
experimental parameters, a wide linearity range and low limit of detection (LOD) were obtained, indicating the proposed sandwich-type immunosensing platform has potential applications for LM detection.

2. Experimental section

2.1 Preparation of PTCA/GNR

Firstly, GNR was prepared according to previous reports with some modification.\textsuperscript{26,27} Briefly, 50.0 mg CNT was dispersed in the concentrated H\textsubscript{2}SO\textsubscript{4}/H\textsubscript{3}PO\textsubscript{4} (9:1) in a glass flask for stirring 1 h before adding 250.0 mg KMnO\textsubscript{4}. The formed mixture was heated to 65 °C and kept for 2 h, then it was poured into ice water containing H\textsubscript{2}O\textsubscript{2} to quench the reaction. After filtering, washing, and drying, the initial graphene oxide nanoribbon (GONR) was obtained. For achieving GNR, ammonia and hydrazine solution were added in to the GONRs dispersion and heated for 10 h at 60 °C under stirring, GNR can be obtained successfully after filtration and washing.

Next, 50.0 mg GNR was ultrasonicated in 100.0 mL ethanol containing 20.0 mg PTCA for 1 h, and the solution was stirred continuously for 2 h at room temperature. Then, the mixture was filtered through a nylon membrane (0.22 mm), washed several times and dried, thus resulting the formation of PTCA/GNR.

2.2 Preparation of SAb-Au NPs-Fc

For preparing SAb-Au NPs-Fc, Au NPs was prepared firstly according to the previous report.\textsuperscript{28,29} Briefly, 0.2 mol L\textsuperscript{-1} sodium citrate (400 μL) was added dropwise to the 0.2 mg mL\textsuperscript{-1} HAuCl\textsubscript{4} solution (12.0 mL) and heated at 60 °C for 2 hour. Then Au NPs was obtained through centrifugation and dried. Next, 20.0 μL SAb was added in the 1.0 mL Au NPs solution to incubate for 2 hours, and then 1.0 mM Fe-SH solution (20.0 μL) was added into SAb-Au NPs solution to obtain SAb-Au NPs-Fc.

2.3 Construction for LA sandwich-type immunosensor
The polished GCE was covered firstly with 12.0 μL PTCA/GNR suspension of (0.5 mg mL⁻¹) and treated with EDC/NHS mixture to activate carboxyl groups. After rinsing and drying, the activated PTCA/GNR/GCE was coated with 12.0 μL PAb to bound effective PAb, and the formed electrode is denoted as PAb/GNR/GCE. Next, PAb/PTCA/GNR/GCE was rinsed with phosphate buffer solution (PBS) to remove the unbound PAb; meanwhile, it was incubated with BSA to prepare BSA/PAb/PTCA/GNR/GCE for preventing the nonspecific and unreacted sites. For detecting LA, the prepared BSA/PAb/PTCA/GNR/GCE above was interacted with different concentrations of LM for 60 min to capture Listeria cells (denoted as LM/BSA/PAb/PTCA/GNR/GCE) and then dipped in PBS for three times to remove the nonspecific proteins and LM cells. Finally, LM/BSA/PAb/PTCA/GNR/GCE was combined with SAb-Au NPs-Fc for 45 min to form a sandwich-type structure and measured with the electrochemical technologies which were carried out via CHI 660E Electrochemical Workstation (China).

3. Results and discussion

3.1 Characterization of PTCA/GNR and Au NPs

The lengthwise unzipping of CNT to form GNR and the produced Au NPs were investigated through transmission electron microscopy (TEM, Figure 1). As exhibited in Figure 1A, CNT shows a typical tube-like nanostructure with a ~30.0 nm diameter. After unzipping CNT, the produced GNR possesses an increased width (~70.0 nm) and the tube-like structure is nearly disappeared (Figure 1B), indicating that CNT was lengthwise cut successfully to nanoribbon structure (GNR). Figure 1C shows the TEM image of Au NPs, it’s found Au NPs has a homogeneous size with ~4.5-nm average diameter via the statistical investigation.

Next, PTCA/GNR was characterized via Fourier transform infrared (FT-IR) spectroscopy. As shown in Figure 2, GNR only exhibits C=C conjugation at ~1563 cm⁻¹ and C-C bands at 1115 cm⁻¹; while for PTCA/GNR, two obvious new peaks at ~3445 and ~1771 cm⁻¹ were observed, which are ascribed to the broad stretching bands O-H and C=O respectively from the carboxyl groups of
PTCA. Then, the dispersibility of GNR and PTCA/GNR in water was studied (Figure 3). It can be found that the dispersion capability of the pure GNR is rather poor and aggregated with each other owing to the hydrophobic surface of GNR. However, PTCA/GNR can be uniformly dispersed to generate a homogeneous and dark solution in water. These characterizations suggest that GNR was functionalized successfully with PTCA and the form PTCA/GNR shows excellent dispersion ability in water.

3.2 Electrochemical studies of the sandwich-type immunosensor

The construction possesses of the immunosensor were investigated by electrochemical impedance spectroscopy (EIS) which were recorded in 5.0 mM [Fe(CN)₆]³⁻/⁴⁻ solution and the related EIS plots of various modified electrodes were shown in Figure 4. From curve a, it can be found that the charge-transfer resistance ($R_{CT}$) of PTCA/GNR/GCE is considerable weak owing to the prominent conductivity of GNR. As for the other modified electrodes including BSA/PAb/PTCA/GNR/GCE, LM/BSA/PAb/PTCA/GNR/GCE and SAb-Au NPs-Fc/LM/BSA/PAb/PTCA/GNR/GCE, the $R_{CT}$ values increase in turn as following: PTCA/GNR/GCE < BSA/PAb/PTCA/GNR/GCE < LM/BSA/PAb/PTCA/GNR/GCE < SAb-Au NPs-Fc/LM/BSA/PAb/PTCA/GNR/GCE, these are resulted from that the related biomolecules (antibody, BSA and LM cells) are non-conductive and can restrain electron channel, thus impeding the electron transfer and increasing the $R_{CT}$ values. These studies suggest that the developed immunosensor layers were successfully assembled.

Next, the feasibility of sandwich-type immunosensor for LM was demonstrated via measuring the differential pulse voltammetry (DPV) response of various electrodes in 0.1 M PBS (pH 7.0) (Figure 5), the experimental results showed an obvious peak current of Fc (~0.247 V) can be observed at SAb-Au NPs-Fc/LM/BSA/PAb/PTCA/GNR/GCE, while for the other electrodes (PTCA/GNR/GCE, BSA/PAb/PTCA/GNR/GCE and LM/BSA/PAb/PTCA/GNR/GCE), there is no peak current presented, indicating that SAb-Au NPs-Fc was captured on the LM/BSA/PAb/PTCA/GNR/GCE surface owing to the antigen-antibody specific reaction between
SAb and LM cells, and suggesting that the proposed sandwich-type immunosensing method for detecting LM is feasible.

### 3.3 Optimization of the sensing conditions

Some important conditions were optimized to obtain the high sensitivity in sensing LM. Firstly, the influence from PBS with different pH values was evaluated (Figure 6A), it’s noted that the current responses of SAb-Au NPs-Fc/LM/BSA/PAb/PTCA/GNR/GCE increase with the increase of pH value from 4.5, obtaining the maximum at 7.0. This may be resulted from that the excessively alkaline or acidic exterior can enable the assembled proteins to be instable, thus PBS of 7.0 was used in the work. Undoubtedly, the amount of PTCA/GNR on the electrode surface is very important toward the sensing performance, hence it needs to be optimized. As shown in Figure 6B, the current signal of the sensor increased when the amount varied from 2.0 to 12.0 μL. When the amount was more than 12.0 μL, the peak current of Fc changed only slightly, so 12.0 μL PTCA/GNR dispersion was used to modify electrode. Next, the interaction time between LM and PAb or SAb were studied (Figure 6C), the results indicate that the optimum time between LM and PAb is 60 min, while 45 min is enough for the interaction between LM and SAb. Furthermore, the amount of SAb introduced in the preparation of SAb-Au NPs-Fc is a critical factor for the sensing capability, thus it was also studied (Figure 6D). As shown in the figure, the current signals of Fc increase with the increase of SAb amount varied from 4.0 to 20.0 μL. When the amount exceed more than 20.0 μL, the peak current show a decrement owing to the competitive immobilization between Fc and SAb with Au NPs, thus 20.0 μL SAb was selected as the optimized amount in preparing SAb-Au NPs-Fc.

### 3.4 Detection of LM

Under the optimized conditions obtained above, the quantitative determination of LM based on the proposed sandwich-type immunosensor was carried out using DPV technology via drawing the calibration curve, which includes the Fc signals change upon the change from the LM concentration (Figure 7). It can be observed that the peak current signal linearly increases with the increase of LM
concentrations varied from 10 and \(2 \times 10^4\) CFU mL\(^{-1}\), and LOD was calculated to be 6 CFU mL\(^{-1}\) (S/N=3), which are more superior than those obtained in many previous works (Table 1); Meanwhile, the developed method herein is more simple and time-saving comparing to the previous standard methods, revealing that the proposed method can be used in the highly sensitive sensing for LM. The excellent sensing performances based on the developed sandwich-type immunosensor can mainly attribute to the high surface and conductivity of GNR and the signal amplification ability of SAb-Au NPs-Fc.

3.5 Specificity the immunosensor

To investigate the specificity of the proposed immunosensor, the response signals of Fc at the sensors by using other similar bacterial pathogens instead of LM were studied. The results show that only LM can produce a significant peak current, while the other bacteria (\(10^4\) CFU mL\(^{-1}\)) including Vibrio parahaemolyticus, Salmonella enteriditis, Shigella, Staphylococcus aureus and Escherichia. coli exhibited negligible effects on the current signals at the concentration of \(3 \times 10^3\) CFU mL\(^{-1}\) (Figure 8), demonstrating that the proposed sandwich-type immunosensor possesses desirable specificity for LM detection.

4. Conclusion

In summary, for achieving the highly sensitive and selective detection of LM, a novel electrochemical immunosensor was proposed in this work by constructing SAb-Au NPs-Fc/LM/BSA/PAb/PTCA/GNR sandwich-type structure. Owing to the high conductivity and large surface of GNR, abundant PAb can be immobilized on the PTCA/GNR surface and the sensing performance was also enhanced; meanwhile, the response signal was amplified further via assembling Fc and SAb on the Au NPs surface to form SAb-Au NPs-Fc which can be captured by LM cells owing to the antigen-antibody specific reaction. After optimizing various conditions, the proposed sandwich-type immunosensor exhibits superior analytical performance coupled with a
wide linear range (10 to $2 \times 10^4$ CFU mL$^{-1}$) and very low LOD (6 CFU mL$^{-1}$). In addition, the immunosensor possesses desirable specificity. It’s thus confirmed the proposed sandwich-type immunoassay has important potential application for LM detection.

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Scheme 1. Scheme Illustrations for constructing SAb-Au NPs-Fc/LM/BSA/PAb/PTCA/GNR sandwich-type electrochemical immunosensor of LM.
Figure 1. The TEM images of CNT (A), GNR (B) and Au NPs (C).
Figure 2. The FT-IR spectra of GNR (a) and PTCA/GNR (b).
Figure 3. The photographs of pure GNR (a) and PTCA/GNR (b) dispersions in water.
Figure 4. The EIS plots of PTCA/GNR/GCE (a), BSA/PAb/PTCA/GNR/GCE (b), LM/BSA/PAb/PTCA/GNR/GCE (c) and SAb-Au NPs-Fc/LM/BSA/PAb/PTCA/GNR/GCE (d).
Figure 5. The DPV response of SAb-Au NPs-Fc/LM/BSA/PAb/PTCA/GNR/GCE in 0.1 M PBS; the LM concentration is $4 \times 10^4$ CFU mL$^{-1}$. 
Figure 6. Influences of the (A) pH value of PBS; (B) amount of PTCH/GNR; (C) interaction time of LM with BSA/PAb/PTCA/GNR/GCE (a) and LM/BSA/PAb/PTCA/GNR/GCE with SAb-Au NPs-Fc (b); (D) amount of Ab₂. The concentration of LM is $4 \times 10^3$ CFU mL$^{-1}$. 
Figure 7. (A) The DPV responses of LM with different concentrations in 0.1 M PBS (from a to g: $10, 8 \times 10^2, 5 \times 10^3, 3 \times 10^3, 8 \times 10^3, 1.4 \times 10^4$ and $2 \times 10^4$ CFU mL$^{-1}$) based on the developed immunosensor; (B) the calibration curve for LM detection.
Figure 8. Specificity of LM detection based on the developed sandwich-type immunosensor.
Table 1. Comparisons for different electrochemical immunosensing platform for LM detection.

| Electrodes                                | Assay label | Measuring method | Linear range / CFU mL$^{-1}$ | LOD / CFU mL$^{-1}$ | Reference |
|-------------------------------------------|-------------|------------------|------------------------------|---------------------|-----------|
| Gold electrode                            | HRP         | Amperometry      | $10^2$-10$^6$                | $10^2$              | 5         |
| Interdigitated array microelectrode       | Urease      | EIS              | 1.9×10$^3$-1.9×10$^6$        | 1.6×10$^3$          | 30        |
| TiO$_2$ nanowire bundle gold microelectrode | Label-free  | EIS              | ___                          | 10$^2$              | 31        |
| Au NPs/screen-printed carbon electrode    | HRP         | Amperometry      | 2.25×10$^2$-2.25×10$^5$      | 2.25×10$^2$         | 32        |
| CNT fibers Electrode                      | HRP         | Cyclic voltammetry | $10^2$-10$^5$              | 1.07×10$^2$         | 33        |
| PTCA/GNR/GCE                              | Fc          | DPV              | 10-2×10$^4$                  | 6                   | This work |

This work